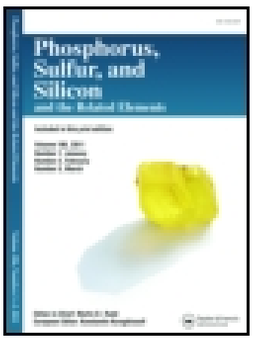

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Whole-cell biotransformation of diethyl 1-hydroxy-1-phenylmethanephosphonate in a different reaction environment

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ABSTRACT

The whole-cell biocatalytic resolutions of enantiomers of racemic 1-butyryloxy-1-phenylmethanephosphonate was performed by *Bacillus subtilis* strain in different media. For this purpose, the well-known medium, which induce lipolytic properties, was tested as well as nutrient broth medium. The enantioselectivity of the hydrolysis reactions was at the same level (50% conversion, ~80% ee) regardless of the used medium, but the time of reaction varied significantly, depending on the medium in which the microorganism was grown and on the reaction medium. The addition of 0.01% of tributyrin shortened the response time in the range from 30 min to 1 h. In addition, when the nutrient broth with tributyrin was used as a medium for grown and for biotransformation reactions, enantioselectivity also increased from 23 to 35.

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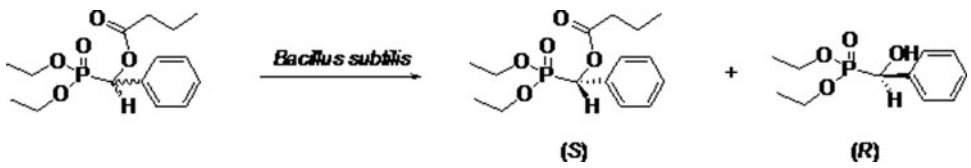
Biocatalysis; enantioselectivity; hydroxyphosphonates

GRAPHICAL ABSTRACT



Introduction

Many research teams are interested in chiral hydroxyphosphonates and obtaining them in an optically pure form because they are a class of organophosphorus compounds applied as building blocks for the synthesis of medicinally important molecules.[1,2] Moreover, substituted hydroxyphosphonic acids constitute a class of mimics of natural hydroxycarboxylic acids[3] and consequently are considered as those that may exhibit promising biological activity. This is indeed the fact and some of these compounds exhibit interesting antibacterial, antiviral or anticancer agents acting as inhibitors of enzymes important for pathogens development.[3,4]

The whole-cell biotransformation is becoming an increasingly popular method, because stereoselective biocatalytic processes constitute a useful alternative to asymmetric synthesis of chiral compounds.[5] Until now, there are only few publications about whole-cell biotransformation of hydroxyphosphonates by microorganisms with lipolytic activity. The lipases produced by bacteria strains can catalyse the hydrolysis of esters of hydroxyphosphonates by means of kinetic resolution providing high enantiomeric excess of both hydroxyphosphonates and their unreacted esters.[6–9] There are many bacterial strains which exhibit lipolytic activity. One of them is the common bacteria *Bacillus subtilis*, which produces two extracellular lipases (LipA and LipB).[10] The biotransformations of hydroxyphosphonates conducted by this strain are highly enantioselective.[7–9]

The activities of most of bacterial lipases are usually induced in a medium that contains fatty acids and oils,[11–13] but also other triglycerides like tributyrin or surfactants can induce lipolytic activity.[14,15] The well-known growth medium for bacteria strain used in biotransformation reactions of hydroxyphosphonates (medium A.2, see paragraph 2.1.1) contained: 10 g of soluble starch, 1 g of yeast extract, 5 g of $(NH_4)_2SO_4$, 2 g of K_2HPO, and 100 μL of tributyrin.[7–9] After 24 h of incubation, the microorganism was centrifuged and transferred to the buffer, wherein the biotransformation reactions were performed. There are reports that the nutrient broth was applied as both medium for growing of bacteria and as a medium for biotransformations.[16] The use of the medium in which the microorganism is growing as the reaction medium appears to be appropriate, because of the simplification of biotransformation procedures. In this work it is studied how the composition of the reaction medium, and the culture medium has an influence on the reaction enantioselectivity. For this purpose, the following media were tested: popular A.2 medium, a similar medium lacking tributyrin, and a classical nutrient broth with and without tributyrin.

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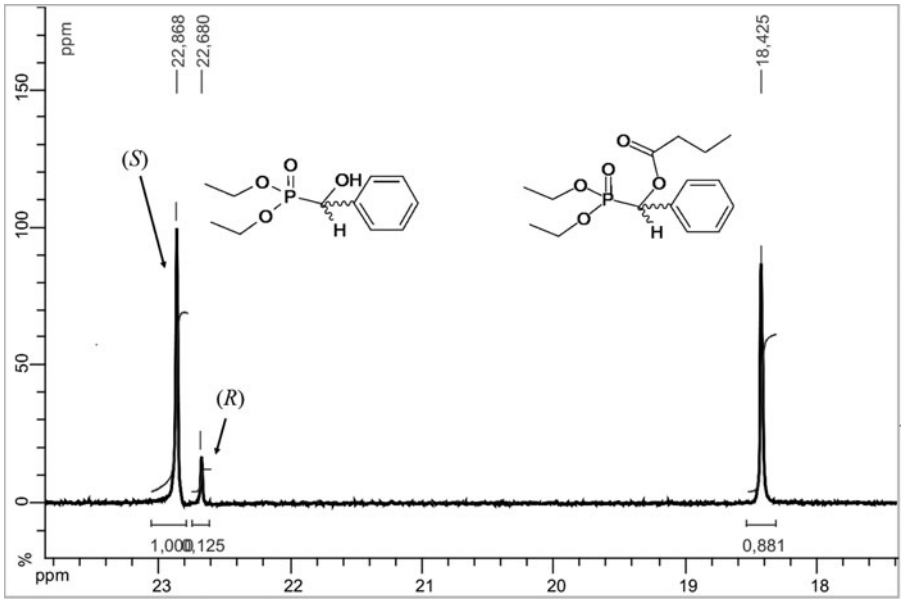

Figure 1. ^{31}P NMR spectrum with quinine after hydrolysis of diethyl 1-butyryloxy-1-phenylmethanephosphonate 2 (5 h, medium A.2, method 2).

Results and discussion

Racemic hydroxyphosphonate (compound **1**) was obtained by the addition of diethyl phosphite to the corresponding aldehyde. After purification, racemic hydroxyphosphonate **1** was converted into *O*-butyryl derivative **2** by simple acylation with butyryl chloride. The ester **2** was then hydrolysed using *Bacillus subtilis* strain as a whole-cell biocatalyst. Such kinetic resolution of this compound by hydrolysis with *Bacillus subtilis* was described previously.[9] The reaction resulted in an enantiomeric excess of 95% at 44% substrate conversion after 8 h. Also *Serrratia liquefaciens, Escherichia coli, Pseudomonas aeruginosa,* and *Pseudomonas fluorescens* were tested following the same procedures of growth of these strains (medium A.2) and biotransformation procedures (phosphate buffer 0.017M pH = 7.0).[9]

In this report the change in composition of the growing medium and procedure of hydrolysis affecting the course of this reaction was studied. Thus, biotransformation reactions were carried out in four different media and additionally by two different methods. Each time, the biotransformation products were analyzed by ^{31}P NMR spectroscopy using quinine as CSA (Figure 1) and by analytical HPLC using a chiral column (Figure 2). Because of the nature of the kinetic resolution only the results when conversion was close to 50% are shown.

An increased reaction time resulted in a decrease of enantiomeric excess of product up to 0% for a conversion being near 100% at 24–48 h depending on the medium and procedure. The enantiomeric excess of unreacted substrate is increasing during the time, but it is connected with a decrease of yield of obtained unreacted ester.

As can be seen from Tables 1–2, the conversion near 50% was obtained faster when the method 1 was used. This procedure is far easier to perform than method 2 because, after incubation, the cells remained in the medium, consequently centrifugation and washing of bacterial cells was omitted. It can therefore be concluded that the separation of cells of bacteria followed by their suspension in the buffer is inefficient and unnecessary. At the same time it can be deduced that the addition of tributyrin reduce time required to reach 50% of degree conversion (in some cases up to 1 h), which suggest that addition of this component assists in producing a more amount of

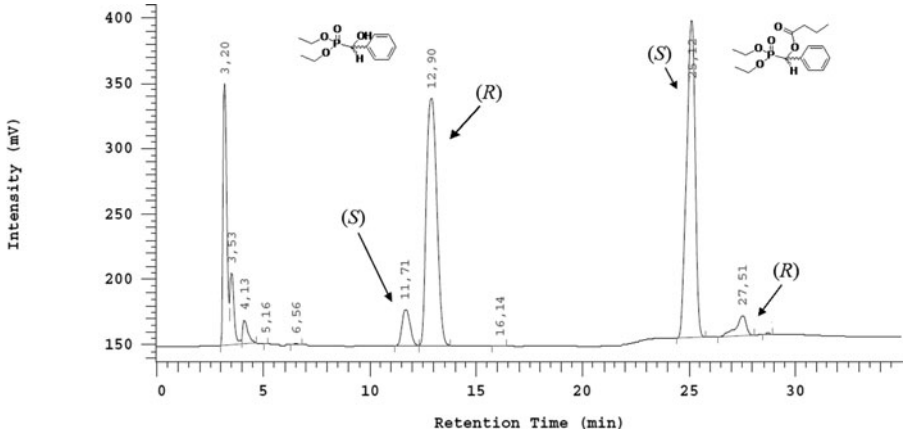

Figure 2. Chromatogram after hydrolysis of diethyl 1-butyryloxy-1-phenylmethanephosphonate 2 (5 h, medium A.2, method 2).

Table 1. Hydrolysis of diethyl 1-butyryloxymethanephenylphosphonate by *Bacillus subtilis* in media A1 and A2.

Medium	Method	Time [h]	Conversion [%]	ee of ester [%]	ee of alcohol [%]	*E*
A.1	1	4	45	64	83	11
		5	50	84	82	29
		6	54	98	77	232
	2	5	45	68	93	17
		5.5	49	75	89	21
		6	52	91	84	56
A.2	1	3	49	72	84	16
		4	50	79	78	20
		5	54	93	73	60
	2	5	50	80	85	24
		5.5	51	82	86	28
		6	59	72	81	15

lipases. Only in one case, when the medium B.2 was used, the time of reaction time increased significantly. Unfortunately, the addition of tributyrin does not affect the enantioselectivity of biotransformation.

Moreover, it can be noticed that the change of the method from medium-based to buffer-based does not increase the time required to achieve 50% of degree conversion and does not improve the enantioselectivity of the reaction.

Finally it can be perceived that the direct use of nutrient broth significantly reduced the time of biotransformation reactions, and that the combination of the use of cell culture media and medium of reaction in one (medium B.1 and method 1) gave the best results.

Experimental

Materials and methods

All chemicals were purchased from Sigma Aldrich (St. Louis, Missouri, United States), POCh (Gliwice, Poland) or BIOCORP (Warszawa, Poland) and were used without further purification.

B. subtilis strain was from our own collection (isolated from soil) and was identified by the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

NMR (Nucleic Magnetic Resonance) spectra were measured on a Bruker Avance^{TM} 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 D, containing 0.03% v/v TMS) or on a Bruker Avance^{TM} DRX 300 instrument operating at 300.13 MHz for ^{1}H; 121.50 MHz for ^{31}P and 75.45 MHz for ^{13}C in $CDCl_3$ (99.8% D with 0.03% v/v TMS). Chemical shifts (δ) are reported in ppm in relation to standards. The biotransformations products were analysed by ^{31}P NMR with quinine used as a Chiral Solvating Agent (CSA). In the case where not all the signals from the enantiomers have been separated, the sample was also analysed on HPLC using LaChrome D-7000 system with chiral column Chiralpak AD 250 × 4.6 mm. Synthesized compounds were purified by gradient column chromatography using Merck Silica Gel 60 (63–230 mesh). Separation of unreacted substrate from the product was done by Medium-pressure Liquid Chromatography system Interchim PuriFlash 430evo on reversed phase column PuriFlash C18-HP with a grain size of 15 microns. Specific rotation was measured on polAAr 31 polarimeter in chloroform ($\lambda = 589$ nm).

Table 2. Hydrolysis of diethyl 1-butyryloxymethanephenylphosphonate by *Bacillus subtilis* in media B1 and B2.

Medium	Method	Time [h]	Conversion [%]	ee of ester [%]	ee of alcohol [%]	*E*
B.1	1	1.5	49	79	86	23
		2	57	92	68	49
		2.5	61	97	57	117
	2	2	51	80	73	20
		2.5	54	90	74	42
		3	56	88	65	31
B.2	1	1	51	86	84	35
		1.5	62	93	58	50
	2	4	52	89	76	39
		4.5	59	96	60	90

Synthesis of diethyl 1-hydroxy-1-phenylmethanephosphonate and diethyl 1-butyryloxy-1-phenylmethanephosphonate

Compounds **1** and **2** were synthesized according to the method described previously.[9]

Microorganism: growth and biotransformations conditions

Bacillus subtilis strain was stored in nutrient agar at a temperature of 4 °C. In order to obtain good conditions for microorganism growth and ensuring satisfied results of biotransformation reactions, several media were tested. They were as follows:

Medium A.1 composed of: 10 g of starch soluble, 1 g of yeast extract, 5 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4 and 1000 mL of distilled water;

Medium A.2:[7] 10 g of starch soluble, 1 g of yeast extract, 5 g of $(NH_4)_2SO_4$, 2 g of K_2HPO_4, 100 μL of tributyrin and 1000 mL of distilled water;

Medium B.1: 8 g of nutrient broth (BIOCORP PS 90) and 1000 mL of distilled water;

Medium B.2: 8 g of nutrient broth (BIOCORP PS 90), 100 μL of tributyrin and 1000 mL of distilled water.

Microorganisms were incubated at 26 °C with shaking at 150 rpm for 24 h. Then biotransformation reactions were prepared in two ways:

Method 1: After incubation cells remained in the medium and the 50 μL of substrate was added.

Method 2: After incubation cells were centrifuged at 3000 rpm for 10 min and washed twice in 0.017 M phosphate buffer, pH 7.0. The washed cells were suspended in 100 mL of 0.017 M phosphate buffer and 50 μL of substrate was added.

Biotransformation reactions were carried out at 26 °C with shaking at 150 rpm. After an appropriate time depending on conversion (close to 50%, see Tables 1 and 2), reactions were stopped by biomass centrifugation followed by extraction of biotransformation products with ethyl acetate (50 mL). Organic layer was dried over anhydrous magnesium sulfate and, after filtration, the organic solvent was evaporated.

Enantioselectivity assignment

The mixtures of biotransformations products (alcohol and unreacted ester) were analyzed by ^{31}P NMR spectroscopy using quinine as a chiral solvating agent and by analytical HPLC with chiral column (CHIRALPAK AD, Diacel, gradient method: 15 min of isocratic flow of 1% of 2-propanol in n-hexane, 5 min from 1% to 4% of 2-propanol in n-hexane, 15 min of isocratic flow of 4% of 2-propanol in *n*-hexane, products retention time: ester: (*S*) –11.5 min, (*R*) – 13 min, alcohol: (*S*) – 25 min, (*R*) – 26.5 min.)

The degree of the enantiomeric excess was expressed as a percentage (%) and defined as:

$$\mathrm{ee} = \frac{P1 - P2}{P1 + P2} \times 100\%$$

where *P*1 and *P*2 are the values of the area under the signals coming from the major and minor enantiomer of the product respectively.

Enantiomeric ratio (*E*) was computed from the following formula:

$$E = \frac{\ln[\mathrm{ee}_p \frac{(1-\mathrm{ee}_s)}{(\mathrm{ee}_s+\mathrm{ee}_p)}]}{\ln[\mathrm{ee}_p \frac{(1+\mathrm{ee}_s)}{(\mathrm{ee}_s+\mathrm{ee}_p)}]}$$

Conclusions

The use of whole cells of *Bacillus subtilis* seems to be a method of choice for obtaining α-hydroxyphosphonates with high enantiomeric excess. The reaction conditions are environmentally friendly and are compatible with the global trend of green chemistry. The use of culture medium as the reaction medium substantially accelerates the biotransformation, probably because enzymes are already produced before introduction of substrate. Additionally, the use of a substantially simpler and commercially available cultivation medium is profitable, because its preparation is much easier and biotransformation reactions proceed faster and more effective.

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