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Application of cyanobacteria for chiral phosphonate synthesis†

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This is the first report on morphologically different strains of cyanobacteria: *Arthrospira maxima, Nostoc cf-muscorum* and *Nodularia sphaerocarpa* used for enantioselective bioreduction of selected, structurally different diethyl esters of oxophosphonic acids. The efficiency of the asymmetric hydrogen transfer was strongly dependent on the chemical structure of the substrates. *Arthrospira maxima* was active only toward diethyl (*S*)-2-oxopropylphosphonate (20% of yield, 99% of ee), whereas the application of *Nostoc cf-muscorum* as a biocatalyst allowed diethyl (*S*)-2-hydroxy-2-phenylethylphosphonate with a high enantiomeric excess (99%) and with 26% conversion degree to be obtained. Employing *Nodularia sphaerocarpa* led to the most spectacular result – diethyl (*S*)-2-hydroxy-2-phenylethylphosphonate with a degree of conversion of 99% and an optical purity of 92%. Enantioselective bioconversion of oxophosphonate with an aromatic side group located in the immediate vicinity of the carbonyl functionality was achieved for the first time. Additionally, flow cytometry showed excellent resistance of the cells of *Nodularia sphaerocarpa* against the examined xenobiotic – 2-oxo-2-phenylethylphosphonate, these cells remain viable at the concentration of 10 mM of the bioconversion substrate compared to the 1 mM described previously for a fungal biocatalyst. The effect of cultivation medium, light source and light cycle (light : dark) on the effectiveness of the biotransformation process was examined.

Introduction

Chiral compounds are important and useful intermediates in the chemical and life science industries. Biocatalytic production methods for such structures have been rapidly applied in industry, mostly because they comply with contemporary expectations of low toxicity and high efficacy for synthetic protocols. 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 in scaling up the invented methods.[1]

Biocatalytic reduction of ketones leading to chiral alcohols and employing non-photosynthetic and heterotrophic microorganisms or their purified enzymes is the method of choice.[2] However, phototrophic prokaryotes such as cyanobacteria have also been identified as a source of reductive activities, but in contrast to other extensively studied bacteria and fungi, this field is still poorly investigated.[3,4]

Biocatalysis is an effective and, in many cases, preferable alternative to the standard synthesis of optically active isomers of valuable chemicals, including phosphonates of a defined structure and absolute configuration.[5] Hydroxyphosphonates are a class of organophosphorus compounds with possible biological activity – they represent a synthetic platform for further applications (*e.g.* aminophosphonic acids and their derivatives) (Scheme 1).[6,7]

This paper examines the reductive potential of cyanobacteria toward xenobiotics of synthetic and medical nature, such as optically pure diethyl β-hydroxyphosphonates obtained as products of bioreduction. Previously described biotransformations of oxophosphonates[8] were effective only towards substrates with the carbonyl functionality not located in the immediate vicinity of the aromatic ring; moreover, these processes only allowed the formation of products on a laboratory scale. Because of the inhibitory properties of phosphonate derivatives (against many enzymes, that is why whole-cell biocatalysts are preferred) – scaling up was always the limiting factor. Considering this, screening for biocatalysts capable of overcoming such problems among organisms of completely different physiology is a possible scientific approach.

Cyanobacteria represent a morphologically diverse and widely distributed group of photosynthetic prokaryotes with significant roles in aquatic and terrestrial ecosystems. These bacteria have gained a lot of attention in recent years, because

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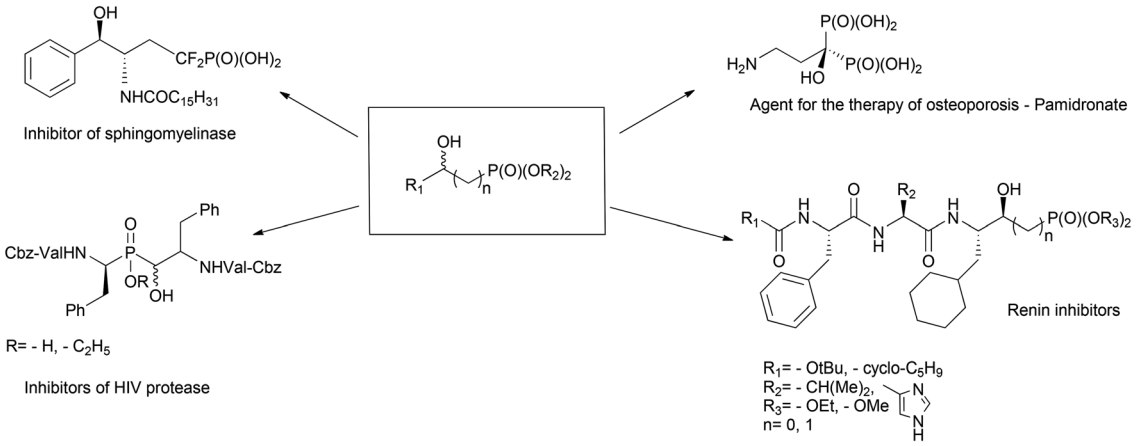


Scheme 1 Selected examples of important biologically active hydroxyphosphonates.[9–12]

of their possible applications in the bioconversion of structurally different substrates into the desired usable products. The potential of microalgae in the transformation of organic compounds is focused mainly on asymmetric reduction, production of biofuel, degradation of pollutants or modification of steroids. These reactions are crucial in the synthesis of intermediates for the production of pharmaceuticals and agrochemicals and are important in environmental safety. Cyanobacteria are applied mainly for the production of chiral compounds.[13]

Morphologically different cyanobacterial strains have been successfully used as whole-cell biocatalysts for a variety of reactions, *e.g.* biotransformation of hydrocortisone by *Nostoc muscorum* PTCC 1636 and asymmetric reduction of prochiral ketones by *Synechococcus elongates* PCC 7942.[14,15]

Additionally, they are considered as an economic alternative to other enzymatic systems, since solar energy can be used directly for bioconversion of xenobiotics; however, technical application of photobiocatalysis is sometimes limited by equipment requirements – photobioreactors enabling the biomass production.

The key advantage of cyanobacteria over other microbes such as the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* that are of common industrial use, is their ability to use light energy to reduce CO_2 to products of interest. Their incomplete metabolism may reduce the probability of by-product formation or future product degradation. In addition diverse mechanisms, for adaptation to changing environmental conditions, make cyanobacteria more biotechnologically useful.

The availability of the genome sequence and the relatively simple application methods mean that unicellular cyanobacteria, *Synechocystis* sp. PCC 6803 and *Synechococcus* sp. PCC 7942, are some of the most important cyanobacterial strains usually chosen as the model organisms for this phylum, whereas filamentous strains of the genera *Leptolyngbya*, *Nostoc*, *Nodularia* or *Arthrospira* are identified as a rich source of versatile secondary metabolites (fatty acids, saccharides, lipopeptide, alkaloids), the production of which is always related to the activity of oxidoreductases of constitutive and/or inducible nature and of different specificities.[16] Literature data suggest that these strains are a potential source of enzymatic activity, as is manifested as their ability to transform the structurally different compounds, *e.g.* steroids, and to degrade organophosphorus compounds such as pesticides (malathion or glyphosate).[17,18]

This article reports novel, effective adoption of phototrophic microorganisms as biocatalysts for the production of chiral β-hydroxyalkylphosphonates of aliphatic and aromatic structures.

Results and discussion

Structurally different substrates were tested: diethyl 2-oxopropylphosphonate (**1**), diethyl 2-oxo-2-phenylethylphosphonate (**2**), and diethyl 2-oxobutylphosphonate (**3**). A wide range of biocatalysts – unicellular photoautotrophic *Synechococcus bigranulatus*, heterocystous photoheterotrophic cyanobacterium *Nostoc cf-muscorum* and filamentous strains of *Nodularia sphaerocarpa*, *Arthrospira maxima, Leptolyngbya foveolarum* and *Geitlerinema* sp. were used for the very first time for asymmetric hydrogen transfer reactions with selected oxophosphonates. These particular whole-cell biocatalysts were chosen on the basis of current scientific data (Introduction).

Several efforts have been made to optimize the bioconversion conditions. Thus, the effect of cultivation medium, light source and light cycle (light : dark) on the effectiveness of the biotransformation process was examined.

Light is the energy source that drives photosynthesis and allows the conversion of nutrients into algal biomass. The availability and intensity of light are the major factors controlling the productivity of photosynthetic cultures. Maximum culture depth and cell density are the key variables which are evaluated to establish the efficient use of light. Light intensity, spectral characteristics and photoperiod are the components of an illumination regime.[19]

The effective reduction of exogenous ketones by cyanobacterial cells is dependent on the photosynthetic activity of the biocatalyst – cell viability.[20] Moreover, literature data indicate that the enzymes involved in such reduction are NAD(P)H-dependent and light is regarded as a crucial regulator of enzyme selectivity in photobiocatalytic reactions. The reduced cofactor, NAD(P)H, generated during photosynthesis, acts as the electron donor, which can be used for the reduction of oxo-xenobiotics to the corresponding chiral alcohols.[2,21,22]

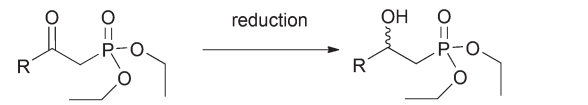
It is well-documented that the oxidoreductase from *Synechococcus* sp. PCC 7942, which is involved in the reduction of multi-halogenated and sterically demanding ketones (4-chloroacetoacetate; 2′,3′,4′,5′,6′-pentafluoroacetophenone) with excellent enantioselectivity (>99.8%, *S*), has been identified as 3-ketoacyl-(acyl–carrier–protein) reductase (EC 1.1.1.100) and this is effective inside the cells under an optimal light regime.[23] Homologous reductases have also been found in other cyanobacterial strains belonging to different taxonomic groups (*Nostocales*, *Oscillatoriales*, *Chroococcales*).[24] These are defined as crucial for the metabolism of many endogenous and xenobiotic compounds, given their variable specificities and activities.

Apart from light energy, nutrient supply is an equally important factor in culturing phototrophic cells. Both these are vital for cell growth.[25]

Among the tested cyanobacterial strains, the following were capable of chiral hydroxyphosphonate synthesis: *Arthrospira maxima*, *Nodularia sphaerocarpa*, *Leptolyngbya foveolarum* and *Nostoc cf-muscorum*. Target diethyl (*S*)-2-hydroxyphosphonates (**1** and **3**) were obtained *via* enantioselective bioreduction, within 7 days, under continuous or cyclic (16 h light : 8 h dark) illumination provided by different light sources: Power Glo or Sun Glo fluorescent lamps. The efficiency and enantioselectivity of the bioconversion for particular biocatalyst species are presented in Table 1. *Leptolyngbya foveolarum* exhibit the lowest catalytic activity – the degree of conversion was up to 5% (Power Glo fluorescent bulb); therefore, this cyanobacterial strain did not remain under consideration (data not shown). Different from other species of bacteria was *Nodularia sphaerocarpa* which was efficient towards aromatic compound **2** (Scheme 2 and Table 1), yielding a 99% conversion degree and 92% optical purity of the (*S*)-2-hydroxy-2-phenylethylphosphonate ($[\alpha]_D^{25}$ +17°, *c* 0.029 in MeOH). This is the first time that such results have been obtained. Such an extraordinary result was surprising and important, because previously reported efforts in those experiments had failed. Phosphonic substrates with carbonyl functionality, which is situated directly next to the aromatic ring (no matter what the location of the acidic group), remained unreacted or the bioconversion resulted in racemic mixtures of the corresponding chiral alcohols.[8,26] Previously applied baker's yeasts were active towards β-oxophosphonates with aromatic side groups, but this activity was limited to the moieties with carbonyl functionalities situated far from the phenyl-side group – located then on γ or δ carbon atoms (diethyl 2-oxo-3-phenylpropylphosphonate and diethyl 2-oxo-4-phenylbutylphosphonate).[8]

This directly proves that cyanobacteria represent a completely different enzymatic profile, in terms of the activity toward xenobiotic substrates having structures with steric hindrance. The experiments allowed us to observe other useful features of *N. sphaerocarpa* – independence from the illumination regime in the case of compound **2** (Table 1).

The importance of the light impact on the effectiveness of the bioreduction and on the biocatalytic properties of tested cyanobacteria is combined with the substrate structures. In terms of aliphatic substrates, previous efforts described in the literature enabled the identification of fungi-baker's yeasts,



compound **1** R= - CH_3
compound **2** R= - C_6H_5
compound **3** R= - CH_2CH_3

corresponding hydoxycompounds
1a, **2a**, **3a**

Scheme 2 Reduction of 2-oxoalkylphosphonates.

Table 1 Seven-day biotransformations under continuous or cyclic (16 h light : 8 h dark) illumination provided by different light sources: fluorescent lamps, Power Glo or Sun Glo

Illumination system	Power Glo lamp				Sun Glo lamp				
	Continuous		Cyclic (16 h light : 8 h dark)		Continuous		Cyclic (16 h light : 8 h dark)		
Substrate	Conv. [%]	ee [%]	Conv. [%]	ee [%]	Conv. [%]	ee [%]	Conv. [%]	ee [%]	Config.
	Nodularia sphaerocarpa								
1	**17 (±2)**	**93 (±2)**	15 (±1)	90 (±1)	22 (±3)	88 (±1)	12 (±1)	93 (±4)	*S*
2	**99 (±0.3)**	**93 (±1)**	**98 (±0.2)**	**95 (±0.2)**	**99 (±0.5)**	**92 (±0.8)**	98 (±0.2)	93 (±0.2)	*S*
3	**27(±3)**	**80 (±1)**	20 (±1)	75 (±0.6)	18 (±2)	84 (±3)	21 (±2)	77 (±4)	*S*
	Arthrospira maxima								
1	16 (±3)	≥99	7 (±1)	≥99	**20 (±2)**	**≥99**	11 (±1)	≥99	*S*
	Nostoc cf-muscorum								
2	18 (±5)	≥99	**26 (±4)**	**≥99**	15 (±2)	≥99	24 (±1)	≥99	*S*

Rhodotorula rubra and *Geotrichum candidum* strains as efficient biocatalysts toward aliphatic diethyl 2-oxopropylphosphonate (**1**) and allowed diethyl 2-hydroxypropylphosphonate (**1a**) with high optical purity (86–99%) and good chemical yield to be obtained.[27–29] As shown in Table 2, biotransformation catalyzed by baker's yeast or *Geotrichum candidum* strains resulted in enantiomer *S*, whereas when *Rhodotorula rubra* was employed, the product of the bioconversion was of an opposite absolute configuration, *R*. Also, another diethyl 2-oxobutylphosphonate (**3**) was bioconverted by *Rhodotorula gracilis* cells into (*R*)-alcohol with a high yield (90.5%) and an excellent optical purity of 99%. This result also proves that the fungal enzymatic system is different, because a butyl derivative is highly toxic to photobiocatalysts (see below). However, the process based on yeast activity required the addition of methyl isopropyl ketone into the biotransformation medium as a part of the cofactor regeneration system. This increases the cost of such solutions.[30] The most important disadvantage of previously reported oxophosphonate bioreduction is that every effort to scale up the invented methods failed until the application of the current photobiocatalysts. Representative experimental data with fungal catalysts are included in Table 2.

The efficiency of the bioreduction of aliphatic substrates **1** and **3** by *N. sphaerocarpa* ranged between 17 and 27% of conversion degree and the optical purity of the products was up to 93% under continuous illumination. The best yield for substrate **3** was obtained under continuous bluish white light: 27% with an ee of 80% of the *S*-isomer ($[\alpha]_D^{25}$ +6°, *c* 0.019 in MeOH) (Table 1), while under periodic illumination, the conversion of this compound declined to 20% and the optical purity of the product (ee) also decreased to 75% (Table 1) – these observations clearly indicate that in the case of aliphatic oxophosphonates light variations are crucial factors affecting the optical purity of the products (Table 1). Further, *Nostoc cf-muscorum* was an efficient biocatalyst towards aromatic substrate **2**, while a filamentous strain of *Arthrospira maxima* was efficient toward aliphatic diethyl 2-oxopropylphosphonate (**1**) and these two strains, regardless of illumination or environmental conditions in place (photoperiod and cultivation medium), remained active and allowed products with an optical purity of up to 99% of the *S*-isomer to be obtained.

Table 2 Efficiency of the biotransformation of diethyl 2-oxopropylphosphonate by fungi

Microorganisms	Yield [%]	ee [%]	Ref.
Baker's yeast	50	99 (*S*)	27 and 28
Rhodotorula rubra	40[a]	99 (*R*)	28
Rhodotorula glutinis	—	—	
Rhodotorula gracilis	—	—	
Geotrichum candidum IFO 4597	78	98 (*S*)[b]	29
Geotrichum candidum IFO 5767	70	86 (*S*)[b]	

— No reaction. [a] Addition of ethyl chloroacetate. [b] Correct absolute configuration of the bioconversion product.

It is noteworthy that the cell biomass and composition of extracellular metabolites also varied with different wavelengths of light. These attributes resulted from the cyanobacterial ability to adjust the contents and relative ratio of their pigments to the light quality.[31] The above metabolic flexibility was observed especially for *Nostoc cf-muscorum*. Continuous illumination may cause overheating or photoinhibition; therefore, the application of periodic illumination, in the case of this strain, contributed to conversion of substrate **2** with a similar yield under both Power Glo and Sun Glo illumination, with the respective conversion degrees of 26% and 24% (Table 1). This indirectly suggests that cell activity is also affected by the amount of energy offered per cycle, and not only by the duration of the photoperiod.

The remarkable results from a 7-day photo-bioreduction of the aromatic substrate, diethyl 2-oxo-2-phenylethylphosphonate (**2**) by *Nodularia sphaerocarpa* were the starting point for further process optimization. It was important to correlate the reaction progress with the duration of the bioconversion to improve the economy of the biotransformation simply by shortening it. As shown in Fig. 1, the maximum degree of conversion of substrate **2** was 95% and this requires only 5 days of biocatalysis, in the case of biomass exposed to continuous daylight illumination.

To achieve positive results also for aliphatic substrates (**1** and **3**), the biotransformation medium was engineered in order to activate enzymatic systems toward the studied xenobiotics. Microalgae have developed diverse mechanisms for sensing and adapting to the changes in their environment. Application of two different mineral media (BG-11 and Z8[32]) allows an evaluation of the significance of micronutrients on the catalytic activity of *Nodularia sphaerocarpa*. Additional supplementation of the media with trace elements is important for enzyme activity. This turned out to be ineffective, as is shown in Table 3. It was assumed that neither the light source nor the varied micronutrient supply was the critical factor in the case of aromatic oxophosphonate, compound **2**, but rather that these factors were essential for the conversion degree and optical purity for the aliphatic substrates (**1** and **3**). Thus, in the presence of biomass cultured in Z8 medium, a significant increase in conversion was obtained for the aliphatic compound diethyl 2-oxopropylphosphonate (**1**), up to 32% for bluish white light, but the optical purity of the product (ee) declined significantly to 81% (*N. sphaerocarpa*).

Another approach included the application of the Z8 medium without a source of nitrogen ($NaNO_3$, $Ca(NO_3)_2{\cdot}4H_2O$) for *N. cf-muscorum* cultivated under bluish white light, and this resulted in a moderate conversion degree of diethyl 2-oxo-2-phenylethylphosphonate (**2**) – 21% (data not shown), but with a high optical purity of the product (over 99%). Similar experiments were conducted for *Arthrospira maxima* strain, cultivated on *Spirulina* medium and BG-11 medium. This allowed the reduction of oxophosphonate **1** by biomass of *A. maxima* cultivated on BG-11 medium, with a degree of conversion of less than 10% (data not shown). This may result from the concentrations of carbonate and bicarbon-

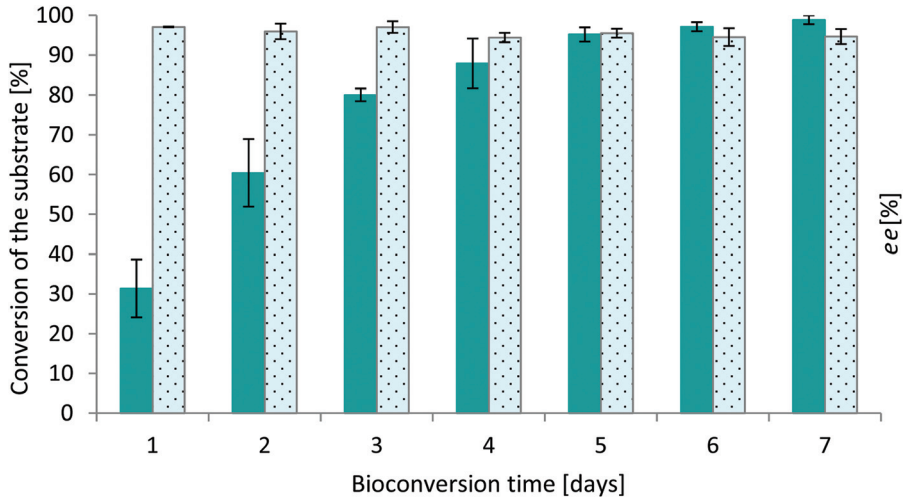


Fig. 1 Effect of biotransformation time on the reduction of compound **2** by *Nodularia sphaerocarpa*.

Table 3 Comparison of the effects of cultivation media on the catalytic activity of *Nodularia sphaerocarpa* (under continuous illumination)

	Power Glo lamp				Sun Glo lamp			
	BG-11		Z8		BG-11		Z8	
Substrate	Conv. [%]	ee [%]	Conv. [%]	ee [%]	Conv. [%]	ee [%]	Conv. [%]	ee [%]
1	17 (±2)	93 (±2)	32 (±1)	81 (±1)	22 (±3)	88 (±1)	24 (±5)	88 (±5)
2	99 (±0.3)	93 (±1)	99 (±0.5)	92 (±0.5)	99 (±0.5)	92 (±0.8)	98 (±2)	93 (±2)
3	27 (±3)	80 (±1)	29 (±4)	84 (±3)	18 (±2)	84 (±3)	22 (±2)	77 (±3)

ate ions in the tested media, which are much higher in *Spirulina* medium, than in BG-11 medium. High alkalinity is mandatory for the growth of *A. maxima* and bicarbonate is used to maintain high pH.[33]

The most important nutrients for autotrophic growth are C, N and P and their supply. The exogenous source of glucose strongly affects respiration and photosynthesis in cyanobacterial cells.[34] Experiments that allowed an evaluation of such effects on the catalytic activity of the tested strains were conducted under bluish white light (Power Glo lamp). Both glucose (0.5 g L^{-1}) and the tested substrates were added at the same time to the cultures of cyanobacteria. Glucose supplementation in combination with a periodic illumination allowed an improvement of only the conversion of diethyl 2-oxo-2-phenylethylphosphonate (**2**) by *N. cf-muscorum* to 24% (Table 4).

Results confirm that the relationship between nutrients and physical factors, such as illumination, and interactions between these factors affect the catalytic activity of photobiocatalysts. Application of different cultivation media as well as various sources of light energy did not contribute to a decrease in the results of the conversion of compound **2** by filamentous strains of *Nodularia sphaerocarpa* used as biocatalysts. These confirm the unique biocatalytic property of *N. sphaerocarpa* and its independence from the tested externals.

Table 4 Effect of glucose on the bio-reduction of 2-oxoalkylphosphonates

	A. maxima			*N. sphaerocarpa*			*N. cf-muscorum*		
Substrate	Conv. [%]	ee [%]	Config.	Conv. [%]	ee [%]	Config.	Conv. [%]	ee [%]	Config.
1	14 (±3)	≥99	*S*	23 (±3)	95 (±2)	*S*	—	—	—
2	—	—	—	98 (±1)	93 (±2)	*S*	24 (±5)	≥99	*S*
3	—	—	—	28 (±3)	81 (±6)	*S*	—	—	—

— No reaction.

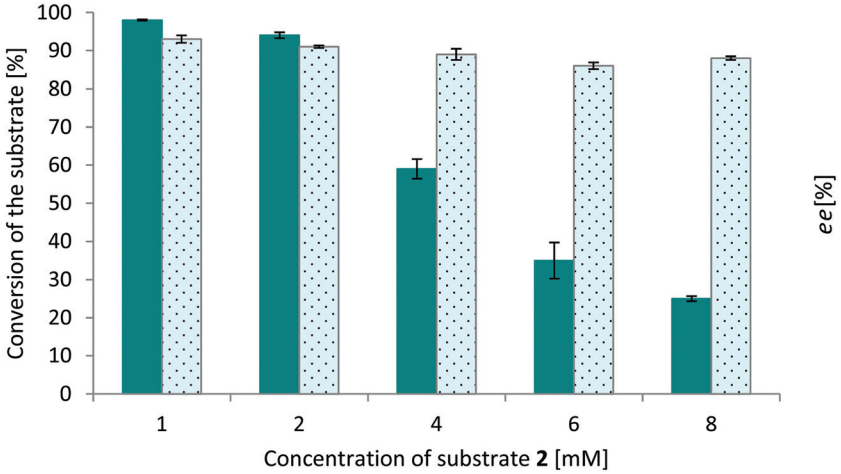


Fig. 2 Effect of substrate **2** concentration on the effectiveness of the *Nodularia sphaerocarpa*-catalyzed bioconversion process.

The limited literature data about cyanobacterial enzymes mean that the mechanisms of bioreduction are presented as possible, hence increasing the limited knowledge in this scientific field. Thus, the low catalytic activity of the tested strains toward aliphatic substrates (**1** and **3**) could be related to several factors, such as the inhibitory features of oxophosphonates and the effect of external factors on the cyanobacterial cell metabolism, such as nutrient availability (microelements, glucose *etc.*), and light sources. These are identified as activators or inhibitors of cellular metabolic pathways. The results of the discussed experiments also confirm this interaction. Oxophosphonates are structural analogs of oxocarboxylic acids, with acidic group replacement. Therefore, it is possible that their diethyl esters compete with the carboxylic substrates (*e.g.* physiological keto esters, keto acids *etc.*) for the active sites of the enzymes involved in their metabolism.[6] Further, it seems that oxidoreductases from *N. sphaerocarpa* exhibit substrate specificity toward aromatic β-ketoesters, because xenobiotic substrates act as inducers for the synthesis of particular enzymes. This could also explain the large tolerance of the discussed algal strain to the increasing concentration of bioconverted oxophosphonate. The lower efficiency of *N. sphaerocarpa* toward the aliphatic substrates is probably a consequence of the inhibitory interaction of such molecules with the native enzymes, possibly involved in their conversion.

Finally, the effectiveness of xenobiotic bioconversion is usually strongly affected by substrate concentrations. Also, in the case under discussion it was observed for compound **2** (Fig. 2, data for *Nodularia sphaerocarpa*) that the conversion degree decreases with an increase in the substrate concentration, but not enantioselectivity. The cell viability also remains almost at the same level, as was confirmed by the flow cytometry studies. It seems that oxophenylphosphonate performs an inhibitory function against selected enzymes responsible for its conversion and that it is involved in secondary cell metabolism, but not primary. This explains why such high xenobiotic concentrations do not affect the viability of the cells. This is important for the future prospects of phosphonate biocatalyzed synthesis, especially in the context of previous studies with fungal biocatalysts, which were effective only up to a concentration of 1 mM of the starting phosphonate derivatives.[8,27,28] An important observation from the flow cytometry studies is the high toxic effect of 1 mM of substrate **3** on the viability of *Nodularia sphaerocarpa* cells. Diethyl 2-oxobutylphosphonate (**3**) presents inhibitory features and a toxic effect on the cyanobacterial cells, which suggests that this xenobiotic interferes in some manner with the primary metabolism and it may be considered a possible anti-algal agent. This result indicates the potential application of aliphatic oxophosphonate-**3** as an algaecide for preventing the excessive growth of microalgae.

An advantage of the described photobiocatalytic system is its good resistance against increasing substrate concentration, which is manifested by a high substrate/biocatalyst ratio, 0.03, compared to other biocatalysts such as baker's yeast (0.006).[8,27] *Nodularia sphaerocarpa* seems to be the key for chiral phosphonic platform synthesis according to environmentally friendly green solutions.

Conclusion

This article presents the effective use of photoautotrophic microorganisms as biocatalysts for the production of chiral (*S*)-2-hydroxyalkylphosphonates. Different from other possible solutions, cyanobacteria represent a diverse and unusual catalytic ability to transform β-oxoalkylphosphonates. This manifests itself, among others, in the capacity to reduce substrates with aromatic side chains located directly next to carbonyl functionality. The application of the strain of *Nodularia sphaerocarpa*, in particular, resulted in diethyl (*S*)-2-hydroxy-2-phenylethylphosphonate, with a degree of conversion of 99% and an optical purity of 92%.

Morphologically different strains were able to reduce substrates to their corresponding alcohols of *S* configuration. The results were strongly dependent on the chemical structure of the substrates and steric hindrance of the functionalities located in the immediate vicinity of the carbonyl group, and they were slightly dependent on the external physical and chemical factors. Thus, another filamentous strain of *Arthrospira maxima* was a source of reductive activity only toward diethyl (*S*)-2-oxopropylphosphonate (**1**), whereas the application of *Nostoc cf-muscorum* as a biocatalyst allowed diethyl (*S*)-2-hydroxy-2-phenylethylphosphonate (**2a**) with a high enantiomeric excess (over 99%) to be obtained.

The very important conclusion which comes from this study is that cyanobacteria were able to survive under increasing substrate concentrations, which suggests the possibility of further technological applications.

Experimental

Chemicals

All chemicals were purchased from commercial suppliers: Sigma, Aldrich, Fluka, and Avantor Performance Materials, Poland, S.A.

Substrate synthesis

Substrates – diethyl 2-oxopropylphosphonate, diethyl 2-oxo-2-phenylethylphosphonate, and diethyl 2-oxobutylphosphonate were synthesized according to standard procedures described in the literature.[35]

Microorganisms

Arthrospira maxima (CCALA 27), *Geitlerinema* sp. (CCALA 138), *Leptolyngbya foveolarum* (CCALA 76), *Nodularia sphaerocarpa* (CCALA 114), *Nostoc cf-muscorum* (CCALA 129), *Synechococcus bigranulatus* (CCALA 187) were purchased from the Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences, Czech Republic.

Cultivation conditions

All species were cultivated in 250 mL Erlenmeyer flasks containing 100 mL of a suitable medium. *Geitlerinema* sp., *Leptolyngbya foveolarum*, *Nodularia sphaerocarpa* and *Synechococcus bigranulatus* were grown in BG-11 medium,[36] *Nostoc cf-muscorum* was grown in BG-11 medium without $NaNO_3$ and *Arthrospira maxima* – in *Spirulina* medium.[37] All of the species of cyanobacteria were cultivated under continuous illumination at 7–12 μmol photons per m^2 per s (Power Glo fluorescent bulb, 8W, Hagen) or at 5–9 μmol photons per m^2 per s (Sun Glo fluorescent bulb, 8W, Hagen) at 29 °C (±1) and under stationary conditions. A Power Glo fluorescent bulb illuminates with bluish white light, whereas a Sun Glo lamp simulates daylight intensity. The experiments were conducted under sterile conditions.

General procedure for β-oxoalkylphosphonate bioreduction – laboratory scale

After 21 days of pre-cultivation, 1 mM of substrate was added to the culture of cyanobacteria. The bioconversion was carried out for 7 days, at 29 °C (±1) under stationary conditions and under continuous or cyclic (16 h light : 8 h dark) illumination provided by different light sources, which allowed for the varying of the transmission spectra (Power Glo or Sun Glo fluorescent lamps). Experiments were completed by the removal of biomass by centrifugation (2800*g*, 25 min). Finally, supernatants were extracted twice with 50 mL of ethyl acetate. Subsequently, the collected organic layers were dried over *anhydrous* $MgSO_4$ and evaporated under reduced pressure, and oil residues were analyzed. The conversion degrees of the substrates and the optical purity of the products were evaluated. Each experiment was replicated in parallel at least five times.

The control experiments were carried out in culture medium without cyanobacteria cells or without the substrate being in the presence of the biocatalyst.

Preparative biotransformations

After 21 days of pre-cultivation, 2 mM (51 mg), 4 mM (102.5 mg), 6 mM (154 mg), 8 mM (205 mg) or 10 mM (256 mg) of substrate **2** was added to the cultures of *N. sphaerocarpa* (100 mL). The bioconversions were carried out for 7 days, at 29 °C (±1) under stationary conditions, and under continuous bluish white light (Power Glo). Experiments were completed by the removal of biomass by centrifugation (2800*g*, 15 min). Finally, supernatants were extracted twice with 50 mL of ethyl acetate. Subsequently, the organic layers once collected were dried over anhydrous $MgSO_4$ and evaporated under reduced pressure. The resulting oils were analyzed. The conversion degrees of the substrates and the optical purity of the products were evaluated. Experiments were performed in triplicate.

Cell viability assay

N. sphaerocarpa cell viability under the experimental conditions was determined by applying a combination of DNA-specific dyes using flow cytometry (Becton Dickinson FACSCalibur). SYTOX Green (Molecular Probes) is a fluorescent stain with a high affinity for nucleic acids that does not penetrate living cells, it passively diffuses into cells with compromised membranes where it preferentially binds to DNA. The fluorescence of SYTOX Green does not overlap chlorophyll autofluorescence. Therefore, SYTOX Green fluorescence and autofluorescence can be used simultaneously as markers for dead and live cells, respectively, in a viability assay.

SYTOX Green dye was prepared freshly by diluting the stock solution (5 mM) 500-fold with distilled water (0.2 μm prefiltered).

Staining procedure

One milliliter of biotransformation medium containing *N. sphaerocarpa* cells was centrifuged (1924*g*, 25 min, 20 °C),

biomass was resuspended in 10 mL of distilled water to a final concentration of cells, which was 4×10^6 per mL. To 0.990 mL of cell suspension, 10 μL of working solution of SYTOX Green (10 μM) was added, yielding a final dye concentration of 0.1 μM. After 10 min of incubation at room temperature in the dark, the experiment was completed by biomass separation *via* centrifugation (1924*g*, 25 min, 20 °C). Finally, the biomass was resuspended in 1 mL of distilled water. Cells were analyzed using a Becton Dickinson FACSCalibur Flow Cytometer equipped with a 488 nm laser. Fluorescence emission was collected using a 530/30 bandpass filter. Live cells were easily distinguished from the dead cell population.

Chemical reduction of substrates

Chemical reduction of substrates was performed according to the literature, using $NaBH_4$ as a reducing agent.[29]

Optical purity assignment

The mixtures of bioconversion products were analyzed by the ^{31}P NMR technique. Spectra were recorded on a Bruker Avance™ 600 instrument operating at 600 MHz. Measurements were obtained in $CDCl_3$ (99.5 at% D) at a temperature of 298 K.

The optical purity of the products was estimated using quinine as a chiral solvating agent, which allowed a shift difference of ^{31}P NMR signals coming from hydroxyphosphonates enantiomer-bioreduction products to be achieved.[38]

The degree of the conversion of the substrate was expressed as a percentage (%) and defined as:

$$\text{conversion} = \frac{\text{PP}}{\text{PP} + \text{PS}} \times 100\%,$$

where PP and PS express the areas under the signals observed on the ^{31}P NMR spectra, coming from the product and substrate of the bioconversion, respectively.

The optical purity of the products (ee) was also computed from the ^{31}P NMR spectrum following the formula:

$$\text{ee} = \frac{E1 - E2}{E1 + E2} \times 100\%,$$

where $E1$ and $E2$ express the values of the area under the signals coming from the major and minor enantiomers of the product, respectively.

Spectroscopic data

Diethyl 2-oxopropylphosphonate (1). ^{31}P NMR (600 MHz, $CDCl_3$) δ (ppm) 19.74; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 1.34 (t, 6 H, J = 7.1 Hz, P(O)(OCH_2CH_3)), 2.33 (s, 3 H, CH_3C(O)), 3.1 (d, 2 H, J = 22.9 CH_2P), 4.1–4.2 (m, 4 H, m, P(O)(OCH_2CH_3)).

Diethyl 2-hydroxypropylphosphonate (1a). ^{31}P NMR (600 MHz, $CDCl_3$) δ (ppm) 30.11; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 1.30 (dd, 3 H, J = 6.1, J = 2.2, CH_3C(OH)), 1.35 (t, 6 H, J = 7.1 Hz, P(O)(OCH_2CH_3)), 1.9–1.98 (m, 2 H, CH_2P), 3.55 (s, 1 H, C(O*H*)), 4.08–4.25 (m, 4 H, P(O)(OCH_2CH_3)).

Diethyl 2-oxo-2-phenylethylphosphonates (2). ^{31}P NMR (600 MHz, $CDCl_3$) δ (ppm) 20.58; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 1.29 (t, 6 H, J = 7.0 Hz, P(O)(OCH_2CH_3)), 3.65 (d, 2 H, J = 22.7, CH_2P), 4.15 (m, 4 H, P(O)(OCH_2CH_3)), 7.3–7.6 (m, 5 H, C_6H_5).

Diethyl 2-hydroxyl-2-phenylethylphosphonates (2a). ^{31}P NMR (600 MHz, $CDCl_3$) δ (ppm) 29.76; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 1.29 (t, 6 H, J = 7.1, P(O)(OCH_2CH_3)), 2.14–2.28 (m, 2 H, CH_2P), 4.05–4.20 (m, 4H P(O)(OCH_2CH_3)), 5.07–5.14 (m, 1H, C(O*H*)), 7.25–7.43 (m, 5H, C_6H_5).

Diethyl 2-oxobutylphosphonate (3). ^{31}P NMR (600 MHz, $CDCl_3$) δ (ppm) 20.08; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 1.04 (t, 3 H, J = 7.2 Hz, CH_3CH_2C(O)), 1.31 (t, 6 H, J = 7.0 Hz, P(O)-(OCH_2CH_3)), 2.65 (q, 2 H, J = 7.2 Hz, CH_3CH_2C(O)), 3.06 (d, 2 H, CH_2P, J = 22.8 Hz), 4.05–4.19 (m, 4 H, P(O)(OCH_2CH_3)).

Diethyl 2-hydroxybutylphosphonate (3a). ^{31}P NMR (600 MHz, $CDCl_3$) δ (ppm) 30.81; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm) 0.95 (t, 3 H, J = 7.2 Hz, CH_3CH_2C(O)), 1.33 (t, 6 H, J = 7.0 Hz, P(O)(OCH_2CH_3)), 1.49–1.62 (m, 2 H, CH_3CH_2CH(OH)), 1.82–2.00 (m, 2 H, CH_2P), 2.22 (s, 1 H, C*H*(OH)), 3.56 (s, 1 H, CH(O*H*)), 4.06–4.18 (m, 4 H, P(O)(OCH_2CH_3)).

Purification of diethyl 2-hydroxyphosphonates (1a, 2a, and 3a)

Diethyl 2-hydroxyphosphonates were purified by high performance liquid chromatography (C18-reversed phase silica gel, particle size 15–25 μm, pore size 100 Å) using a mixture of water–acetonitrile (10 : 2, v/v) as an eluent, and flow rate 3 mL min^{-1}.

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