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Reductive capabilities of different cyanobacterial strains towards acetophenone as a model substrate – Prospect of applications for chiral building blocks synthesis

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ABSTRACT

Bioreductive capabilities of four morphologically different strains of cyanobacteria have been assessed in this work. *Arthrospira maxima, Leptolyngbya foveolarum, Nodularia sphaerocarpa* and *Synechococcus bigranulatus* were applied as catalysts for the reduction of acetophenone to the corresponding chiral phenylethyl alcohol. The process was modified regarding substrate concentration, duration of pre-cultivation period, duration of biotransformation, light regime and glucose addition to the culture media. Obtained results clearly showed that cyanobacteria were active towards acetophenone what resulted in the substrate reduction to (*S*)-1-phenylethanol with high enantiomeric excess. The reaction efficiency increased with the biotransformation time, but the higher concentration of substrate limited the process yield. Also, all tested strains performed reaction with the highest efficacy under continuous light regime. The most active strains – *N. sphaerocarpa* and *S. bigranulatus* carried out the conversion of 1 mM acetophenone with high efficiency of respectively 97.6% and 96.2% after 13 days of biotransformation. *A. maxima* reached 45.8% of conversion after 13 days of biotransformation whereas *L. foveolarum* did not exceed 20%. The enantiomeric excesses were respectively 98.8%- *A. maxima*, 91.7%- *L. foveolarum*, 72.6%- *S. bigranulatus* and *N. sphaerocarpa* 16.2%.

1. Introduction

Cyanobacteria are widely distributed group of photosynthetic microorganisms, which are present in many terrestrial and aquatic environments. They are applied in many fields of biotechnology such as pharmaceutical, cosmetic and food industry since they are a prolific source of biologically active compounds with diverse chemical structures. Among these chemicals are anticancer, antibacterial, antifungal, antiviral or algicidal factors [19,1,21]. Also, cyanobacteria are useful tools in the biofuels production thanks to their supreme photosynthetic capabilities [18]. Recently they have been identified as a promising biocatalyst source [25]. However their full potential in this area remains undiscovered [5].

Cyanobacteria are superior to other industrialized biocatalysts like fungi or bacteria due to their photosynthetic abilities. First, they do not require carbon source in the cultivation media, since light is their main energy source for reductive CO_2 assimilation. Secondly, their metabolism is very flexible and can be a source of high value products. Additionally, blue-green algae are very adaptable and can be easily genetically modified, which further enlarges their biotechnological potential as well as commercial applications possibilities [13].

Biocatalysis is a powerful tool in chemical synthesis because it offers easier and environmentally friendly approach to synthesize optically pure chemicals of high added value. Production of chiral compounds by bacterial and fungal biocatalysts have been applied in industry due to numerous advantages over classical chemical synthesis – it provides cost-effective method of obtaining desired product, simultaneously remaining non-toxic and highly effective and selective.

Application of cyanobacteria in biocatalysis focused mainly on the asymmetric reduction of aldehydes and ketones to corresponding chiral alcohols [14,17,12,8]. However, they have also been shown to hydroxylate 4-androstenedione [2] or degrade side chain of hydrocortisone [24]. Additionally, cyanobacteria have been applied in the biotransformations of monoterpene compounds [4]. Recently we discovered the potential of cyanobacteria to enantioselective reduction of diethyl esters of oxophosphonic acids using free [10] and immobilized [11] cells.

Application of cyanobacteria in biocatalysis is limited to small

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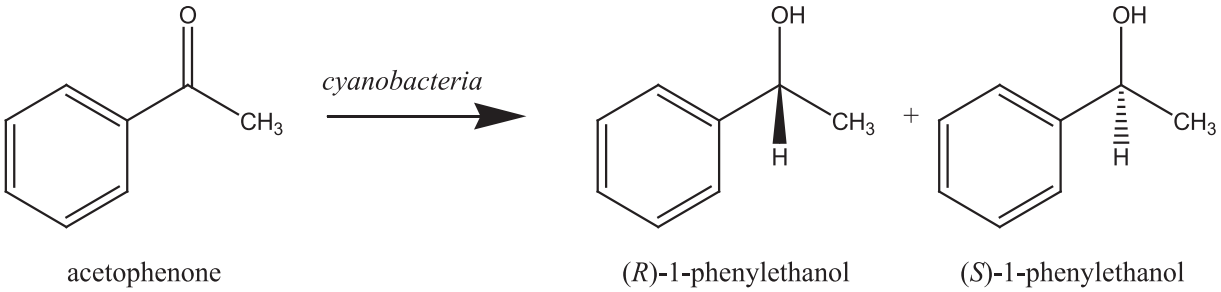

Fig. 1. Reduction of acetophenone to (*R*)-1-phenylethanol and (*S*)-1-phenylethanol.

number of strains, although widespread occurrence in environment and diverse metabolic pathways. Therefore, this field of science is not fully explored and developing it seems to be important. Four, morphologically different strains of cyanobacteria, were tested for their biocatalytic capability of acetophenone reduction (Fig. 1) to evaluate the bioreductive potential of this photoautotrophic microorganisms. Acetophenone is a convenient model – easily available and very well described chemical compound. The optically active products of its reduction are used in large quantities in industry, being an interesting building elements that can be used in chemical synthesis or as a benchmarks for analysis. Additional advantage of work with this compound is the availability of simple analytical methods of detection and quantification both for substrate as well as for products.

2. Materials and methods

2.1. Chemicals

Acetophenone, (*R*)- and (*S*)-1-phenylethanol were purchased from Sigma Aldrich. Media components were purchased from Sigma Aldrich, Chempur, Avantor Performance Materials. Glucose and ethyl acetate were purchased from Avantor Performance Materials.

2.2. Microorganisms

Cyanobacterial strains: *Arthrospira maxima* (CCALA 27), *Leptolyngbya foveolarum* (CCALA 76), *Nodularia sphaerocarpa* (CCALA 114) and *Synechococcus bigranulatus* (CCALA 187) were obtained from the Culture Collection of Autotrophic Organisms (CCALA) (Institute of Botany, Academy of Sciences of the Czech Republic).

2.3. Cultivation

All microorganisms were cultivated in 250-ml conical flasks containing 100 mL of BG-11 medium [20] except from *Arthrospira maxima*, which was grown on Spirulina medium [3]. Cultures were grown under sterile, stationary conditions, at 25 °C, under continuous or periodic illumination at 7–12 μm photons per m^3, provided by fluorescent SunGlo 8 W (Hagen) lamp.

Optical density (OD_{750}) of growing cultures were monitored by absorbance measurements, performed on Perkin Elmer Lambda XLS spectrophotometer at wavelength 750 nm compared to control, which was non-inoculated cultivation medium.

2.4. Biotransformation procedure

After pre-cultivation period, appropriate substrate was added to the cyanobacterial culture, using aseptic techniques. Biotransformation procedure was performed under varying conditions regarding final concentration of substrate (1–4 mM), duration of the biotransformation (1–13 days), optical density of pre-culture (OD_{750} 0.5 – 4) and illumination system (continuous or periodic 16 h light: 8 h dark). In case of periodic illumination system, the substrate – acetophenone was added to the pre-culture alongside with the sterile glucose solution to final concentration of 0.5 g/mL. Control experiments were carried out on pure cultivation media and the growing cultures without addition of substrate.

2.5. Products analysis

Experiments were terminated by centrifugation of the biomass (2300*g*, 20 min, 17 °C) and extraction of supernatant with ethyl acetate (2 × 50 mL). The organic layer was dried over anhydrous $MgSO_4$ for overnight, and then solvent was evaporated under reduced pressure. Product of biotransformation was analyzed by gas chromatography (Agilent Technologies Co. Ltd. 7890A), equipped with Chiralsil DEX CB column 25 m × 0.25 mm and flame ionization detector with nitrogen as make-up gas and helium as carrier gas. Spilt ratio was 35/1. Column was heated from 110 °C to 120 °C at the rate of 2.5 °C/min and subsequently to 200 °C at the rate of 10 °C/min. Inlet temperature – 250 °C. Retention times: acetophenone – 4.11 min; (*R*)-1-phenylethanol – 6.04 min; (*S*)-1-phenylethanol – 6.28 min.

Enantiomeric excess (*e.e.*) was calculated based on the GC spectra with formula:

$$e.\,e. = \frac{E_1 - E_2}{E_1 + E_2}$$

where E_1 and E_2 were respective percentage fractions of enantiomers in the mixture.

3. Results and discussion

Biotransformation procedure was performed using four different strains of cyanobacteria: unicellular *Sunechocccus bigranulatus,* filamentous non-heterocystous *Arthrospira maxima, Leptolyngbya foveolarum,* as well as heterocystous *Nodularia sphaerocarpa*. The selection of strains is caused by previously performed screening tests, which indicated the strains with the highest activity. These strains differ regarding morphology and physiology what could be a contributing reason of variation in obtained results - differences in process efficiency and enantiomerical excesses of the products. The basic parameters of process were modified, and the influence of such changes were monitored to set the most effective protocols of bioconversion, starting from the examination of the pre-culture cultivation, before adding the acetophenone. Best results were obtained after addition the substrate into the microbial culture being in optimal growth phase, during which metabolism is most robust. Thus, the cells are relevant to perform the process with the highest yield. Table 1 shows, that the differences between the activities of different pre-cultures are not so big, but for the next experiments pre-culture of OD_{750}: 1.8. was chosen as the best average result for all strains, because the results of bioconversion with this pre-culture were repeatable. Also, the older pre-cultures probably contain more not viable and as a consequence non-active cells. Additionally, cultures of high density are self-shadowing, resulting with less light amount reaching live microorganisms, so slowing dawn the metabolic rate. The duration of the culturing ranges between 15 and

Table 1
Bioconversion of acetophenone carried out with different cyanobacteria pre-cultures.

Microorganism	OD_{750}	Conversion [%]	*e.e.* [%]	Configuration
Synechococcus bigranulatus	1	6.3 (± 0.6)	72.2 (± 7.6)	*S*
	1.8	7.1 (± 0.6)	66.9 (± 8.8)	*S*
	2.5	7.5 (± 0.1)	66.2 (± 4.0)	*S*
Arthrospira maxima	1	20.3 (± 5.9)	89.1 (± 8.3)	*S*
	1.8	23.7 (± 7.9)	87.2 (± 9.7)	*S*
	2.5	24.7 (± 3.6)	87.7 (± 2.6)	*S*
Nodularia sphaerocarpa	1	89.4 (± 3.2)	45.3 (± 5.7)	*S*
	1.8	85.7 (± 10.2)	45.3 (± 7.1)	*S*
	2.5	84.5 (± 5.4)	36.9 (± 4.3)	*S*
Leptolyngbya foveolarum	1	27.8 (± 10.5)	95.7 (± 0.3)	*S*
	1.8	31.3 (± 16.4)	95.0 (± 0.4)	*S*
	2.5	25.0 (± 4.8)	92.7 (± 3.3)	*S*

25 days, but the point of the substrate addition was set every time regarding the OD value.

The elaboration of the optimal biotransformation conditions also included the adjusting concentration of substrate – acetophenone, which varied in the range of 1–4 mM (Fig. 2) in process carried out for seven days, employing the pre-culture (OD_{750}: 1.8). Results indicate that the increase substrate concentration had a negative impact on the process efficiency and in most cases, on enantioselectivity of the reaction. Only in case of one biocatalyst – *N. sphaerocarpa*, the enantiomeric excess increased alongside with the increased concentration of substrate, but, this was the catalyst of the poorest selectivity and even the best selectivity achieved by this strain was worse than in case of all

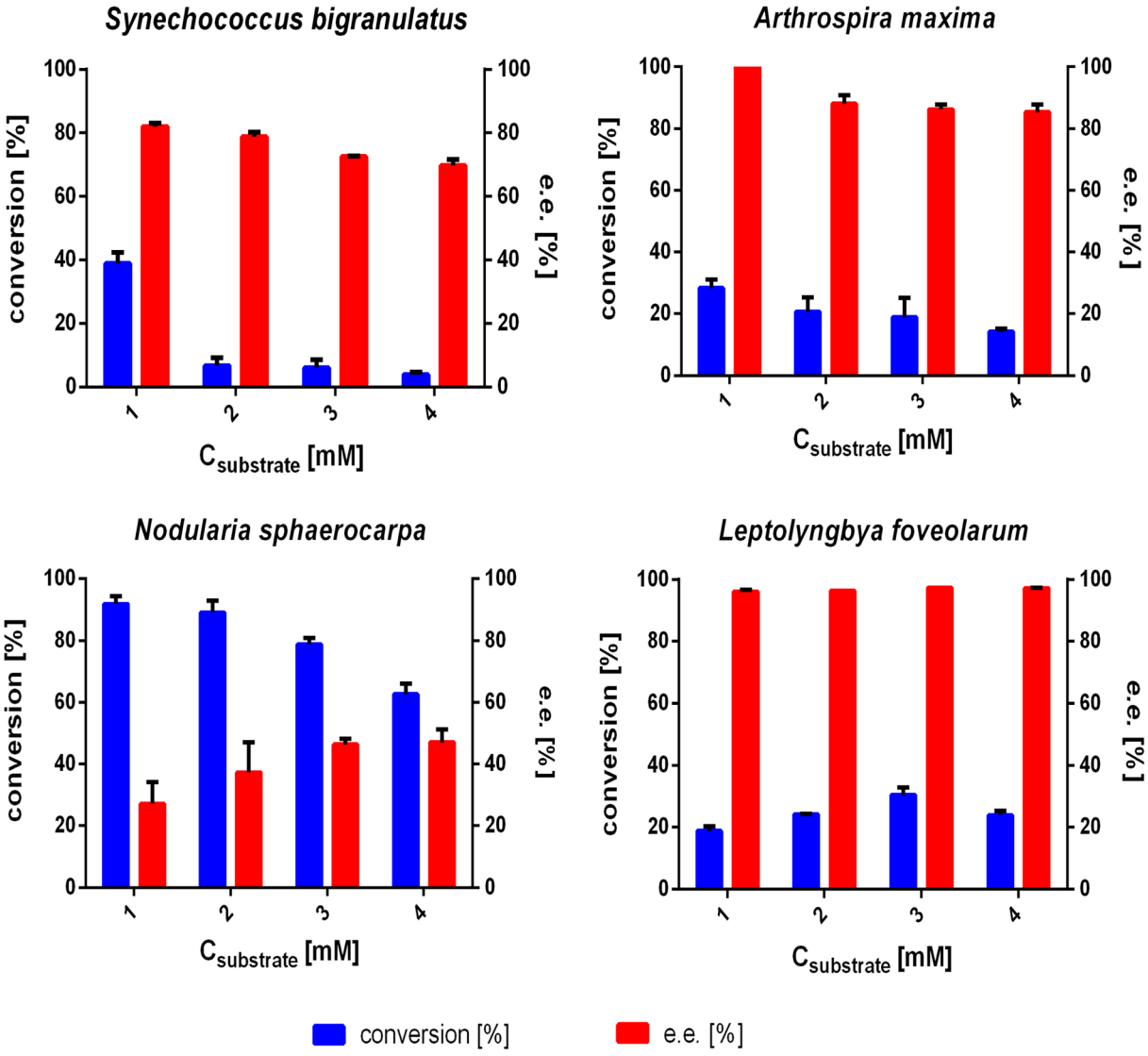

Fig. 2. Influence of substrate concentration on efficiency and enantioselectivity (*e.e.*) of acetophenone 7- days reduction.

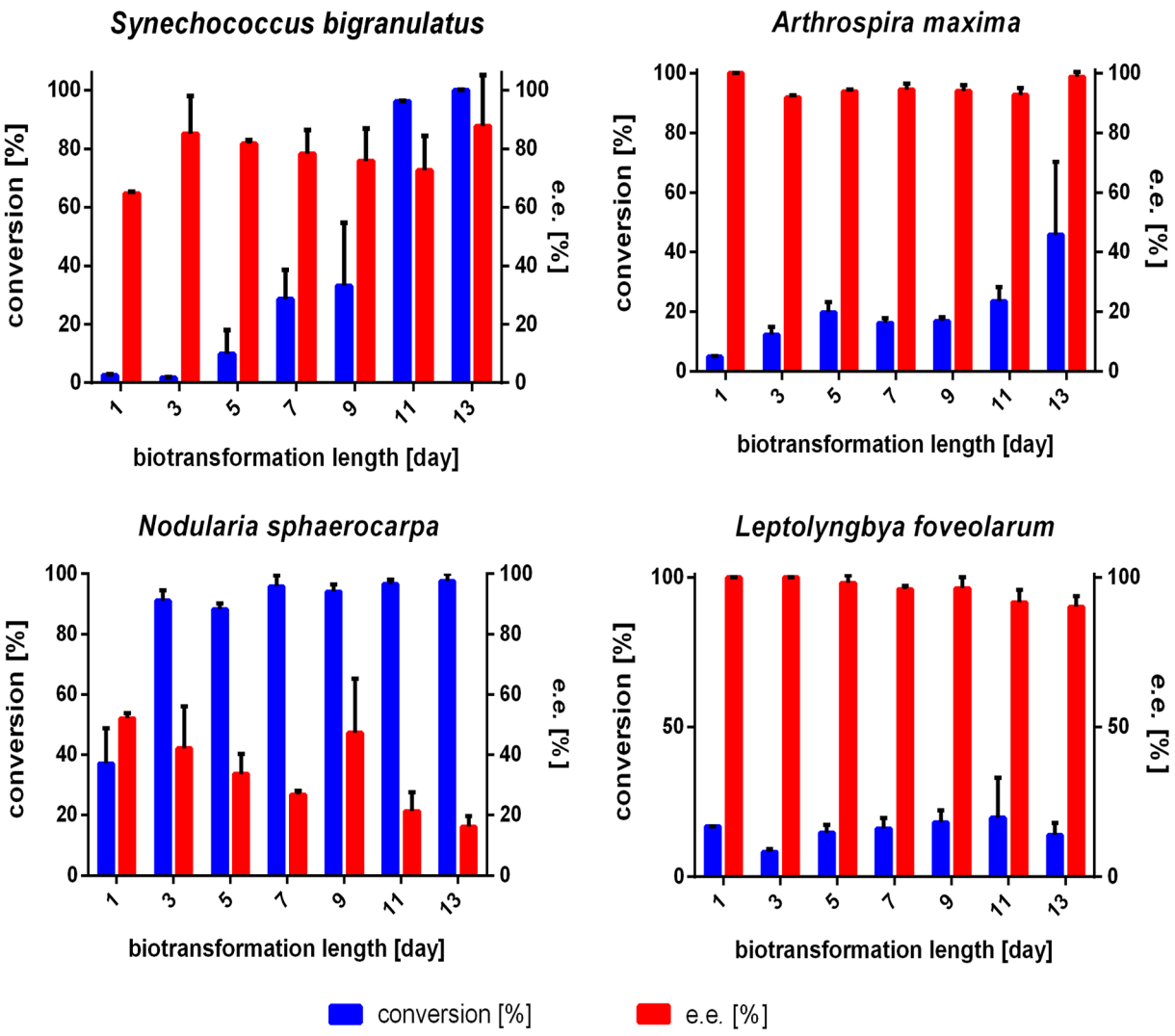

Fig. 3. Influence of duration time of biotransformation on its efficiency and enantioselectivity.

other strains. This phenomenon can be ascribed to the toxicity of acetophenone towards viable cells, including cyanobacterial ones.

Described above preliminary results achieved after 7- days of bioconversion, indicate that by monitoring the progress of bioreduction, the optimal duration of the process can be selected. Therefore, the particular bioconversions were carried out in the time range varying between 1 and 13 days, adding the substrate (1 Mm, after the pre-culture – 21 days under continuous illumination, until OD_{750} 1.8) preculture of Under the same conditions, except the bioconversion duration, the next results, were as follows: In case of *S. bigranulatus* and *A. maxima*, longer bioconversion process increased the product yield (Fig. 3) while efficiency of *N. sphaerocarpa* bioconversion exceeded 80% after the three day of biotransformation, *L. foveolarum* allowed to achieve conversion of around 20% just after one day of growth at the presence of acetophenone. Surprisingly, such dependency was not noticed in the terms of enantioselectivity. For *A. maxima* and *L. foveolarum*, enantiomeric excess was close to 100% after 1 day of bioconversion and the value remained quite table with time extending. In case of *S. bigranulatus,* after first day of bioconversion conversion degree was up to only 2% with 65% of *e.e.* While the efficacy of the process increased gradually within 11 days, *e.e.* reached 85% after three days and further improvement was not observed. Surprisingly, in case of *N. sphaerocarpa*, conversion reached 90% after third day but with poor *e.e.* (about 40%), which started decreasing with time extending. This results can be a consequence of increasing concentration of phenylethyl alcohol, which is known for its toxic impact of some organisms [6].

One of the most important factors determining the efficiency of growth and therefore biocatalytic activity of phototrophic organisms is light regime. The impact of light regime on the biotransformation efficiency and enantioselectivity of cyanobacteria towards ketones has been already described in the literature and identified as an meaningful parameter [9]. In this study, pre-cultured cyanobacteria (21 days, until OD_{750} 1.8, light regime 16:8 dark) were applied for the conversion of 1 Mm of the substrate under different light conditions: continuous and periodic ones (16 h light: 8 h dark – mimic the natural conditions).. Thus, continuous illumination may lead to overheating of the culture and as a consequence, photoinhibition can be occurred. In the experiment, previously tested fluorescent lamps were applied (not harmful to the cells) [10]. Obtained results show, that under continuous light regime cyanobacterial strains performed the bioconversion process with higher or the same efficiency as under periodic illumination. The most significant difference can be noted for unicellular strain *Synechococcus bigranulatus* – conversion degree was nearly four times higher under continuous illumination. In case of *Arthrospira maxima*, periodic illumination regime decreased the reaction efficacy twofold and the *e.e.* was poor up to 8.5% (Table 2). Two remaining strains did not behave differently under particular illumination regime, either in case of reaction yields nor the enantioselectivity.

Illumination conditions for photobiocatalysts are crucial ones not

Table 2
Effect of continuous light regime and glucose addition on the reduction of acetophenone.

Microorganism	Illumination regime	Conversion [%]	*e.e.* [%]	Configuration
Synechococcus bigranulatus	Continuous	38.9 (± 3.5)	82.0 (± 1.1)	*S*
	Periodic	9.5 (± 1.0)	88.3 (± 10.2)	*S*
	Periodic + glucose	6.4 (± 0.3)	61.1 (± 3.7)	*S*
Arthrospira maxima	Continuous	28.4 (± 2.8)	100.0 (± 0.0)	*S*
	Periodic	12.9 (± 1.6)	91.5 (± 0.1)	*S*
	Periodic + glucose	16.6 (± 8.0)	92.9 (± 3.2)	*S*
Nodularia sphaerocarpa	Continuous	91.9 (± 2.5)	27.1 (± 7.1)	*S*
	Periodic	89.6 (± 0.7)	31.0 (± 8.6)	*S*
	Periodic + glucose	88.4 (± 2.6)	27.9 (± 2.3)	*S*
Leptolyngbya foveolarum	Continuous	18.8 (± 1.5)	96.0 (± 0.7)	*S*
	Periodic	18.5 (± 8.4)	98.3 (± 2.4)	*S*
	Periodic + glucose	22.4 (± 7.4)	78.5 (± 2.1)	*S*

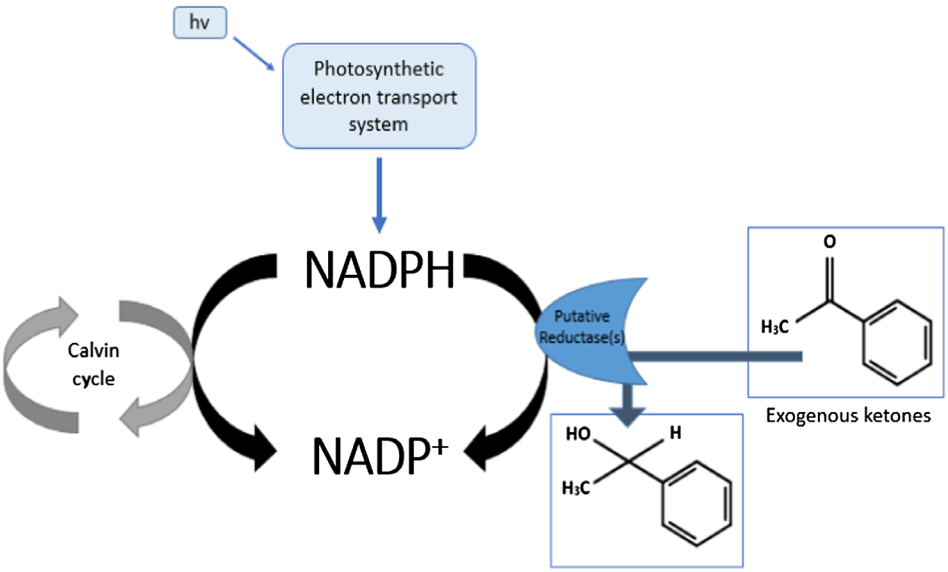

Fig. 4. Reduction of exogenous ketones using NADPH [22] as a part of electron borrowing system in photosynthetic cells.

only as an energy source but also due to the interactions between light reactions and redox balance (e.g., $NADP^+$/NADPH) inside the cells. Cyanobacterial enzymes, which are responsible for the anabolic CO_2 reduction, depend on the presence of reduced form of cofactor – nicotinamide adenine dinucleotide phosphate (NADPH). Light phase of photosynthesis is a source of NADPH, which is also utilized for reduction of exogenous ketones and production of appropriate alcohols, what was noticed in previous works [15,16,22] (see Fig. 4). Thus in discussed case of acetophenone reduction, light phase of photosynthesis seemed to be crucial, considering the substrate as a part of hydrogen flow in cofactor regeneration systems, especially that cyanobacterial, photosynthetic, enzymatic system is used to work with exogenous carbon sources.

Another important factor is the presence of glucose in the cultivation media. It influences photosynthesis processes by affecting the growth of cyanobacteria, especially in case of heterotrophic or mixotrophic strains [7]. The meaning of glucose for the bioconversion of acetophenone was therefore investigated. Sterile glucose solution was added alongside with the acetophenone to cyanobacterial pre-cultures growing under cyclic light regime. The final glucose concentration was 0.5 g/L. Based on the obtained results (Table 2), it can be concluded, that the efficiency of studied bioreduction is not sensitive to the presence of glucose (in the frame of using solutions) and the activity of photobiocatalysts remained more than less stable [23]. It is understandable because the glucose impact is further more spectacular in case of heterotrophic organisms, they, in many cases depend on the organic carbon source and the manipulations of its concentration can switched on the alternative (e.g., secondary) metabolic pathways. Presented study proved that in case of light-dependent organisms applied for bioconversion purposes, the presence of glucose is not important.

4. Conclusions

A comparative research on the reductive abilities of different photobiocatalytic systems towards model compound (acetophenone) was performed. According to obtained results, cyanobacterial enzymatic systems are active towards applied compound, however, the efficiency and enantioselectivity of processes differ depending on the growth rate of biocatalyst, substrate concentration, and light regime. This reductive activity is in a correlation with the natural process of hydrogen borrowing cycle during the cofactors regeneration, which is crucial for photosynthesis and also other pathways in viable cell. Applied ketone can be considered as an exogenous electron and proton acceptor until the concentration of phenylethyl alcohol remain non-toxic to the cells. Finally, our knowledge about the correlation between the external conditions impacting the viability of the cyanobacterial cells and their photobiocatalytic activity towards different substrates is still very narrow. That is why this work is an important contribution to this field of biotechnology.

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