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EFFECT OF THE ADDITIVE OF PYRROLOQUINOLINE QUINONE ON WASTE GLYCEROL BIOCONVERSION TO DIHYDROXYACETONE

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Summary. The aim of this study was to determine the effect of selected concentrations (1, 3, 10 or 15 μM) of pyrroloquinoline quinone (PQQ) on waste glycerol bioconversion to dihydroxyacetone (DHA). The reaction was catalyzed by an immobilized (in sodium alginate) cell extract obtained from *Gluconobacter oxydans*. Concentrations of glycerol and DHA were determined with the gas chromatography method in time 0, 24, 48 and 72 h. In all analyzed variants of the study, the highest consumption of glycerol was observed after 24 h of the process. Immobilized cell extract had the highest enzymatic activity during the first 24 h of bioconversion. Further course of bioconversion had no significant effect on changes in glycerol concentration. Concentration of DHA in all analyzed variants was 9.3 $g \cdot L^{-1}$ (after 24 h) and did not change significantly till the end of the process. Immobilized cell extract showed enzymatic activity towards glycerol in both the first and second cycle of bioconversion. No significant influence of applied doses of PQQ on the bioconversion of waste glycerol to DHA have been observed.

Key words: waste glycerol, dihydroxyacetone, PQQ, glycerol dehydrogenase

INTRODUCTION

Glycerol dehydrogenase (GlyDH, EC 1.1.99.22) catalyzes glycerol oxidation to dihydroxyacetone (DHA) i.a. in cells of acetic acid bacteria *Gluconobacter oxydans* [Van Lare and Claus 2007, Hu et al. 2017]. GlyDH isolated from *Gluconobacter* cells is the

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most active at temperatures of 23–25°C and at pH values of pH 7.0–8.0, and the most stable at temperatures of 33–37°C and pH values of 8.5–9.0 [Lapenaite et al. 2005]. Glycerol dehydrogenase has one prosthetic group – pyrroloquinoline quinone (PQQ) – and has no heme subunit, while ubiquinone is its electron acceptor [Adachi and Yakushi 2016]. Glycerol dehydrogenase is constituted by two subunits – peripheral SldA and transmembrane SldB [Matsushita et al. 2003].

Pyrroloquinoline quinone is soluble in water, aromatic orthoquinone from the third class of redox cofactors [Wei et al. 2016, Ikemoto et al. 2017]. It is composed of glutamate and tyrosine, which are most probably provided by PqqA protein [Hölscher and Goörisch 2006]. The coenzyme PQQ synthesis protein PqqB is engaged in PQQ transport to the periplasmic space wherein dehydrogenases are accumulated [Metlitzky et al. 2012]. The coenzyme PQQ synthesis protein PqqC catalyzes the last stage of PQQ synthesis [Magnusson et al. 2004], whereas the coenzyme PQQ synthesis protein PqqD participates in coding a 29-amino acid peptide claimed to be a precursor of PQQ biosynthesis [Wei et al. 2016].

Bacteria from the species *G. oxydans* are 0.6 to 0.8 µm long Gram-negative mesophilic bacilli [Raspor and Goranovic 2008]. Their optimal growth temperatures range from 25 to 30°C and optimal pH values from 5.5 to 6.0 [Gupta et al. 2001]. *Gluconobacter oxydans* strains are used i.a. for microbiological synthesis of DHA which is a main active compound in self-tanning cosmetics and also a semi-product for the synthesis of many organic chemicals and surface-active agents [De Vero et al. 2010, Antolak and Kręgiel 2015].

Waste glycerol formed during biodiesel production is a compound, which should be valorized to prevent its excess accumulation in the ecosystem. Its content in the generated waste ranges from 70 to 98% [Yang et al. 2012, Tan et al. 2013], whereas the remaining part of the waste includes contaminants such as methanol, methyl esters of fatty acids and salts left after the transesterification process [Ayoub and Abdullah 2012, Leoneti et al. 2012].

MATERIAL AND METHODS

This study was conducted with *G. oxydans* ATCC 621 strain, which was stored, multiplied and GlyDH activated according to procedures described by Stasiak-Różańska et al. [2017]. The medium used for bioconversion contained: waste glycerol 30 $g \cdot L^{-1}$ (Bio-Agra Oil, Tychy), Ca^{2+} 0.16 ML^{-1}, Mg^{2+} 0.16 ML^{-1}, PQQ 0.02 ML^{-1} and 10 mM Tris-HCl buffer with pH 7.5 (according to the modified method of Lapenaite et al. [2005]). Cell extract (CE) was prepared according to Stasiak-Różańska et al. [2017], with the following modification: water and physiological saline were replaced with 10 mM Tris-HCL buffer. Cell extract was suspended in 50 cm^3 of a preincubation solution containing: 10 mM Tris-HCl buffer (pH 9.0), 8 mM Ca^{2+} ions, 8 mM Mg^{2+} ions and selected concentration of PQQ (1, 3, 10 or 15 µM). The control (reference) medium did not contain PQQ. Cell extract was preincubated at 23°C for 1.5 h. Afterwards, it was immobilized in sodium alginate according to Stasiak-Różańska et al. [2017]. Bioconversion was carried out at 23°C with shaking (200 rpm) for 72 h. Samples were collected every 24 h to

determine glycerol and DHA concentrations. The bioconversion process was conducted in three independent series. Concentrations of glycerol and DHA were determined using a gas chromatograph (Thermo SCIENTIFIC Trace 1300) with a flame-ionization detector and a ZB-WAX plus column.

Obtained results were analyzed statistically (Statistica 12). Two-way analysis of variance was conducted. Tukey test (at a significance level of $\alpha = 0.05$) was used to compare the significance of differences between mean values.

RESULTS AND DISCUSSION

The aim of this study was to evaluate the effect of PQQ concentrations during preincubation of CE on a waste glycerol bioconversion into DHA. The reaction was conducted with using an immobilized CE with GlyDH activity. It was hypothesized that preincubation of CE in PQQ solutions may facilitate the process of enzyme reconstruction and may have a positive effect upon the course of waste glycerol bioconversion to DHA. The effect of PQQ concentration on changes in glycerol concentration during the first cycle of bioconversion was presented in Figure 1.

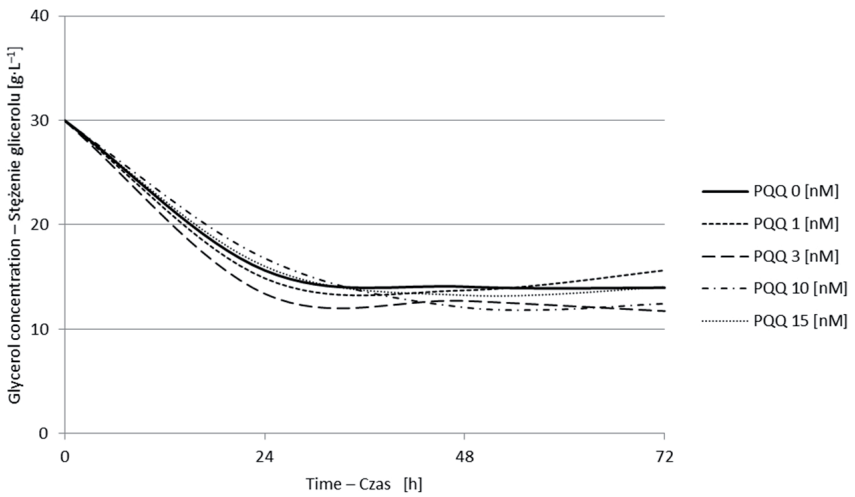

Fig. 1. Time profiles of glycerol consumption during the first cycle of bioconversion with the use of a cell extract preincubated in solutions with various concentrations of PQQ

Rys. 1. Zmiany stężeń glicerolu podczas pierwszego cyklu biokonwersji z zastosowaniem preparatu komórkowego, inkubowanego w roztworach o zróżnicowanym dodatku PQQ

Glycerol concentration in the control sample (0 μM PQQ) reached 15.6 ±0.16 $g \cdot L^{-1}$ after 24 h of the reaction. The lowest value of substrat concentration (13.39 ±0.009 $g \cdot L^{-1}$) was determined in the culture media with CE preincubated in a solution with 3 μM PQQ. In the same time, a slightly higher concentration of the substrate (14.85 ±0.009 $g \cdot L^{-1}$) was assayed in the media containing immobilized CE that had earlier been preincubated

in a 1 μM PQQ solution (Fig. 1). The lowest consumption of glycerol was noted in the media with CE preincubated in PQQ solutions with concentrations of 10 and 15 μM (16.8 ±0.11 and 16.0 ±0.06 $g \cdot L^{-1}$, respectively) – Figure 1. On the next day of the process (48 h), glycerol concentration decreased in all experimental variants. The lowest substrate consumption (compared to assayed after 24 h) was observed in the medium containing CE preincubated in a 10 μM PQQ solution and reached ca. 4.7 $g \cdot L^{-1}$ (a decrease from 16.8 ±0.11 after 24 h to 12.08 ±0.03 after 48 h). In the medium with CE preincubated in the solution with the highest applied PQQ concentration (15 μM), the difference in glycerol concentrations determined after 24 and 48 h of the process accounted for 2.79 $g \cdot L^{-1}$ (Fig. 1). In the same time, substrate concentration in the control medium (0 μM PQQ) decreased by 1.56 $g \cdot L^{-1}$. Compared to the first day of the process, a decrease was also observed in glycerol concentration in the other experimental variants (1 and 3 μM PQQ), however it did not exceed 1.1 $g \cdot L^{-1}$ (Fig. 1). Extension of the bioconversion process to 72 h caused no significant changes in glycerol concentration in the control medium (it decreased by ca. 0.1 $g \cdot L^{-1}$ compared to the concentration determined after 48 h). In turn, glycerol concentration decrease by ca. 1 $g \cdot L^{-1}$ (from 12.7 ±0.13 after 48 h to 11.72 ±0.13 after 72 h) was noted in the culture medium with CE preincubated in a 3 μM PQQ solution, whereas no significant changes were observed in substrate concentration in the remaining experimental variants (Fig. 1).

Irrespective of PQQ concentration in the preincubation solutions, the mean concentration of DHA determined in all culture media after 24 h of the bioconversion process reached 9.3 ±0.02 $g \cdot L^{-1}$ and did not change till the end of the process. Differences in DHA concentrations observed in particular variants were statistically insignificant.

Once the first cycle of bioconversion had been completed, the immobilized CE was rinsed with a TrisHCl buffer (pH 9.0) and put into fresh solutions of waste glycerol to start the second cycle of the process. The effect of PQQ concentration on changes in glycerol concentration during the second cycle of bioconversion was presented in Figure 2.

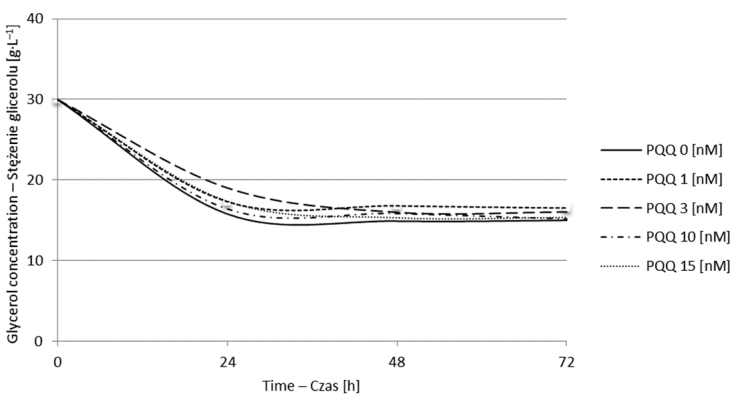

Fig. 2. Time profiles of glycerol consumption during the second cycle of bioconversion with the use of a cell extract preincubated in solutions with various concentrations of PQQ

Rys. 2. Zmiany stężeń glicerolu podczas drugiego cyklu biokonwersji z zastosowaniem preparatu komórkowego, inkubowanego w roztworach o zróżnicowanym dodatku PQQ

After 24 h of the second cycle of bioconversion, glycerol concentration in the control medium (0 μM PQQ) reached 15.8 ±0.025 $g \cdot L^{-1}$. In the same period, mean substrate concentrations in the other analyzed media were higher and reached 17 ±0.57, 19 ±0.058, 16 ±0.096 and 17 ±0.031 $g \cdot L^{-1}$ in the media with the cell extract preincubated in solutions of 1, 3, 10 and 15 μM PQQ, respectively (Fig. 2). After another 24 h substrate concentration in the control medium decreased by ca. 1 $g \cdot L^{-1}$, compared to that determined after 24 h of the process. A similar observation was made in the medium containing CE preincubated in 1 μM PQQ solution, however in this case glycerol concentration decreased by ca. 0.5 $g \cdot L^{-1}$ (Fig. 2). After 48 h of the reaction, glycerol concentration decreased by ca. 3, 0.6 and 2 $g \cdot L^{-1}$ in the media containing CE preincubated in 3, 10 and 15 μM PQQ solutions, respectively (Fig. 2). After 72 h of the second cycle of bioconversion, no significant differences were noted in glycerol concentration compared to its values determined after 48 h of the process. Finally, glycerol concentration decreased by ca. 15 $g \cdot L^{-1}$ in the control medium, likewise in the media with CE preincubated in 10 and 15 μM solutions of PQQ. In the other media glycerol concentration was lower by 13–14 $g \cdot L^{-1}$ from the initial one.

The mean DHA concentration determined in the culture media after 24 h of the second cycle of bioconversion reached 9.30 $g \cdot L^{-1}$ (like in the first cycle), regardless of PQQ concentration used for CE preincubation. It remained at the same level till the end of the process. The statistical analysis showed no significant effect of PQQ concentration on the amount of DHA produced during bioconversion. Two homogenous groups were identified. The first one included results obtained in time T_0 of bioconversion for each of the studied variant of PQQ concentrations (0, 3, 10 and 15 μM), whereas the other one included results achieved for each tested variant after 24, 48 and 72 h of bioconversion. Differences between the remaining results were too small and therefore impaired further statistical analysis.

Sainz et al. [2016] demonstrated that GlyDH isolated from *G. oxydans* 621H and preincubated in a solution containing 3 mM $CaCl_2$ and 0.1 μM PQQ exhibited the highest activity after 24 h and its activity decreased after 48 and 96 h [Sainz et al. 2016]. Presumably, a similar situation occurred in our study, wherein the highest consumption of glycerol (in the first and the second cycle of bioconversion) was observed in the first 24 h of the process. Lapenaite et al. [2005] demonstrated that a solution used for GlyDH preincubation, enabling enzyme reconstruction and providing its stable activity, should contain 1 μM PQQ, 8 mM Ca^{2+} and 8 mM Mg^{2+}.

Glycerol consumption was not equivalent with DHA concentration increase, which suggests that the substrate could be conversed to other than DHA compounds. It is supposed that other membrane-bound enzymes were active in *G. oxydans* cell during bioconversion and might catalyzed glycerol conversion to, e.g. glyceric acid, 5-keto-D--gluconate, glycerin aldehyde or glycerol carbonate. Cells of acetic acid bacteria contain many dehydrogenases (i.a. alcoholic, polyol or aldehyde dehydrogenase) that could be present in the obtained cell extract and could exhibit an activity against glycerol and other compounds of the waste glycerol fraction [Peters et al. 2013]. Pyrroloquinoline quinone can be a cofactor also for enzymes different from GlyDH (e.g. kinases, oxidases, hydratases or decarboxylases). It is presumably, that some amounts of PQQ (dedicated to GlyDH) could have been attached by other enzymes which were present in the CE [Richter et al. 2010, Almeida et al. 2012, Kumar 2015, Yakushi et al. 2018]. In study

conducted with a CE from *G. oxydans* not preincubated with PQQ, the highest DHA concentration was reached after 48 h and accounted for ca. 9 $g \cdot L^{-1}$ [Stasiak-Różańska et al. 2017]. Successive bioconversion led to its decrease in the culture medium. In the second cycle of bioconversion, the highest concentration of DHA was determined after the first 24 h and reached 8.7 $g \cdot L^{-1}$. In the subsequent days of the process, DHA concentration decreased as well [Stasiak-Różańska et al. 2017]. In the presented study, DHA concentration maintained at a stable level (9.3 $g \cdot L^{-1}$), which could be due to the stabilization of the reaction medium by using a solution of waste glycerol in a buffer (TrisHCl), and not in water as in the study by Stasiak-Różańska et al. [2017].

Mean efficiency of the first cycle of waste glycerol bioconversion to DHA was shown in Figure 3.

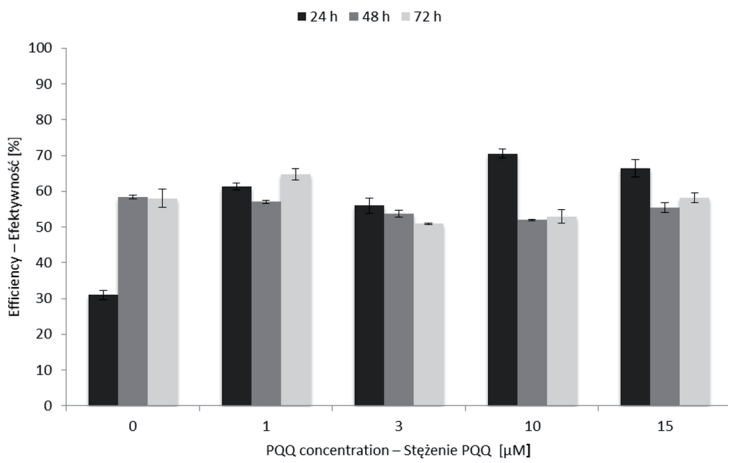

Fig. 3. Effect of PQQ concentration on the efficiency of the first cycle of glycerol bioconversion to DHA

Rys. 3. Wpływ dodatku PQQ na efektywność pierwszego cyklu biokonwersji glicerolu do DHA

The efficiency of the bioconversion conducted with a CE preincubated in a control solution free of PQQ, reached ca. 30% after 24 h, increased during next hours untill for ca. 58% after 48 and 72 h (Fig. 3). Cell extract preincubation in a 1 µM PQQ solution allowed reaching process efficiency of ca. 60% within the first 24 h of the first cycle of bioconversion. In the subsequent measurements (48 and 72 h), the efficiency increased from 57 to 65% (Fig. 3). The results conducted in the culture medium containing CE preincubated in a 3 µM PQQ solution were at 50–56% (Fig. 3). The highest efficiency of the first cycle of bioconversion, i.e. 70.5 and 66.5%, were determined after 24 h in the culture media in which the process was conducted with CE preincubated in 10 and 15 µM PQQ solutions, respectively (Fig. 3). After 48 h bioconversion efficiency decreased in both cases to 51–55%, whereas further extension of the process had no effect on results (Fig. 3).

Mean efficiency of the second cycle of waste glycerol bioconversion to DHA was shown in Figure 4.

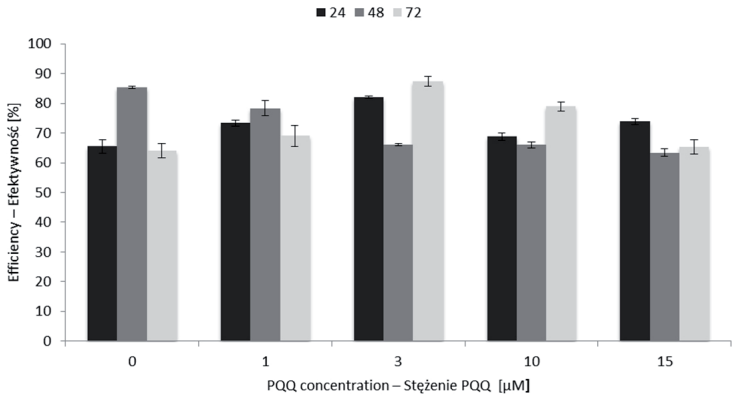

Fig. 4. Effect of PQQ concentration on the efficiency of the second cycle of glycerol bioconversion to DHA

Rys. 4. Wpływ dodatku PQQ na efektywność drugiego cyklu biokonwersji glicerolu do DHA

In the second cycle bioconversion yield in the analyzed culture media was higher than in the first cycle (Figs. 3, 4). In the control medium (0 μM PQQ), the mean process efficiency reached 65.5% after 24 h and increased to ca. 85% after 48 h. Continuation of the process for another 24 h caused it efficiency to decrease to ca. 64% (Fig. 4).

Conducting the second cycle of bioconversion with the cell extract preincubated in a 1 μM PQQ solution enabled reaching process efficiency at ca. 73, 78 and 69% after 24, 48 and 72 h, respectively (Fig. 4). The yields of the second stage of bioconversion in the culture media with CE preincubated in a 3 μM solution of PQQ were high and accounted for ca. 82, 66 and 87% after 24, 48 and 72 h, respectively (Fig. 3).

CONCLUSIONS

- The immobilized CE showed GlyDH activity in both the first and second cycle of waste glycerol bioconversion, irrespective of PQQ concentration used for preincubation.
- The use of various PQQ concentrations for preincubation of CE with GlyDH activity had no significant effect on the concentration of obtained DHA.
- The use of the higher PQQ concentrations (10 and 15 μM) for CE preincubation probably caused a better and faster reconstitution of GlyDH, which in turn allowed reaching a higher yield of the bioconversion process.
- The highest glycerol consumption was observed after 24 h of the process, which suggests that CE showed the highest enzymatic activity during the first day of the process.
- The use of a buffer (pH 9.0) in the bioconversion medium could have a positive effect on stabilization of the obtained DHA.

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WPŁYW DODATKU PIROLOCHINOLINOCHINONU NA BIOKONWERSJĘ GLICERYNY ODPADOWEJ DO DIHYDROKSYACETONU

Streszczenie. Celem badań była ocena wpływu wybranych stężeń (1, 3, 10 lub 15 μM) pirolochinolinochinonu (PQQ) na przebieg biotransformacji glicerolu odpadowego do dihydroksyacetonu (DHA). Katalizatorem reakcji był immobilizowany w alginianie sodu preparat komórkowy o aktywności dehydrogenzy glicerolowej, otrzymany z *Gluconobacter oxydans*. Stężenia glicerolu i DHA oznaczono metodą chromatografii gazowej w czasie 0, 24, 48 i 72 h. We wszystkich analizowanych wariantach doświadczenia największe zużycie glicerolu obserwowano po 24 h procesu. Immobilizowany preparat komórkowy charakteryzował się największą aktywnością enzymatyczną w trakcie pierwszej doby procesu. Dalsze prowadzenie biotransformacji nie miało istotnego wpływu na zmiany zawartości glicerolu w podłożu. Zawartość DHA we wszystkich wariantach doświadczenia wynosiła po 24 h 9.3 $g \cdot l^{-1}$ i nie uległa istotnym zmianom do końca trwania procesu. Immobilizowany preparat komórkowy wykazywał aktywność enzymatyczną względem glicerolu zarówno w pierwszym, jak i w drugim cyklu biotransformacji. W żadnym z analizowanych wariantów doświadczenia nie zaobserwowano istotnego wpływu zastosowanych dawek PQQ na biokonwersję gliceryny odpadowej do DHA.

Słowa kluczowe: glicerol odpadowy, dihydroksyaceton, PQQ, dehydrogenaza glicerolowa