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Research article

Utilization of a waste glycerol fraction using and reusing immobilized *Gluconobacter oxydans* ATCC 621 cell extract

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ABSTRACT

Background: Depletion of petroleum resources has enforced the search for alternative sources of renewable energy. Introduction of biofuels into the market was expected to become a solution to this disadvantageous situation. Attempts to cover fuel demand have, however, caused another severe problem—the waste glycerol generated during biodiesel production at a concentration of approximately 10% w/w. This, in turn, prompted a global search for effective methods of valorization of the waste fraction of glycerol.
Results: Utilization of the waste fraction at 48 h with an initial glycerol concentration of 30 $g \cdot L^{-1}$ and proceeding with 62% efficiency enabled the production of 9 $g \cdot L^{-1}$ dihydroxyacetone at 50% substrate consumption. The re-use of the immobilized biocatalyst resulted in a similar concentration of dihydroxyacetone (8.7 $g \cdot L^{-1}$) in two-fold shorter time, with an efficiency of 85% and lower substrate consumption (35%).
Conclusions: The method proposed in this work is based on the conversion of waste glycerol to dihydroxyacetone in a reaction catalyzed by immobilized *Gluconobacter oxydans* cell extract with glycerol dehydrogenase activity, and it could be an effective way to convert waste glycerol into a valuable product.

1. Introduction

Fuel demand has been intensively growing worldwide in recent years. At the same time, increasing attention has been paid to the protection of natural environment [1,2]. It was, therefore, necessary to find alternative sources of renewable energy. The replacement of petroleum with biofuel was expected to become a successful solution to this situation. Instead, it has contributed to the generation of waste glycerol, which today poses a severe environmental problem [1]. It is estimated that with each year, the global production of biodiesel will increase by 42% with each year. Experts of the fuel market have estimated that by 2016, the level of biodiesel production would reach 140 million tons, which means generation of approximately 14 million tons of waste glycerol [3].

The development of biodiesel production methods with reduced generation of waste glycerol is currently at the laboratory stage, and many years would pass till their application on the industrial scale [4,5]. It is, therefore, advisable to search for effective and environmentally safe methods for the utilization of waste glycerol. Many of the so far proposed methods were based on the widely understood metabolic potential of microorganisms that converted the waste glycerol into valuable and industrially desirable chemical compounds [6].

Some recent papers [1,3] present new possibilities of microbiological utilization of waste glycerol including 1,3-propanediol production by a *Citrobacter freundii* strain in culture medium with waste glycerol [7]. Similar investigations have been successfully conducted with *Klebsiella pneumoniae* and *Clostridium butyricum*, with proven capability to produce 1,3-propanediol from waste glycerol [8,9,10]. Attempts were also undertaken to convert glycerol into ethanol [11], citric acid [12], and polyhydroxyalkanates [13].

Another interesting idea of waste glycerol management may be its transformation into dihydroxyacetone (DHA), a compound with vast industrial applications. Presently, DHA is applied in food, cosmetic, and pharmaceutical industries and in medicine, among others, and its application possibilities are currently being extended [14,15,16,17]. The annual production of DHA reaches approximately 2000 tons [18]. The most common method of its industrial production involves glycerol biotransformation by the free cells of the acetic acid bacteria *Gluconobacter oxydans*. This method has, however, some technological disadvantages that are difficult to eliminate [18]. The main problems of biotechnological production of DHA include susceptibility of *G. oxydans*

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to a high concentration of glycerol and inhibition of the oxidation of this compound by increasing DHA concentration in the culture medium [19].

G. oxydans ATCC 621 is a strain of acetic acid bacteria that oxidizes glycerol to DHA in a reaction catalyzed by glycerol dehydrogenase (GlyDH, EC 1.1.99.22). This enzyme is strictly adhered to the cytoplasmic membrane of *G. oxydans* [20,21], and its action depends on the presence of PQQ cofactor [22]. Because of a strongly hydrophobic character and low stability of the purified fraction of GlyDH, it is difficult to determine the spatial structure of this enzyme [23,24,25]. The optimal temperature of GlyDH action ranges from 23°C to 25°C and the optimal pH from 7.0 to 7.5 [23,24,26].

Our previous study [27] demonstrated the feasibility of applying immobilized *G. oxydans* cell extract with GlyDH activity for the biotransformation of glycerol into DHA. The present work reports on the attempt to utilize the waste fraction of glycerol after biodiesel production for DHA generation in a reaction catalyzed by immobilized *G. oxydans* cell extract with GlyDH activity. Another objective of this study was to check whether the same cell extract can be used repetitively for a number of cycles.

2. Materials and methods

2.1. Biological materials

The study was conducted with the acetic acid bacteria *G. oxydans* ATCC 621 (Manassas, NY).

Waste glycerol from biodiesel production originated from BIOAGRA-OIL S.A. plant (Tychy, Poland). Characteristic of the waste glycerol used in our experiments were as follows: 24°Blg, pH 6.12, concentration of glycerol 659,5 $g \cdot L^{-1}$, dry substance content 69.76%, medium content of individual elements in $mg \cdot g^{-1}$: Ca (0.33), K (0.07), Mg (0.04), Na (19.06), and P (0.08).

2.2. Culture media

Composition of culture medium for the storage of *G. oxydans* in $g \cdot L^{-1}$ (incubated for 48 h at 28°C): yeast extract (5), peptone (3), mannitol (25), and agar (15).

Composition of inoculation medium in $g \cdot L^{-1}$ (pH 5.0, incubated for 24 h at 28°C): yeast extract (30) and ethanol (20).

Composition of culture medium for GlyDH activation in $g \cdot L^{-1}$ (pH 5.0, incubated for 48 h at 28°C): yeast extract 5, glycerol 20, $(NH_4)_2SO_4$ 5, [28].

Reagents were obtained from Avantor Performance Materials Poland. The culture media were sterilized at a temperature of 121°C for 20 min.

2.3. Preparing of cell extract

After *G. oxydans* culture in the activation medium (the end of stationary phase), the resultant biomass was centrifuged and rinsed. Wet biomass (0.44 g) was suspended in 60 mL/cm^3 sterile distilled water and subjected to ultrasonic disintegration (210 W, 18 kHz, 4°C, 5 min) in an Omni Ruptor 4000 apparatus with Titanium 3/8¨ DiaSolid tip [29,30].

2.4. Immobilization

The disintegrated cell extract was mixed (1:1, v/v) with sodium alginate (40 $g \cdot L^{-1}$, Fluka) and added to a 0.2 M solution of $CaCl_2$ (Avantor Performance Materials Poland) using a syringe with a needle of 0.9 mm diameter. The immobilized cell extract was incubated in $CaCl_2$ at 4°C for 3 h [31].

2.5. Biotransformation of the waste fraction of glycerol to DHA

The immobilized cell extract was transferred into 150 cm^3 waste glycerol fraction, diluted with distilled water to obtain a glycerol concentration of 30 $g \cdot L^{-1}$, pH 7.5. The experiment was conducted in 500 cm^3 flasks on a reciprocating shaker (200 rpm) at 23°C for 168 h. Subsequently, the immobilized cell extract was separated, rinsed with sterile distilled water, and immediately transferred to flasks containing a fresh waste fraction of glycerol.

2.6. Determination of glycerol concentration

Glycerol concentration was determined with the use of Free Glycerol Reagent (FGR, Sigma-Aldrich F6428). The FGR (0.80 cm^3) was mixed with 0.01 cm^3 of the analyzed sample and the mixture was incubated for 5 min at 37°C. Blank and standard samples, instead of the exact experimental sample, contained 0.010 cm^3 of water and Glycerol Standard Solution (Sigma-Aldrich, G7793), respectively. Absorbance (A) of the exact sample, blank sample, and standard was recorded at 540 nm. Glycerol concentration [g/100 cm^3] was computed from the formula $(A_{sample} - A_{blank}) / (A_{standard} - A_{blank}) \times 0.26$.

2.7. Determination of DHA concentration

A 2 cm^3 volume of 3,5-dinitrosalicylic acid (Fluka) was added to 2 cm^3 analyzed sample. The mixture was incubated at 100°C for 10 min and then cooled and transferred quantitatively into 20 cm^3 water. Absorbance of the sample was measured at the wavelength $\lambda = 550$ nm against a control that contained water instead of the sample. DHA concentration was determined from the regression equation of absorbance dependency on the concentration of standard solutions [32].

2.8. Reaction efficiency

The efficiency of the reaction was calculated according to the stoichiometric equation of a chemical reaction, indicating that 90 $g \cdot mol^{-1}$ DHA was produced from 92 $g \cdot mol^{-1}$ of glycerol.

Biotransformation was performed in three independent series, and each determination was carried out in three replications.

2.9. Statistical analysis

Standard deviation was calculated for all experimental results.

3. Results and discussion

3.1. Utilization of the waste fraction of glycerol with the use of immobilized G. oxydans ATCC 621 cell extract with GlyDH activity

Changes in DHA and glycerol concentration, substrate consumption, and efficiency of the utilization of the waste glycerol fraction with the use of immobilized *G. oxydans* cell extract with GlyDH activity are presented in Table 1.

After 24 h of the process, the mean concentration of DHA formed reached 6.9 ± 0.07 $g \cdot L^{-1}$, the mean concentration of glycerol in the solution reached 22 ± 0.06 $g \cdot L^{-1}$, and the reaction proceeded with a mean efficiency of 90% in the first cycle, which was the highest noted (Table 1). In the following cycle, the substrate consumption reached 27% (Table 1). After 48 h of biotransformation, the mean DHA concentration increased compared to the previous measured value and reached 8.9 ± 0.03 $g \cdot L^{-1}$ (Table 1). In the next cycle, the content of glycerol remaining in the waste fraction decreased by half compared to the initial concentration. After 48 h, the mean efficiency of utilization decreased by approximately 28% and reached 62% (Table 1). Successive reactions did not increase but slightly decreased the DHA concentration (Table 1). The mean glycerol content in the

Table 1
Changes in DHA and glycerol concentrations (arithmetic mean of free independent series ± standard deviation), substrate consumption, and utilization efficiency of the waste glycerol fraction with the use of immobilized *G. oxydans* cell extract.

Time [h]	0	24	48	72	96	120	144	168
DHA [g·L^{-1}]	0 ± 0.00	6.9 ± 0.07	8.9 ± 0.03	8.3 ± 0.0	7.7 ± 0.01	7.5 ± 0.06	7.2 ± 0.08	7.4 ± 0.03
Glycerol [g·L^{-1}]	30 ± 0.00	22 ± 0.06	15 ± 0.02	11 ± 0.10	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
Substrate consumption [%]	0	27	50	63	100	100	100	100
Reaction efficiency [%]	0	90	62	44	28	26	25	24

waste fraction after 72 h of valorization accounted for 11 ± 0.10 g·L^{-1} (Table 1). The greatest glycerol consumption (63%) was determined after 72 h of utilization (Table 1).

Results obtained demonstrated that GlyDH present in the cell extract exhibited the highest activity in the first 24 h of the process. These results are consistent with literature data [18]. GlyDH is an enzyme that is bound to the cytoplasmic membrane of *G. oxydans* and whose active center is in the periplasmic space. Glycerol is directly oxidized to DHA, which, in turn, is released outside the cell [18]. The purified GlyDH fraction proved to be less stable and lost its activity more rapidly than the fraction whose enzyme was bound to the cytoplasmic membrane [33]. It was also demonstrated that the purified enzyme lost 70% of its activity on the third day of storage [24]. Owing to the low stability of the purified enzyme, it has not been thoroughly characterized so far [34]. The activity of GlyDH depends on the active acidity of the medium. It was determined that GlyDH (isolated from the *Gluconobacter* sp. 33 and separated from the membranes) exhibited the highest activity in a pH range of 7.0–7.5 and highest stability at pH 8.5–9.5 [24]. In the present study, the initial pH of the reaction was pH 7.5. After 168 h of utilization, the active acidity of the waste glycerol decreased to 4.0. Acetic acid bacteria oxidize glycerol to DHA. A glycerol aldehyde is, simultaneously, formed in this reaction that is then converted into glyceric acid, which possibly decreases the medium pH to 4.0 [18]. This change in active acidity during utilization could immediately decrease the GlyDH activity and, consequently, result in lesser increases of DHA concentration. In future studies, utilization of waste glycerol should be conducted under conditions that would ensure stable pH of the waste fraction that is optimal for the activity of GlyDH (*e.g.* in a biofermenter). No studies have been published so far on the characteristics of GlyDH from the strain used in our study (*G. oxydans* ATCC 621). Therefore, it can be assumed that the optimal parameters of this enzyme activity may differ from those reported in research works for GlyDH isolated from other strains of acetic acid bacteria [25,33].

The efficiency of biotransformation after 72 h reached 44%, and in the successive measuring intervals, it did not exceed 28% (Table 1). After 100 h, no glycerol was found in the waste fraction (Table 1). Similar results were obtained by Celik et al. [35], who demonstrated that *G. oxydans* NBRC12528 oxidized glycerol to DHA only in the first 24 h of the reaction. Further biotransformation did not cause any increase in DHA concentration but only converted the remaining glycerol into glyceric acid [35,36].

In addition to the predominating glycerol, the waste glycerol usually contains many impurities including residues of methanol, NaOH, fats, oils, esters, small quantities of sulfur compounds, proteins, and minerals [35]. The composition of the waste glycerol fraction depends, among other things, on the type of catalyst applied for biodiesel production, efficiency of transesterification, efficiency of biodiesel, and catalyst recovery and on conditions of separation of the polar and nonpolar fraction [1,37]. Some contaminants of the waste fraction, particularly free fatty acids, may inhibit many processes of bacterial fermentation, *e.g.*, production of 1,3-propanodiol in cells of *C. butyricum* [10,38,39], and may negatively affect many bacterial metabolic processes [40].

In the present study, although DHA concentration did not increase after 48 h of utilization, some part of glycerol was consumed (Table 1). Apart from GlyDH, the immobilized cell extract contained other enzymes, including enzymes bound to the cytoplasmic membrane, which (despite the application of conditions optimal for the activity of GlyDH) could exhibit some activity against glycerol and catalyze some oxidation reactions, *e.g.*, oxidation of alcohols and polyols, in a stereo- or regioselective manner [41]. Cells of acetic acid bacteria contain at least eight characterized and two so far not characterized dehydrogenases bound to the cytoplasmic membrane [20]. These include, among others, alcohol dehydrogenase [42,43], inositol dehydrogenase [44], aldehyde dehydrogenase [20,45], sorbitol dehydrogenase [46,47], D-glucone dehydrogenase [48], glucose dehydrogenase [49], lactate dehydrogenase [50], and GlyDH [25,51]. It cannot be excluded, therefore, that the mentioned dehydrogenases were active during waste glycerol biotransformation to DHA. The presence of these enzymes in the immobilized cell extract could, to some extent, affect the reactions proceeding during the utilization of the glycerol waste (glycerol could be transformed into other compounds than DHA) and thus also affect the process efficiency.

The process of biotransformation was continued for 168 h to ensure that DHA content did no decrease with time, *e.g.*, as a result of further transformations. From the 96th h, DHA concentration was 7.7 ± 0.01 g L^{-1}, and in the last measuring period, *i.e.*, after 168 h, it was 7.4 ± 0.30 g L^{-1} (Table 1). These results demonstrate the stability of the produced dihydroxyacetone. Earlier studies [52,53] indicated that DHA was the most stable at pH 4.0, and such a value of active acidity was determined in our study after completed utilization.

3.2. Utilization of the waste glycerol fraction with re-used immobilized G. oxydans ATCC 621 cell extract with GlyDH activity

The second part of the study was conducted to verify the feasibility of re-using the catalytic activity of GlyDH in the immobilized *G. oxydans* cell extract for the utilization of the waste fraction of glycerol. Changes in DHA and glycerol concentrations, substrate consumption, and efficiency of utilization observed with the re-applied cell extract are summarized in Table 2.

After 24 h of utilization with the re-used cell extract, the mean concentration of DHA reached 8.7 ± 0.06 g·L^{-1} (Table 2). This value was higher by 1.8 g·L^{-1} than the value determined after the same time during utilization with the cell extract applied for the first time (Table 1). Glycerol consumption during 24 h of the second utilization cycle (with re-applied cell extract) reached 35% and was higher by 8% than that in the first cycle (Table 1, Table 2). Utilization efficiency after 24 h reached 85% (Table 2). It is speculated that the results obtained could be influenced by the earlier activation of GlyDH induced by a low glycerol concentration in the culture medium [54] and transferring of the cell extract from lower pH (the mean pH after 168 h reached 4.0) to pH 7.5 (pH of the freshly prepared glycerol waste). After 48 h of the second utilization cycle, the medium concentration of DHA was 7.6 ± 0.06 g·L^{-1} and accounted for 19 ± 0.14 g·L^{-1} glycerol content in the waste (at 36% substrate consumption), whereas process efficiency reached 73% (Table 2) and was higher by 11% than the efficiency determined after 48 h of the first utilization cycle (Table 1). After 48 h of reaction with the re-applied cell extract, no increase was observed in DHA concentration in the post-reaction mixture (similar to that during the first cycle). The above results suggest that waste glycerol may be

Table 2
Changes in DHA and glycerol concentrations (arithmetic mean of three independent series ± standard deviation), substrate consumption, and efficiency of utilization of the waste fraction with re-application of the immobilized *G. oxydans* cell extract.

Time [h]	0	24	48	72	96	120	144	168
DHA [g·L^{-1}]	0.0 ± 0.00	8.7 ± 0.06	7.6 ± 0.06	6.8 ± 0.05	6.9 ± 0.03	6.6 ± 0.03	6.6 ± 0.02	6.5 ± 0.01
Glycerol [g·L^{-1}]	30 ± 0.00	19 ± 0.16	19 ± 0.14	19 ± 0.14	19 ± 0.14	19 ± 0.14	19 ± 0.14	19 ± 0.14
Substrate consumption [%]	0	35	36	36	36	36	36	36
Reaction efficiency [%]	0	85	73	65	66	63	63	62

effectively utilized during the first 48 h of the process. It is an important parameter to consider for the future development and improvement of waste valorization method for DHA production. An attempt should be undertaken in future studies to utilize waste glycerol in a semi-continuous mode, with fresh portion of waste being fed every 48 h and the resultant product being collected, under optimal conditions for GlyDH activity (temperature 23°C and pH 7.5).

In contrast to the first cycle of utilization, the re-used cell extract did not cause further transformation of glycerol, which was indicated by the glycerol concentration (19 ± 0.14 g·L^{-1}) maintained at the same level till the end of experiment (Table 2). Glycerol that was completely transformed during biotransformation with immobilized cell extract (Table 1) was present in the mixture media at a concentration of approximately 11 g·L^{-1} during biotransformation with the re-applied immobilized *G. oxydans* cell extract (Table 2). It is believed that the immobilized cell extract used in the first reaction contains enzymes that have sufficient concentration of cofactors (GlyDH is PQQ-dependent enzyme) and therefore were fully active and converted to glycerol. Reuse of immobilized cell extract without cofactor supplementation possibly reduces the activity of the enzymes involved in the biotransformation of glycerol or causes their complete inactivation. Small changes in DHA concentration in successive hours of the utilization cycle (ranging from 6.8 ± 0.05 g·L^{-1} in the 72nd h to 6.5 ± 0.01 g·L^{-1} in the 168th h) obviously influenced the process efficiency despite the unchanging concentration of the product (Table 2).

The concentration of DHA obtained in the second cycle of utilization could be affected by the PQQ content in the reaction medium. It is assumed that during the first biotransformation process, the concentration of PQQ was sufficient to maintain high activity of GlyDH in the immobilized cell extract. During the second cycle of utilization (conducted with the use of the same immobilized extract), the content of PQQ could be too low to ensure the proper functioning of GlyDH. GlyDH is an enzyme whose activity depends on the presence of PQQ as cofactor [24]. The proposed method for the utilization of waste glycerol should be modified in the future considering the appropriate concentration of PQQ in the medium of action of the immobilized cell extract with GlyDH activity. The efficiency of biotransformation could also be influenced by the selection of the carrier and by method of cell extract immobilization. Partial or complete saturation of the active sites of GlyDH with the substrate could occur during the oxidation of glycerol, which might impair the course of the reaction. The release of DHA into the culture medium could be impaired by the resistance of the alginate carrier, which had to be overcome by diffusing molecules of the substrate and the product. Some limitations resulting from insufficient aeration of the reaction medium could not be excluded as well. Another study [55] performed an experiment with better success of re-use of immobilized enzyme. They adsorbed GlyDH onto magnetically separable mesoporous silica with 38 nm mesocellular pores connected through 18 nm window mesopores (ADS) and further crosslinked through a simple glutaraldehyde treatment to prepare nanoscale enzyme reactors of GDH (NER). The residual activities of the free GDH and ADS could no longer be detected after 8 and 22 d, respectively, while the NER maintained 64% of its initial activity even after a 24-d incubation. The time-dependent conversion of glycerol to DHA was measured for both ADS and NER not only by analyzing the generation of NADH spectrophotometrically but also by HPLC measuring the increase in the concentration of DHA. Magnetically separable NER maintained 39% of its initial activity after seven cycles of re-application, while the residual activity of ADS dropped to 13% of its initial activity after only two re-applications [55].

Oxidation of glycerol to dihydroxyacetone catalyzed by immobilized cell extract requires further optimization because efficiency of DHA production is not sufficient. In the traditional method using whole-cell *G. oxydans*, glycerol and DHA can inhibit the metabolic activity of *G. oxydans* and consequently inhibit the production of DHA. The traditional method requires cell proliferation every time the reaction starts. Furthermore supplementation of culture media is needed to create optimal conditions for *G. oxydans* growth, *e.g.*, magnesium or some amino acids [56]. Crystallization of the final product from media is difficult, and the first step is the separation of the bacteria cells. Obtaining DHA with immobilized cell extract involves the use of the enzyme. At the same time *G. oxydans* cells are inactivated after disintegration. Therefore, supplementation of media with growth components and earlier activation of GlyDH (which shortens the preparation stage of the process) are not necessary. Immobilization allows the easy separation of the biocatalyst from the reaction mixture. This study also showed that the immobilized cell extract does not lose GlyDH activity, and it can be re-used in the next cycle of glycerol oxidation, which is an advantage of the proposed method because in the future (after optimization these method), it can shorten the process and increase the cost-effectiveness.

Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] Yang F, Hanna MA, Sun R. Value-added uses for crude glycerol—A byproduct of biodiesel production. Biotechnol Biofuels 2012;5:13. http://dx.doi.org/10.1186/1754-6834-5-13.
[2] Glithero NJ, Ramsden SJ, Wilson P. Barriers and incentives to the production of bioethanol from cereal straw: A farm business perspective. Energ Policy 2013;59:161–71. http://dx.doi.org/10.1016/j.enpol.2013.03.003.
[3] Anand P, Saxena RK. A comparative study of solvent-assisted pretreatment of biodiesel derived crude glycerol on growth and 1,3-propanediol production from *Citrobacter freundii*. New Biotechnol 2012;29:199–205. http://dx.doi.org/10.1016/j.nbt.2011.05.010.
[4] Kai T, Mak GL, Wada S, Nakazato T, Takanashi H, Uemura Y. Production of biodiesel fuel from canola oil with dimethyl carbonate using an active sodium methoxide catalyst prepared by crystallization. Bioresour Technol 2014;163:360–3. http://dx.doi.org/10.1016/j.biortech.2014.04.030.
[5] Witoon T, Bumrungsalee S, Vathavanichkul P, Palitsakun S, Saisriyoot M, Faungnawakij K. Biodiesel production from transesterification of palm oil with methanol over CaO supported on bimodal meso-macroporous silica catalyst. Bioresour Technol 2014;156:329–34. http://dx.doi.org/10.1016/j.biortech.2014.01.076.
[6] Almeida JRM, Fávaro LCL, Quirino BF. Biodiesel biorefinery: Opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. Biotechnol Biofuels 2012;5:48–64. http://dx.doi.org/10.1186/1754-6834-5-48.
[7] Hiremath A, Kannabiran M, Rangaswamy V. 1,3-Propanediol production from crude glycerol from jatropha biodiesel process. New Biotechnol 2011;28:19–23. http://dx.doi.org/10.1016/j.nbt.2010.06.006.
[8] Mu Y, Xiu ZL, Zhang DJ. A combined bioprocess of biodiesel production by lipase with microbial production of 1,3-propanediol by *Klebsiella pneunoniae*. Biochem Eng J 2008;40:537–41. http://dx.doi.org/10.1016/j.bej.2008.02.011.

[9] Oh BR, Seo JW, Heo SY, Hong WK, Luo LH, Joe M, et al. Efficient production of ethanol from crude glycerol by a *Klebsiella pneumonia* mutant strain. Bioresour Technol 2011;102:3918–22. http://dx.doi.org/10.1016/j.biortech.2010.12.007.
[10] Chatzifragkou A, Papanikolaou S, Dietz D, Doulgeraki AI, Nychas GJE, Zeng AP. Production of 1,3-propanediol by *Clostridium butyricum* growing on biodiesel-derived crude glycerol through a non-sterilized fermentation process. Appl Microbiol Biotechnol 2011;91:101–12. http://dx.doi.org/10.1007/s00253-011-3247-x.
[11] Ito T, Nakashimada Y, Senba K, Matsui T, Nishi N. Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. J Biosci Bioeng 2005;100:260–5. http://dx.doi.org/10.1263/jbb.100.260.
[12] Papanikolaou S, Aggelis G. Biotechnological valorization of biodiesel derived glycerol waste through production of single cell oil and citric acid by *Yarrowia lipolytica*. Lipid Technol 2009;21:83–7. http://dx.doi.org/10.1002/lite.200900017.
[13] Chanprateep S. Current trends in biodegradable polyhydroxyalkanoates. J Biosci Bioeng 2010;110:621–32. http://dx.doi.org/10.1016/j.jbiosc.2010.07.014.
[14] Nguyen BC, Kochevar IE. Factors influencing sunless tanning with dihydroxyacetone. Br J Dermatol 2003;149:332–40. http://dx.doi.org/10.1046/j.1365-2133.2003.05434.x.
[15] Brown DA. Skin pigmentation enhancers. J Photochem Photobiol B 2001;63:148–61. http://dx.doi.org/10.1016/S1011-1344(01)00212-3.
[16] Bauer R, Katsikis N, Varga S, Hekmat D. Study of the inhibitory effect of the product dihydroxyacetone on *Gluconobacter oxydans* in a semi-continuous two-stage repeated-fed-batch process. Bioprocess Biosyst Eng 2005;28:37–43. http://dx.doi.org/10.1007/s00449-005-0009-0.
[17] Bicker M, Endres S, Ott L, Vogel H. Catalytical conversion of carbohydrates in subcritical water: A new chemical process for lactic acid production. J Mol Catal A Chem 2005;239:151–7. http://dx.doi.org/10.1016/j.molcata.2005.06.017.
[18] Habe H, Fukuoka T, Morita T, Kitamoto D, Yakushi T, Matsushita K, et al. Disruption of the membrane-bound alcohol dehydrogenase-encoding gene improved glycerol use and dihydroxyacetone productivity in *Gluconobacter oxydans*. Biosci Biotechnol Biochem 2010;74:1391–5. http://dx.doi.org/10.1271/bbb.100068.
[19] Stasiak-Różańska L, Błażejak S, Gientka I. Effect of glycerol and dihydroxyacetone concentrations in the culture medium on the growth of acetic acid bacteria *Gluconobacter oxydans* ATCC 621. Eur Food Res Technol 2014;239:453–61. http://dx.doi.org/10.1007/s00217-014-2238-4.
[20] Prust C, Hoffmeister M, Liesegang H, Wiezer A, Fricke WF, Ehrenreich A, et al. Complete genome sequence of the acetic acid bacterium *Gluconobacter oxydans*. Nat Biotechnol 2005;23:195–200. http://dx.doi.org/10.1038/nbt1062.
[21] Gätgens C, Degner U, Bringer-Meyer S, Herrman U. Biotransformation of glycerol to dihydroxyacetone by recombinant *Gluconobacter oxydans* DSM 2343. Appl Microbiol Biotechnol 2007;76:553–9. http://dx.doi.org/10.1007/s00253-007-1003-z.
[22] Nakano M, Suzuki H, Imamura T, Lau A, Lynch B. Genotoxicity of pyrroloquinoline quinone (PQQ) disodium salt (BioPQQ™). Regul Toxicol Pharmacol 2013;67:189–97. http://dx.doi.org/10.1016/j.yrtph.2013.07.007.
[23] Ma L, Lu W, Xia Z, Wen J. Enhancement of dihydroxyacetone production by a mutant of *Gluconobacter oxydans*. Biochem Eng J 2010;49:61–7. http://dx.doi.org/10.1016/j.bej.2009.11.011.
[24] Lapenaite I, Kurtinaitiene B, Razumiene J, Laurinavicius V, Marcinkeviciene L, Bachmatova I, et al. Properties and analytical application of PQQ-dependent glycerol dehydrogenase from *Gluconobacter* sp. 33. Anal Chim Acta 2005;549:140–50. http://dx.doi.org/10.1016/j.aca.2005.06.025.
[25] Matsushita K, Fujii Y, Ano Y, Toyama H, Shinjoh M, Tomiyama N, et al. 5-Keto-D-gluconate production is catalyzed by a quinoprotein glycerol dehydrogenase, major polyol dehydrogenase, in *Gluconobacter* species. Appl Environ Microbiol 2003;69:1959–66. http://dx.doi.org/10.1128/AEM.69.4.1959-1966.2003.
[26] Adachi O, Matsushita K. 1997. USA Patent nr 56143774.
[27] Stasiak-Różańska L, Błażejak S. Production of dihydroxyacetone from an aqueous solution of glycerol in the reaction catalyzed by an immobilized cell preparation of acetic acid bacteria Gluconobacter oxydans ATCC 621. Eur Food Res Technol 2012;235:1125–32. http://dx.doi.org/10.1007/s00217-012-1846-0.
[28] Hekmat D, Bauer R, Fricke J. Optimization of the microbial synthesis of dihydroxyacetone from glycerol with *Gluconobacter oxydans*. Bioprocess Biosyst Eng 2003;26:103–16. http://dx.doi.org/10.1007/s00449-003-0338-9.
[29] Kapucu H, Gülsoy N, Mehmetoğlu Ü. Disruption and protein release kinetics by ultrasonication of *Acetobacteri peroxydans* cells. Biochem Eng J 2000;5:57–62. http://dx.doi.org/10.1016/s1369-703X(99)00065-0.
[30] VanLare IJ, Claus GW. Purification and properties of NAD(P)-independent polyol dehydrogenase complex from the plasma membrane of *Gluconobacter oxydans*. Can J Microbiol 2007;53:504–8. http://dx.doi.org/10.1139/W07-006.
[31] Wang S, Wang J, Zhou X, Guo Y, Fang B. The Improvement of stability, activity, and substrate promiscuity of glycerol dehydrogenase substituted by divalent metal ions. Biotechnol Bioprocess Eng 2013;18:796–800. http://dx.doi.org/10.1007/s12257-013-0125-7.
[32] Chen J, Chen J, Zhou C. HPLC methods for determination of dihydroxyacetone and glycerol in fermentation broth and comparison with a visible spectrophotometric method to determine dihydroxyacetone. J Chromatogr Sci 2008;46:912–6. http://dx.doi.org/10.1093/chromsci/46.10.912.
[33] Ameyama M, Shinagawa E, Matsushita K, Adachi O. Solubilization, purification and properties of membrane-bound glycerol dehydrogenase from *Gluconobacter industrius*. Agric Biol Chem 1985;49:1001–10. http://dx.doi.org/10.1080/00021369.1985.10866858.
[34] Laurinavičius V, Razumiene J, Kurtinaitiene B, Lapenaite I, Bachmatova I, Marcinkevičiene L, et al. Bioelectrochemical application of some PQQ-dependent enzymes. Bioelectrochemistry 2002;55:29–32. http://dx.doi.org/10.1016/s1567-5394(01)00128-1.
[35] Celik E, Ozbay N, Oktar N, Calik P. Use of biodiesel byproduct crude glycerol as the carbon source for fermentation processes by recombinant *Pichia pastoris*. Ind Eng Chem Res 2008;47:2985–90. http://dx.doi.org/10.1021/ie071613O.
[36] Habe H, Fuknoka T, Kitamoto D, Sakaki K. Biotransformation of glycerol to D-glyceric acid by *Acetobacter tropicalis*. Appl Microbiol Biotechnol 2009;81:1033–9. http://dx.doi.org/10.1007/s00253-008-1737-2.
[37] Hájek M, Skopal F. Treatment of glycerol formed by biodiesel production. Bioresour Technol 2010;101:3232–45. http://dx.doi.org/10.1016/j.biortech.2009.12.094.
[38] Chatzifragkou A, Papanikolaou S. Effect of impurities in biodiesel-derived waste glycerol on the performance and feasibility of biotechnological processes. Appl Microbiol Biotechnol 2012;95:13–27. http://dx.doi.org/10.1007/s00253-012-4111-3.
[39] Pflügl S, Marx H, Mattanovich D, Sauer M. Heading for an economic industrial upgrading of crude glycerol from biodiesel production to 1,3-propanediol by *Lactobacillus diolivorans*. Bioresour Technol 2014;152:499–504. http://dx.doi.org/10.1016/j.biortech.2013.11.041.
[40] Samul D, Leja K, Grajek W. Impurities of crude glycerol and their effect on metabolite production. Ann Microbiol 2014;64:891–8. http://dx.doi.org/10.1007/s13213-013-0767-x.
[41] Peters B, Mientus M, Kostner D, Junker A, Liebl W, Ehrenreich A. Characterization of membrane-bound dehydrogenases from *Gluconobacter oxydans* 621H via whole-cell activity assays using multideletion strains. Appl Microbiol Biotechnol 2013;97:6397–412. http://dx.doi.org/10.1007/s00253-013-4824-y.
[42] Adachi O, Tayama K, Shinagawa E, Matsushita K, Ameyama M. Purification and characterization of particulate alcohol dehydrogenase from *Gluconobacter suboxydans*. Agric Biol Chem 1978;42:2045–56. http://dx.doi.org/10.1080/00021369.1978.10863306.
[43] Kondo K, Horinouchi S. Characterization of the genes encoding the three-component membrane-bound alcohol dehydrogenase from *Gluconobacter suboxydans* and their expression in *Acetobacter pasteurianus*. Appl Environ Microbiol 1997;63:1131–8. [cited date July 15, 2016]. Available from: https://www.ncbi.nlm.nih.gov/pubmed/9055427.
[44] Hölscher T, Weinert-Sepalage D, Görisch H. Identyficafion of membrane-bound quinoprotein inositol dehydrogenase in *Gluconobacter oxydans* ATCC 621H. Microbiology 2007;153:499–506. http://dx.doi.org/10.1099/mic.0.2006/002196-0.
[45] Adachi O, Matsushita K, Shinagawa E, Ameyama M. Crystallization and properties of NADP-dependent aldehyde dehydrogenase from *Gluconobacter melanogenus*. Agric Biol Chem 1980;44:155–64. http://dx.doi.org/10.1080/00021369.1980.10863918.
[46] Shinagawa E, Matsushita K, Adachi O, Ameyama M. Purification and characterization of D-sorbitol dehydrogenase from membrane of *Gluconobacter suboxydans* var. α. Agric Biol Chem 1982;46:135–41. http://dx.doi.org/10.1080/00021369.1982.10865042.
[47] Toyama H, Soemphol W, Moonmangmee D, Adachi O, Matsushita K. Molecular properties of membrane-bound FAD-containing D-sorbitol dehydrogenase from thermotolerant *Gluconobacter frateurii* isolated from Thailand. Biosci Biotechnol Biochem 2005;69:1120–9. http://dx.doi.org/10.1271/bbb.69.1120.
[48] Shinagawa E, Matsushita K, Adachi O, Ameyama M. D-Gluconate dehydrogenase, 2-keto-D-gluconate yielding, from *Gluconobacter dioxyacetonicus*: Purification and characterization. Agric Biol Chem 1984;48:1517–22. http://dx.doi.org/10.1080/00021369.1984.10866357.
[49] Ameyama M, Shinagawa E, Matsushita K, Adachi O. D-Glucose dehydrogenase of *Gluconobacter suboxydance*: Solubilization, purification and characterization. Agric Biol Chem 1981;45:851–61. http://dx.doi.org/10.1080/00021369.1981.10864633.
[50] Ma C, Gao C, Qiu J, Hao J, Liu W, Wang A, et al. Membrane-bound l- and d-lactate dehydrogenase activities of a newly isolated *Pseudomonas stutzeri* strain. Appl Microbiol Biotechnol 2007;77:91–8. http://dx.doi.org/10.1007/s00253-007-1132-4.
[51] Adachi O, Fujii Y, Ghaly MF, Toyama H, Shinagawa E, Matsushita K. Membrane-bound quinoprotein D-arabitol dehydrogenase of *Gluconobacter suboxydans* IFO 3257: A versatile enzyme for the oxidative fermentation of various ketoses. Biosci Biotechnol Biochem 2001;65:2755–62. http://dx.doi.org/10.1271/bbb.65.2755.
[52] Pantini G, Ingoglia R, Brunetta F, Brunetta A. Sunless tanning products containing dihydroxyacetone in combination with a perfluoropolyether phosphate. Int J Cosmet Sci 2007;29:201–9. http://dx.doi.org/10.1111/j.1467-2494.2007.00381.x.
[53] Burkhart CG, Burkhart CN. Dihydroxyacetone and methods to improve its performance as artificial tanner. Open Dermatol J 2009;3:42–3. http://dx.doi.org/10.2174/1874372200903010042.
[54] Hu ZC, Liu ZQ, Zheng YG, Shen YC. Production of 1,3-dihydroxyacetone from glycerol by Gluconobacter oxydans ZJB09112. J Microbiol Biotechnol 2010;20:340–5.
[55] Kumar GS, Wee Y, Lee I, Sun HJ, Zhao X, Xia S, et al. Stabilized glycerol dehydrogenase for the conversion of glycerol to dihydroxyacetone. Chem Eng J 2015;276:283–8. http://dx.doi.org/10.1016/j.cej.2015.04.039.
[56] Dikshit PK, Moholkar VS. Optimization of 1,3-dihydroxyacetone production from crude glycerol by immobilized *Gluconobacter oxydans* MTCC 904. Bioresour Technol 2016;216:1058–65. http://dx.doi.org/10.1016/j.biortech.2016.01.100.