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INVESTIGATIONS INTO THE OPTIMIZATION OF PARAMETERS OF GLYCEROL BIOTRANSFORMATION TO DIHYDROXYACETONE WITH THE USE OF IMMOBILIZED CELLS OF *GLUCONACETOBACTER XYLINUS*

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Key words: *Gluconacetobacter xylinus,* glycerol biotransformation, dihydroxyacetone, glycerol, glycerol dehydrogenase

The *Gluconacetobacter xylinus* species, belonging to a group of acetic bacteria, is capable of partial oxidation of glycerol. Dihydroxyacetone (DHA), being a product of that transformation, has been used in the food industry (as a sweetener), cosmetic industry (production of self-tanning creams) and in dermatology (treatment of leukoderma). The objective of the study reported herein was to determine values of the initial concentration of glycerol in the culture medium, active acidity of the medium and time of culture assuring the highest quantity of DHA produced, all three being optimal for the course of glycerol biotransformation to DHA. The biotransformation process was conducted with the use of *G. xylinus* cells immobilized in calcium alginate. The study demonstrated that in the culture medium with pH 5.0 and initial concentration of glycerol reaching 100 g/L, the concentration of DHA after 36 h of biotransformation accounted for 10.9 g/L. The highest content of the product in the culture media with active acidity of 7.0 or 8.0 and containing 100 g of glycerol/L at the beginning of the process, reached 10.5 g/L (after 36 h) and 10.7 g/L (after 34 h of the process), respectively. Elongating the biotransformation process to over 36 h resulted in a decrease in DHA concentration, which could have been due to its phosphorylation. Results obtained in this research demonstrated that the efficiency of glycerol biotransformation to DHA was determined, first of all, by the initial concentration of substrate and, to a slightly lesser extent, by acidity of the culture medium and process duration.

INTRODUCTION

Dihydroxyacetone (1,3-dihydroxy-2-propanoen, $C_3H_6O_3$, abbreviation: DHA) is one of the major intermediate products of glycerol and carbohydrates metabolism by acetic acid bacteria [Tkač *et al*., 2001] and, simultaneously, the simplest representative of ketoses [Ferroni *et al*., 1999]. In nature, this compound occurs in sugar beets and sugar cane. As a result of crystallization it attains the form of white powder with a sweet, refreshing taste [Schmid *et al*., 2007; Erni *et al*., 2006; Rabinowitch, 1925].

A growing demand for dihydroxyacetone and new possibilities of its application emerging in a variety of industry branches, encourage the development of more effective, faster and cheaper methods of its acquisition. Dihydroxyacetone has been used in food production as a sweetener and diet supplement for sportsmen [Omar *et al*., 2005; Cortez *et al*., 1991]. In treatment of cyanide-induced intoxications it has been applied as a respiration-activating agent [Niknahad & Ghelichkhani, 2002; Beasley & Glass, 1998]. It may additionally be used in treatment of diabetes and as an immediate product for the production of some medicines and dyes [Levy, 2000; Claret & Bories, 1994]. Furthermore, dihydroxyacetone is capable or forming colour complexes with arginine occurring in human epidermis [Schmid *et al*., 2007; Petersen *et al*., 2003, Ohrem & Voss, 1995]. For this reason, it has been claimed an indispensable component of the so-called "self-tanning creams" produced in ever increasing quantities and of preparations used in leukoderma treatment [Rogers, 2005; Draelos & Zoe, 2002; Fesq *et al*., 2001].

Chemical methods of DHA acquisition, though more expensive than the microbiological ones, are more frequently applied today [Hekmat *et al*., 2003]. The main restrictions of implementing methods based on microorganisms (primarily bacteria of *Gluconobacter* and *Gluconacetobcter* genera) include difficulties in selecting the optimal parameters of DHA biosynthesis. It is due to the fact that ill-adjusted initial concentration of glycerol and an increasing concentration of the synthesized DHA may exert an inhibiting effect on metabolism of acetic bacteria cells, which in turn leads to diminished efficiency of the reaction [De Muynck *et al*., 2007; Ohrem & Voss, 1995].

Biotransformation of glycerol to DHA mediated by acetic bacteria is catalyzed by glycerol dehydrogenase (EC 1.1.99.22) [Matsushita *et al*., 2003; Hauge *et al*., 1954]. The quantity of dihydroxyacetone synthesized in that reaction may be subject to a considerable decline as affected by the activity of dihydroxyacetone kinase (EC 2.7.1.29) responsible for phosphorylation of DHA [Erni *et al*., 2006; Hauge *et al*., 1954]. Thus, in order to assure a high efficiency of the process, it should be stopped at the specific, right moment. Of great significance is, therefore, also the selection of the growth phase of bacteria used in that process [De Muynck *et al*., 2007], for studies have demonstrated that

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with proper parameters of culture maintained (including: pH, aeration, composition of production medium), only the cells being in the final stationary phase are capable of rapid and effective biosynthesis of DHA [De Muynck *et al*., 2007; Claret & Bories, 1994]. Crystallization of high-purity dihydroxyacetone from a post-production mixture poses further difficulties linked with the presence of bacterial metabolites, claimed to substantially deteriorate the quality of the end product [Holst *et al*., 1985].

In view of the above restrictions in running glycerol biotransformation to DHA with microorganisms, the search for novel technological solutions that would enable utilizing the biochemical potential of acetic bacteria seems to be substantiated.

The reported study involved immobilization of *Gluconacetobacter xylinus* cells from the terminal stationary phase. This treatment enabled reducing the content of cellular metabolites synthesized during the phase of logarithmic growth in the post-production mixture. It additionally assured a high activity of glycerol dehydrogenase and facilitated the separation of biomass from the mixture containing the product of biotransformation. The objective of the study reported herein was to determine values of the initial concentration of glycerol in the culture medium, active acidity of the medium and time of culture assuring the highest quantity of DHA produced, all three being optimal for the course of glycerol biotransformation to DHA.

MATERIAL AND METHODS

Biological material

In the study use was made of acetic acid bacteria of *Gluconacetobacter xylinus* species which originated from a collection of pure cultures of the Department of Food Biotechnology and Microbiology, Warsaw Agricultural University, Warsaw, Poland.

Microbiological media

Three culture media were used in the study.

– Culture medium no. 1 composed of: yeast extract, 30 g/L and ethyl alcohol, 20 mL; pH 5.0. The culture medium was consolidated with agar addition of 8 g/L. The medium in the form of a slant was used for storage of *G. xylinus* strain at a temperature of 4°C, and in the liquid form – for proliferation of biomass [Burbianka *et al*., 1987].

– Culture medium no. 2 composed of: glycerol, 20 mL; yeast extract, 5 g/L; $(NH_4)_2SO_4$, 5 g/L; pH 5.0 [Hekmat *et al*., 2003; Nabe *et al*., 1979]. The medium was used for multiplication of *G. xylinus* biomass and simultaneous activation of glycerol dehydrogenase.

– Culture medium no. 3 composed of: yeast extract, 5 g/L; $(NH_4)_2SO_4$, 7.5 g/L; and KH_2PO_4, 2.5 g/L, [Wei *et al*., 2007; Tkač *et al*., 2001; Wethmar & Deckwer, 1999], enriched with glycerol at concentrations of 30, 50, 70 or 100 g/L; pH 5.0, 7.0 or 8.0. The culture medium was used to run glycerol biotransformation into dihydroxyacetone by *G. xylinus*. This was the so-called "production medium".

The culture media were sterilized at a temperature of 121°C for 20 min.

Preparation of inoculum (I) and inoculum (II)

In order to prepare inoculum (I), 100 mL of liquid culture medium no. 1 were collected into 500-mL bottom-flat round flasks, and the culture media were inoculated with the biomass of acetic bacteria from the slant. The culture of inoculum was run at a temperature of 28°C for 96 h.

In order to prepare inoculum (II), 90 mL of liquid culture medium no. 2 were collected into respective 500-mL bottom-flat round flasks and inoculated with 10 mL of inoculum (I). To prevent the formation of a cellulose film that would hinder the collection of biological material, glass beads 0.8 cm in diameter were added to the culture medium no. 2 (8 beads per flask). The culture was run at a temperature of 28°C, on a reciprocating shaker with a frequency of 200 cycles/min (E. Bühler SM-30 Control, Germany) until optic density has reached OD=1.6 (λ=600 nm), which usually occurred after *ca.* 48 h.

Immobilization of *Gluconacetobacter xylinus* cells

To this end, 100 mL of inoculum (II) were centrifuged for 10 min at 5000 ×*g* (Eppendorf 5804R centrifuge, Germany), whereas cell precipitate was rinsed with physiological salt and re-centrifuged (conditions as above). Bacterial cells were suspended in 10 mL of deionised water and mixed with a solution of sodium alginate (40 g of sodium alginate/L) at 1:1 ratio (v/v). From the cell suspension in sodium alginate beads of equal size were formed by direct instilling (using a syringe and a needle 0.1 cm in diameter) into 0.2 mol/L solution of calcium chloride (II). The immobilizate was left in the solution of calcium chloride (II) for 20 min for hardening, next separated from the solution and rinsed with aseptic distilled water [Tkač *et al*., 2001].

Biotransformation of glycerol to DHA by immobilized cells of *Gluconacetobacter xylinus*

The immobilized cells of *G. xylinus* were transferred into 100-mL post-production culture media no. 3. Cultures were run in 500-mL bottom-flat flasks at a temperature of 28°C on a reciprocating shaker with a frequency of 200 cycles/min (E. Bühler SM-30 Control, Germany). The biotransformation process spanned for 96 h.

Determination of DHA content in the production medium with the spectrophotometric method

The method is based on reducing capability of dihydroxyacetone. DHA reduces nitro groups of 3,5-dinitrosalicilic acid to amine groups, and is oxidized to 1,3-dihydroxypropionic acid itself. The resultant derivatives of 3,5-dinitrosalicilic acid display orange colour, the intensity of which depends on the concentration of the reducing compound being determined [Toczko & Grzelińska, 2001].

A solution of 3,5-dinitrosalicilic acid (g/L) was prepared by mixing: 10 g of 3,5-dinitrosalicilic acid, 200 mL of 2 mol/L NaOH, and 300 g of sodium-potassium tartrate.

The sample examined (2 mL) was mixed with 3,5-dinitrosalicilic acid at the ratio of 1:1 (v/v) and incubated at 100°C for 10 min. Next, the samples were cooled and transferred quantitatively to 20 mL of water. Absorbance

was measured at a wavelength of 550 nm (Bio-Rad Smart Spec 3000 spectrophotometer, USA) against a reagent sample containing respective production medium instead of the exact sample. The concentration of DHA was read out from a standard curve with regression equation: A = 0.000435354*c-0.0179081.

Statistical analysis

Results obtained were subjected to a statistical analysis using Statgraphics Centurion XV computer software. Three-way analysis of variance was conducted and multiple comparisons were made with Tukey's LSD test (Leas Significant Differences) at a significance level of $\alpha=0.05$ [Mądry, 2003]. The analysis verified the effect of the initial content of glycerol in the production media, active acidity of the culture media and time of biotransformation on the concentration of synthesized DHA.

RESULTS AND DISCUSSION

The optimal concentration of glycerol needed for its efficient biotransformation to dihydroxyacetone ranges from 10 to 100 g/L. A higher concentration of glycerol may lead to impaired growth of acetic bacteria as a result of hyperosmotic stress as well as to diminished efficiency of biotransformation [Ohrem & Voss, 1995].

In the reported study, an attempt was made to conduct biotransformation at pH 5.0, which is optimal for the metabolic activity of *G. xylinus* [De Ley *et al*., 1984], and at such an active acidity that would assure a high activity of glycerol dehydrogenase. Literature data [Mishra *et al.*, 2008] report that in the case of this enzyme the optimal pH accounts for 7.0. Some other works show also that glycerol dehydrogenase exhibits the highest stability at the active acidity of culture medium reaching 8.0 [Adachi & Matsushita, 1997]. In view of these facts, in the presented study acidity of the production media accounted for pH 5.0 as well as for pH 7.0 and 8.0. Special attention was paid additionally to the optimization of time of running the biotransformation process. Transformation of glycerol to DHA at a temperature of 28°C usually takes 72 hours [Hekmat *et al*., 2003]. The use of free cells in an experiment may result in a negative effect of the product formed on glycerol dehydrogenase as well as in damage of cytoplasmic proteins responsible for diffusion of glycerol from the culture medium to the interior of bacterial cells [Deppenmeier *et al*., 2002]. The application of immobilized cells of acetic bacteria in the process of biotransformation may restrict this undesirable effect, thus allowing to extend the process of glycerol transformation into dihydroxyacetone [Deppenmeier *et al*., 2002]. The concentration of DHA being formed was monitored for 96 h. The production media differed in the initial concentration of glycerol (30, 50, 70 or 100 g/L) and in the initial value of pH (pH 5.0, 7.0 or 8.0). In total, the efficiency of biotransformation was determined for twelve production media with various composition. Three independent series of determinations were conducted for each medium, and each determination was performed in three replications.

Effect of the initial concentration of glycerol in the production medium on the concentration of dihydroxyacetone

Based on a three-way analysis of variance it was established that, at a significance level of $\alpha=0.05$, the content of dihydroxyacetone was significantly influenced by the initial concentrations of glycerol applied in the study, *i.e.* 30, 50, 70 and 100 g/L. Figure 1 depicts the mean concentration of DHA as affected by concentrations of glycerol applied for different pH values (5.0, 7.0, 8.0) and time of biotransformation (96 h).

A null hypothesis H_0, assuming that the applied concentrations of glycerol did not differentiate mean concentrations of the formed DHA, was verified in the study.

F_{emp} read out from the table of analysis of variance (Table 1) accounted for 27186.43, whereas $F_{\alpha ve}$ read out from statistical tables for t-Student's distribution [Zieliński, 1996] accounted for 3.1824, hence $F_{emp} \geq F_{\alpha ve}$. This result enabled rejection of the null hypothesis. In addition, the study demonstrated that at the significance level of $\alpha=0.05$, the varied dose of glycerol in the production media affected the concentration of dihydroxyacetone to a significant extent.

Effect of the initial active acidity of production media on the concentration of dihydroxyacetone

In the reported study, the three-way analysis of variance demonstrated that, at a significance level of $\alpha=0.05$, the content of dihydroxyacetone was significantly influenced by the initial active acidity of production media, accounting for 5.0, 7.0 or 8.0. Figure 2 presents the mean concentration of DHA as affected by pH value of the culture media for different concentrations of glycerol (30, 50, 70 or 100 g/L) and time of biotransformation (96 h).

The statistical hypothesis H_1 assumed that the initial pH 5.0, pH 7.0 or pH 8.0 did not differentiate concentrations of dihydroxyacetone synthesized during biotransformation. The H_1 hypothesis was subjected to statistical testing with the use of the F test. The F_{emp} value read out from Table 1 was higher than the $F_{\alpha ve}$ value read out from statistical tables for t-Student's distribution ($F_{emp}=1453.85$, $F_{\alpha ve}=4.3027$). Hence, the H_1 hypothesis was rejected at the significance level of $\alpha=0.05$, which indicates that the initial value of pH in the production medium exerted a significant effect on the concentration of DHA.

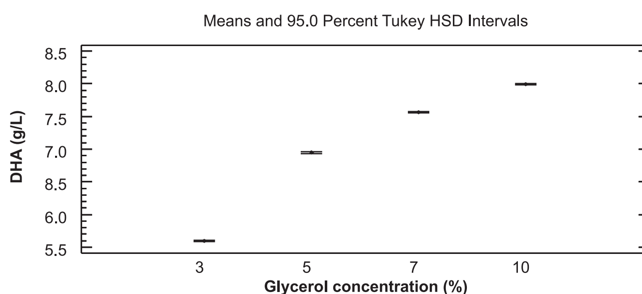

FIGURE 1. Mean values of DHA concentration at various concentrations of glycerol in the production medium at $\alpha=0.05$.

TABLE. 1. Results of three-way analysis of variance of dihydroxyacetone concentration depending on glycerol concentration, active acidity of production medium and time of biotransformation process.

Factors	Sums of squares of standard deviations	Degrees of freedom	Mean squares of standard deviations	F_{emp}	p-Value	$F_{\alpha ve}$
			Main effects			
A: glycerol (%)	2.11847E9	3	7.06157E8	27186.43	0.0000	3.1824
B: pH	7.55263E7	2	3.77631E7	1453.85	0.0000	4.3027
C: time (h)	1.8565E10	23	8.07173E8	31075.47	0.0000	2.0687
			Interactions			
AB	3.40964E7	6	5.68273E6	218.78	0.0000	2.4469
AC	5.70575E8	69	8.2692E6	318.36	0.0000	1.9997
BC	1.35074E7	46	2.936380	11.30	0.0000	2.0130
ABC	3.03435E7	138	219880	8.47	0.0000	1.9600
Others	5.98455E7	2304	25974.6	-	-	-
Sum	2.14674E10	2591	4.49826	-	-	-

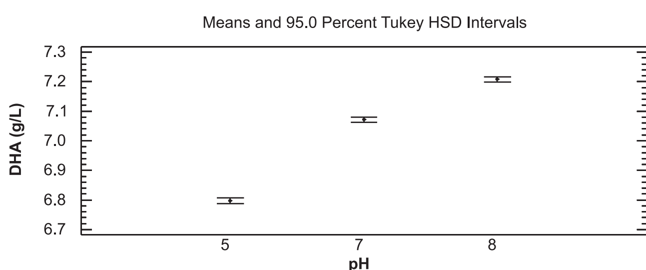

FIGURE 2. Mean values of DHA concentration at various active acidity of the production medium at α=0.05.

Effect of the time of biotransformation on the concentration of dihydroxyacetone

Based on a three-way analysis of variance it was established that, at a significance level of $\alpha=0.05$, the content of dihydroxyacetone was significantly influenced by the time span of the biotransformation process (96 h). Figure 3 depicts the mean concentration of DHA as affected by the time of biotransformation for varied pH values (5.0, 7.0, 8.0) and concentrations of glycerol (30, 50, 70 and 100 g/L) in the production media.

The formulated statistical hypothesis H_2, which assumed that the time of conducting the biotransformation process did not differentiate mean values of dihydroxyacetone concentrations, was subjected to statistical testing with the use of the F test, at a significance level of $\alpha=0.05$.

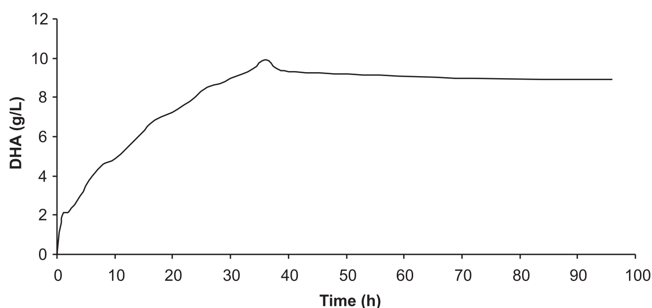

FIGURE 3. Mean values of DHA concentration as affected by duration of biotransformation process at α=0.05.

The F_{emp} value read out from Table 1 ($F_{emp}=31075.47$) was higher than the $F_{\alpha ve}$ value read out from statistical tables for t-Student's distribution ($F_{\alpha ve}=2.0687$). For $F_{emp} \geq F_{\alpha ve}$, the H_2 hypothesis was rejected, which demonstrated that the time of the biotransformation process had a significant effect on the concentration of DHA.

Effect of the interaction of factors of biotransformation medium on the concentration of dihydroxyacetone

Another four hypotheses were formulated: H_3, H_4, H_5, and H_6, for which statistical reasoning was conducted with the F test at a significance level of $\alpha=0.05$.

The H_3 hypothesis assumed that there was no interaction between glycerol concentration and medium pH, meaning that all potential mean values of DHA concentration for levels of one of the two factors were respectively equally independent of the level of the second factor. In that case, $F_{emp}=218.78$ (Table 1) and $F_{\alpha ve}=2.4469$, hence $F_{emp} \geq F_{\alpha ve}$, which enabled rejection of the H_3 hypothesis.

The H_4 hypothesis assumed that there was no interaction between glycerol concentration and time of biotransformation, meaning that all potential differences in the concentration of DHA for levels of one of the factors were respectively equally independent on the levels of the second factor. In that case, $F_{emp}=318.36$ (Table 1) and $F_{\alpha ve}=1.9997$, thus $F_{emp} \geq F_{\alpha ve}$, which allowed us to reject the H_4 hypothesis.

The H_5 hypothesis assumed that there was no interaction between pH value of the medium and time of biotransformation, meaning that all potential mean values of DHA concentration for levels of one of the two factors were respectively equally independent of the levels of the second factor. In that case, $F_{emp}=11.30$ (Table 1) and $F_{\alpha ve}=2.0130$, hence $F_{emp} \geq F_{\alpha ve}$ and the H_5 hypothesis was rejected.

The H_6 hypothesis assumed that there was no interaction between the concentration of glycerol, pH of the medium and time of biotransformation, meaning that there was no modification of two out of three examined factors by the third factor.

In that case, $F_{emp}=8.47$ (Table 1) and $F_{\alpha ve}=1.9600$, thus $F_{emp} \geq F_{\alpha ve}$, which allowed us to reject the H_6 hypothesis.

In the light of the above results, it was concluded that:

– interaction of the applied concentrations of glycerol in the production media and pH values of the media was statistically significant,

– interaction of the applied concentrations of glycerol and time of biotransformation was statistically significant,

– interaction of pH value of the production media and time of biotransformation was statistically significant,

– interaction of the applied concentrations of glycerol, pH values and time of biotransformation was statistically significant.

Evaluation of the effect of biotransformation parameters on the concentration of dihydroxyacetone in production media

In order to determine optimal conditions for the process of glycerol biotransformation to DHA with the use of immobilized cells of *G. xylinus*, the concentration of the product being formed was compared as affected by the applied parameters of incubation in the production media.

Once running glycerol biotransformation to DHA at pH 5.0, the concentration of dihydroxyacetone produced was the highest at the initial content of glycerol reaching 100 g/L and accounted for 10.9 g/L after 36 h of the reaction (Figure 4). After the first 16 h of the process, the concentration of the produced dihydroxyacetone was alike in the media containing 50, 70 and 100 g glycerol/L (Figure 4). The least and, simultaneously, the slowest increase in DHA concentration was observed in the production medium with the initial concentration of glycerol reaching 30 g/L.

In the media with active acidity of 7.0 or 8.0, assuring high activity and stability of glycerol dehydrogenase, the content of DHA was also the highest at the initial concentration of glycerol reaching 100 g/L and accounted for 10.5 g/L (after 36 h) and for 10.7 g/L (after 34 h), respectively (Figures 5 and 6).

Irrespective of medium pH applied, the concentration of the synthesized dihydroxyacetone was observed to increase along with the increasing initial concentration of glycerol in the production medium. The optimal time span of the process reached 36 h and its successive extension led to diminished concentrations of the product (Figures 4-6). This phenomenon might have been due to the inclusion of glycerol and dihydroxyacetone into the pentose phosphate pathway and further transformations of these compounds [Mishra *et al.*, 2008].

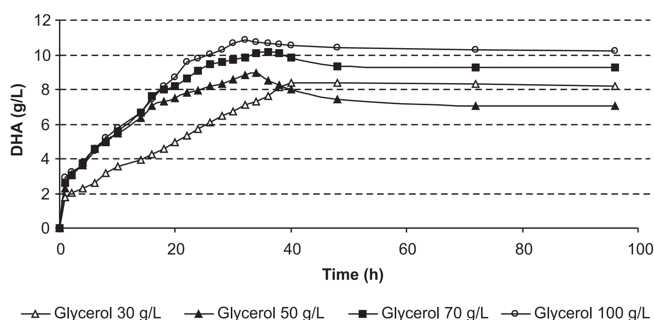

FIGURE 4. Effect of substrate concentration and process duration on the efficiency of glycerol biotransformation to dihydroxyacetone in the medium with pH 5.0 using immobilized cells of *G. xylinus*.

The application of the initial concentration of glycerol of 30 g/L as well as pH 7.0 or pH 8.0 resulted in a higher concentration of the product formed as compared to the media with the same composition but lower pH (pH 5.0). A similar tendency was observed in the case of results obtained for the production medium containing 50 g of glycerol/L with active acidity of 5.0, 7.0 and 8.0 (Figure 7).

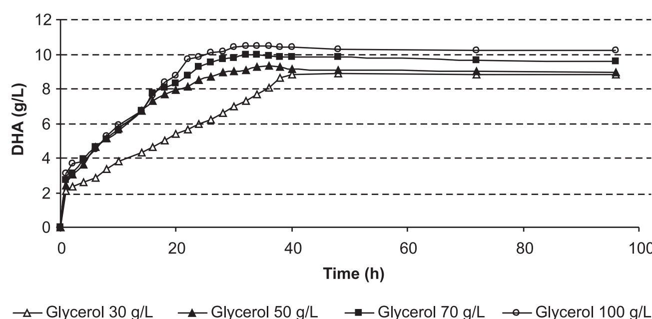

FIGURE 5. Effect of substrate concentration and process duration on the efficiency of glycerol biotransformation to dihydroxyacetone in the medium with pH 7.0 using immobilized cells of *G. xylinus*.

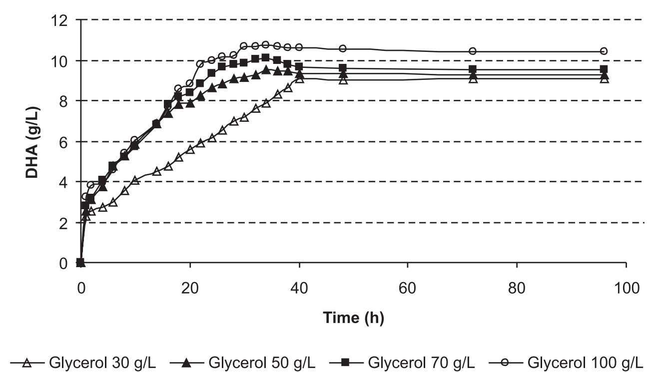

FIGURE 6. Effect of substrate concentration and process duration on the efficiency of glycerol biotransformation to dihydroxyacetone in the medium with pH 8.0 using immobilized cells of *G. xylinus*.

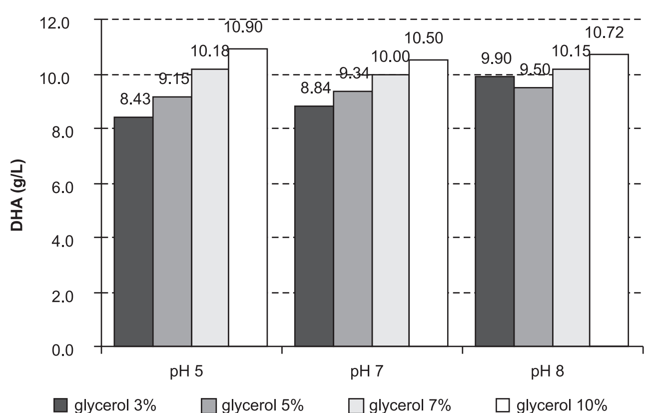

FIGURE 7. Concentration of dihydroxyacetone as affected by the value of active acidity and glycerol concentration in the production medium.

The highest concentrations of dihydroxyacetone obtained in the production media containing 100 g of glycerol/L did not differ significantly between one another, irrespective of the pH values of media applied. Similar observations were made for the other mean contents of DHA in the production media. At substrate concentrations applied in the study (30, 50, 70 or 100 g/L), irrespective of pH, the differences noted between concentrations of DHA turned out to be minute, though statistically significant. In the production medium with pH 5.0 and initial concentration of glycerol reaching 50 g/L, the maximum concentration of DHA accounted for 9.15 g/L (after 28 h). In the same medium but with pH 7.0, the highest noted content of DHA (differing significantly) was higher by 0.19 g/L and reached 9.3 g/L after 36 h of biotransformation. In turn, in the medium with pH 8.0 containing 50 g of glycerol/L, the concentration of dihydroxyacetone increased by 0.16 g/L (after 34 h) as compared to its concentration determined in the production medium with pH 7.0. In the case of the production media with the initial glycerol concentration of 50 g/L and various pH (5.0 or 8.0), the highest concentrations of DHA produced differed by 0.34 g/L and accounted for 9.15 and 9.50 g/L, respectively (Figure 7).

The above results indicate that the initial concentration of glycerol differentiated the content of dihydroxyacetone formed to a various extent and that in the biotransformation process it played a more significant role than active acidity of the production media.

The study discussed herein attempted to determine optimal conditions that would assure reaching a high concentration of dihydroxyacetone in the production media. It did not involve determinations of glycerol content in the course of the process for it has been assumed that the substrate was utilized completely. Assuming that – quantitatively – glycerol was oxidized to dihydroxyacetone, it may be concluded that the highest efficiency of biotransformation reaction was reached in the case of the medium with the initial glycerol concentration of 30 g/L. In that case, irrespective of active acidity of the media applied, the efficiency of the reaction reached 30%. Along with an increase in the initial concentration of glycerol in the medium to 50 or 70 g/L, the mean efficiency of the reaction accounted for *ca.* 19% and *ca.* 14% at the pH values applied. The lowest reaction efficiency (*ca.* 11%) was noted in the culture medium with the initial concentration of glycerol reaching 100 g/L. Thus, the application of the lower initial concentration of glycerol enabled running the biotransformation process with a higher efficiency as compared to the reaction proceeding in the media with the higher initial concentration of glycerol. This observation is consistent with findings of other authors [Wei *et al*., 2007]. It cannot be excluded, however, that in all discussed cases only part of the substrate was not transformed into DHA, hence the efficiency of the biotransformation process might have been higher.

The study demonstrates that the increase in glycerol concentration in the culture medium is accompanied by a decrease in process efficiency. The same conclusion was drawn in 1995 by Hauge and colleagues who carried out biotransformation with the use of free cells of acetic bacteria [Hauge *et al*., 1995]. In their study, the concentration of glycerol in the medium was 60 g/L and active acidity reached 5.0, and the efficiency of the process accounted for 97% after 30 h.

In the year 1926, Virtanen and Barlund conducted one of the first experiments exploiting free cells of acetic bacteria for biosynthesis of DHA. They demonstrated that in a 30-day stationary culture these microorganisms were oxidizing glycerol to dihydroxyacetone with 84% efficiency. The culture medium applied contained 30 g of glycerol/L and its pH reached 5.0 [Rainbow, 1961]. A few years later Kluyver noticed that aeration of culture shortened considerably the time of biotransformation and that at the initial concentration of glycerol ranging from 20 to 50 g/L, reaction efficiency may account for 95% [Kluyver, 1931]. Investigations into the feasibility of applying immobilized cells of acetic bacteria in that process have begun in the 1980ies. In order to optimize conditions of this transformation, a comparison was made between processes with the use of free and immobilized cells of acetic bacteria [Raška *et al*., 2007; Nabe *et al*., 1979]. These experiments were conducted for *Acetobacter xylinum* strain immobilized in polyacrylamide gel [Nabe *et al.*, 1979] and demonstrated that pH in the range of 4.0-5.0 was optimal for the immobilized cells, whereas that of 5.5 – for the free cells. The efficiency of glycerol biotransformation to DHA with the use of the immobilized cells of *A. xylinum* was higher even by 18% than the efficiency of reaction proceeding with the free cells and reached *ca.* 80% after 40 h of the process [Nabe *et al*., 1979].

The results presented above indicate explicitly that in the optimization of the process of glycerol oxidation to dihydroxyacetone, apart from determination of the initial dose of substrate, of equal importance is also appropriate selection of the other conditions of the reaction, *e.g.* time of reaction should not exceed 40 h, otherwise dihydroxyacetone kinase is activated, which leads to phosphorylation of DHA, whilst active acidity of culture media (though it did not affect significantly reaction efficiency) should be optimal for acetic bacteria metabolism and reach 5.0.

Apart from the parameters of glycerol biotransformation to DHA discussed in this work (concentration of substrate, active acidity, time span of the process), efficiency of the reaction may also be affected by other factors, including *e.g.*: size of beads formed during immobilization of bacterial cells in calcium alginate (the smaller the beads, the greater the surface of gaseous exchange and substrate-enzyme-product reaction), type of carrier used for immobilization (carriers displaying greater porosity enable better gaseous exchange), parameters of aearation or culture volume [Mishra *et al*., 2008]. Efficiency of that type of reactions may be increased by the addition of vitamins or cations to the culture medium that enhance the activity of glycerol dehydrogenase. Wethmar & Deckwer [1999] observed that the addition of a vitamin mix (*i.a.* vitamin B12, folic acid, nicotinic acid, riboflavin) to the production medium enabled reaching DHA concentration at a level of 15.5 g/L.

SUMMARY

Contemporarily, a number of organic compounds are produced with microbiological methods that, successively,

displace expensive chemical synthesis. Control over biotechnological procesess by assuring appropriate parameters of active acidity, temperature or composition of production medium enables conducting highly efficient reaction at low financial inputs. An example of such a reaction is biotransformation of glycerol to dihydroxyacetone, proceeding with acetic bacteria of the genus *Gluconacetobacter xylinus* [Bauer *et al*., 2005].

Results of the study reported herein allow concluding that the applied concentrations of glycerol (30 g/L, 50 g/L, 70 g/L, 100 g/L), level of active acidity of production medium (5.0, 7.0, 8.0) and time span of the biotransformation process have a significant effect on the concentration of synthesized DHA. Furthermore, it has been established that elongation of glycerol biotransformation to DHA to over 36 h resulted in diminished concentration of DHA in the production medium. This may point to the inclusion of DHA into successive metabolic pathways proceeding in cells of *G. xylinus*.

The increase in the concentration of DHA upon the application of lower initial concentrations of glycerol in the production media and pH value optimal for stability and activity of glycerol dehydrogenase, afford a possibility of reducing costs of DHA production. Immobilization of *G. xylinus* cells certainly facilitates the later recovery of a high-quality product from the post-production mixture and enables conducting a few cycles of the production process without the necessity of re-proliferation of biomass.

Another solution improving the efficiency of the described process may be immobilization of bacterial enzymes responsible for the process of glycerol transformation to dihydroxyacetone. The enzymatic preparation prepared in this way may be applied for transformation of an aqueous solution of glycerol into DHA. This procedure would eliminate contaminations of bacterial origin, which in turn would facilitate considerably the subsequent crystallization of DHA. It would additionally enable reducing costs linked with multi-stage proliferation of acetic bacteria cells. In the future, it is worth undertaking attempts of inhibiting the activity of enzymes present in the biomass of acetic bacteria and responsible for further transformation of the synthesized dihydroxyacetone. The sequence of *G. xylinus* genes participating in glycerol transformation to dihydroxyacetone has not been recognized so far, therefore they may be inactivated with non-oriented methods of mutagenization, including the application of physical (*e.g.* UV radiation) or chemical (*e.g.* nitrosoguanidine) factors, followed by the search for mutants characterized by a higher capability for glycerol transformation into DHA than in the parental strain.

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