APPLICATION OF IMMOBILIZED CELL PREPARATION OBTAINED FROM BIOMASS OF *GLUCONACETOBACTER XYLINUS* BACTERIA IN BIOTRANSFORMATION OF GLYCEROL TO DIHYDROXYACETONE

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Introduction. Dihydroxyacetone (DHA), being a product of glycerol oxidation by acetic acid bacteria, is an important compound widely applied in the cosmetic, food, and pharmaceutical industry, as well as in medicine. Biotransformation of glycerol to DHA is catalyzed by glycerol dehydrogenase (GlyDH, EC 1.1.1.6) bound with the cytoplasmic membrane of bacteria. An attempt was undertaken in this study to conduct glycerol biotransformation with immobilized fractions of a cell preparation with GlyDH activity. The content of dihydroxyacetone obtained with the cell preparation was compared with its content achieved in the reaction with immobilized viable cells of *G. xylinus*.

Material and methods. Cell walls of *Gluconacetobacter xylinus* bacteria were disintegrated enzymatically. The resultant preparation was immobilized on calcium alginate or first separated into two fractions (precipitate and supernatant) by centrifugation and then immobilized. DHA content was determined colorimetrically after the reaction with 3,5--dinitrosalicilic acid. Glycerol content was assayed with the refractometric method.

Results. After 20 days of the process, the concentration of DHA obtained with immobilized whole cells reached 25 g/l. In turn, the content of DHA obtained in the same period with immobilized fractions of the cell preparation accounted for 16.9 g/l and 8.95 g/l (depending on the fraction applied).

Conclusions. DHA may be obtained in the process independent of *G. xylinus* metabolic activity using a preparation which displays the catalytic activity of glycerol dehydrogenase and obtained as a result of disintegration of live bacterial cells. The application of such a preparation may in the future eliminate technological problems posed by the presence of bacterial cells and their metabolites in the culture medium.

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

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INTRODUCTION

Immobilization consists in immobilizing cells of microorganisms, enzymes or other proteins inside or on the surface of a carrier in a way that enables preserving their catalytic activity [Jack and Zajic 2006]. The major advantage of immobilization is easy separation of biological material from the reaction medium containing the desired product. It thus enables the final filtration or centrifugation, which considerably accelerates the production process of a variety of compounds [Martynenko and Gracheva 2003].

The selection of a carrier for immobilization is determined by the type of biological material and methods of immobilization [Survase et al. 2010]. Porous materials are used the most frequently, for their enable a free contact of substrate with an immobilized biocatalyst, including carrageenan, polyacrylamide gel and calcium alginate [Raška et al. 2007, Garbayo et al. 2004]. Another advantage resulting from the application of such technological solutions is cost-effectiveness of the reaction. Immobilized cell biomass may be re-applied several times without the necessity of its re-proliferation. Nowadays, immobilized cells of selected bacteria are used, among others, for the production of vinegar, ethyl alcohol, propionic acid, citric acid, as well as in the dairy industry and in fruit and vegetable processing.

Despite multiple advantages of using the immobilized biological material in biotransformation processes, these reactions still need to be improved. Restrictions result mainly from partial loss of the activity of immobilized cells or proteins, material leaching from a carrier, and clogging of carrier pores which impairs the contact between a substrate and a biocatalyst [De Muynck et al. 2007].

Dihydroxyacetone (1,3-dihydroxy-2-propanone, DHA) is a ketotriose with reducing properties. It has been found applicable in cosmetology as the main constituent of self-tanning creams [Mishra et al. 2008, Biondi et al. 2007]. The presence of DHA in this type of products enables achieving the effect of tan without the necessity of exposing skin to detrimental action of sunrays. The effect of skin browning in contact with dihydroxyacetone is due to the non-enzymatic reaction of amine groups of arginine present in epidermis with carbonyl groups of reducing sugars [Shipar 2006]. This process results in the formation of mixtures of aroma compounds and high-molecular melanoidin pigments [Petersen et al. 2003]. Dihydroxyacetone is also applied in food production as a sweetener and as a dietary supplement for sportsmen [Omar et al. 2005, Cortez et al. 1991]. Furthermore, in treatment of cyanide-induced intoxications it serves as a respiration-stimulating agent [Niknahad and Ghelichkhani 2002]. It may also be used in therapy of diabetes and as an intermediate product in the production of some drugs and dyes [Levy 2000]. As provided by the Ministry of Science and Higher Education in the Report elaborated in 2007 by the Interdisciplinary Expert Group for Development of Bioeconomy, dihydroxyacetone is currently obtained exclusively with the biotechnological method, which involves biotransformation of glycerol by a strain of acetic acid bacteria – *Gluconobacter oxydans* ATCC 621. Literature data [Deppenmeier et al. 2002, Ruzheinikov et al. 2001] indicate that also other genera of bacteria belonging to the family *Acetobacteriaceae* may in part oxidize glycerol to DHA. This capability is, however, determined by the presence of glycerol dehydrogenase (GlyDH, EC 1.1.1.6) bound with the cytoplasmic membrane of bacteria, being an oxido-reducing enzyme which catalyzes this biotransformation [Matsushita et al. 2003].

Attempts have been made in the past to produce dihydroxyacetone using strains of the genera *Acetobacter suboxydans* and *Gluconobacter melanogenus* [Claret and Boris 1994, Charney and Montclair 1978, Flickinger and Perlman 1977]. A research conducted by Nabe et al. [1979] indicates, however, that the highest activity of glycerol dehydrogenase was found for the strain *Gluconacetobacter xylinus* (*Acetobacter xylinum*).

Until recently, investigations addressing this problem [Raška et al. 2007, Tkač et al. 2001] have been conducted with the use of free or immobilized cells of acetic acid bacteria capable of glycerol transformation into DHA, and their authors have usually been focused on the optimization of process parameters that would assure a high yield of biotransformation. Studies aimed at reducing costs of this transformation [Mishra et al. 2008, Hekmat et al. 2003] have consisted in re-application of free or immobilized cell biomass and in adjusting the main parameters of the process. Special attention has been paid to the degree of biocatalysis medium aeration and to the initial concentration of substrate. Attempts have also been undertaken to replace glycerol with waste products of the agricultural industry [Wei et al. 2007, Claret et al. 1992].

The presented study is the first ever attempt to apply an immobilized cell preparation with the activity of glycerol dehydrogenase, obtained as a result of enzymatic disintegration of cell walls of acetic acid bacteria of *Gluconacetobacter xylinus* strain, for biotransformation of glycerol to dihydroxyacetone. It enabled making the course of the reaction independent of the metabolic activity of free cells of acetic acid bacteria. In addition, the immobilization facilitated the separation of the final product from the post-reaction mixture, which improved the entire process of biotransformation.

AIM OF THE STUDY

The aim of this study was to produce a cell preparation from biomass of *Gluconacetobacter xylinus* bacteria by means of enzymatic disintegration of their cell walls, and to immobilize it in calcium alginate. Next, analyses were carried out to determine whether the resultant immobilized preparation may serve as a biocatalyst in the reaction of glycerol oxidation to dihydroxyacetone, and to identify which of its fractions were characterised by the highest capability to catalyze this transformation. The concentration of dihydroxyacetone obtained as a result of biotransformation conducted with the immobilized cells of *G. xylinus,* was further compared to DHA concentration obtained with the immobilized cell preparation of the examined strain of acetic acid bacteria.

MATERIAL AND METHODS

Biological material

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

Culture and production media

Culture medium in the form of a slant for *G. xylinus* storage: Wort Agar 55 g/l (Merck KGaA, catalogue No. 1054480500), acetic acid 10 $cm^3 \cdot l^{-1}$, ethyl alcohol 30 g/l, and pH = 5.0. The incubation was carried out at a temperature of 28°C for 24-48 h. Afterwards, the strain was stored at a temperature of 4°C and refreshed every 21 days.

Liquid culture medium from biomass proliferation (preparation of inoculum): yeast extract – 30 g/l, ethyl alcohol – 20 g/l, pH = 5.0. The medium was inoculated with the biomass from the slant. Incubation was run in 50 cm^3 of the culture medium, in 500 cm^3 bottom-flat round flasks at a temperature of 28°C for 24 h, with reciprocating aeration applied with the intensity of 200 cycle/min. To prevent the production of bacterial cellulose, non-cut glass beads 5.0 mm in diameter were added to the culture medium (20 beads per flask).

Liquid culture medium for biomass proliferation and activation of glycerol dehydrogenase: glycerol – 20 g/l, yeast extract – 5 g/l, $(NH_4)_2SO_4$ – 5 g/l, pH = 5.0. The medium was inoculated with the inoculum at the ratio of 1:50 (v/v). Incubation was run in 150 cm^3 of the culture medium, in 500 cm^3 bottom-flat round flasks at a temperature of 28°C for 48 h (until reaching $OD_{600} \approx 1.2$). The other parameters of the incubation were the same as these described for inoculum preparation.

Production media for biotransformation: glycerol – 30 g/l or 50 g/l, pH = 5.0 (for immobilized cells of *G. xylinus*) or 7.5 (for immobilized cell preparation).

All media were sterilized at a temperature of 121°C for 15 min.

Enzymatic disintegration of *G. xylinus* cells

The strain was multiplied and glycerol dehydrogenase was simultaneously activated. The biomass was centrifuged (10 min, 13,000×g at a room temperature) and rinsed 4 times with sterile distilled water. Next, 50 µl of protease inhibitors were added to 1 g of centrifuged and rinsed biomass, and the biomass was suspended in 15 cm^3 of sterile distilled water. Afterwards, 0.15 g of lysozyme was added (MP Biomedicals, catalogue No. LYSO 005), and the biomass was vortexed and incubated for 2 h at a temperature of 37°C, with mixing every 5 min.

Determination of the concentration of cellular proteins with the Lowry's method

After enzymatic disintegration of *G. xylinus* cells, the concentration of cellular proteins was determined with a modified Lowry's method [Lowry et al. 1951]. This method is based on the colour reaction of peptide bonds with copper ions of the Folin-Ciocalteau reagent. In this reaction, phosphomolybdic acid and phosphotungstic acid are reduced to phosphomolybdic blue under the influence of tyrosine, tryptophan and cysteine. The intensity of colour of the complex thus formed depends on protein concentration in the sample. The analysed 1 cm^3 sample was mixed with 5 cm^3 of a copper reagent (i.e. 98 cm^3 of Na_2CO_3 solution in 0.1 M NaOH (20 g/l); 1 cm^3 of an aqueous solution of sodium potassium tartrate (20 g/l); 1 cm^3 of an aqueous solution of $CuSO_4 \cdot 5 H_2O$ (10 g/l), and incubated at a room temperature for 10 min. Next, 0.5 cm^3 of Folin--Ciocalteau reagent was added and the sample was incubated again at a room temperature for 30 min. Absorbance was measured at a wavelength of 750 nm against a control sample, i.e. water with the addition of lysozyme. Protein concentration in the sample

was read out from a standard curve with regression equation: $A = 0.00581594 + 0.00167696 \cdot c$.

Immobilization of *G. xylinus* cells in calcium alginate

Cells of acetic acid bacteria *G. xylinus* from 48-h culture on the medium activating glycerol dehydrogenase were centrifuged (10 min, 13,000×g at a room temperature), rinsed 4 times with sterile distilled water, and re-centrifuged. The precipitate was suspended in 15 cm^3 of sterile distilled water, and then mixed with a sodium alginate solution (40 g/l) at the ratio of 1:1 (v/v). From bacterial suspension in sodium alginate, beads of the same size were formed by direct instilling (using a syringe and a needle 0.9 mm in diameter) to a 0.2 M solution of calcium chloride(II). The immobilizate was left in the solution of calcium chloride(II) at a temperature of 4°C for 20 min for hardening, and then rinsed with sterile distilled water.

Immobilization of a cell preparation from *G. xylinus* in calcium alginate

Immobilization was applied to three types of the cellular fraction obtained as a result of enzymatic digestion of *G. xylinus* cell walls: suspension obtained immediately after disintegration, cell precipitate (suspended in 15 cm^3 of sterile distilled water) obtained after centrifugation of disintegrated cells (90 min, 13,000×g, 4°C), and the resultant supernatant. The process was conducted at a temperature of 4°C as described earlier.

Biotransformation of glycerol to dihydroxyacetone

The immobilized biological material was transferred to 500 cm^3 bottom-flat round flasks containing 150 cm^3 of an appropriate production medium. Biotransformation was conducted at a temperature of 28°C (for immobilized cells of *G. xylinus*) or at 23°C (for the immobilized cell preparation) for 4 or 20 days, with aeration through reciprocating shaking with the intensity of 200 cycle/min.

Determination of dihydroxyacetone concentration with the colorimetric method

This method is based on the reducing properties of dihydroxyacetone. DHA is capable of reducing nitric groups of 3,5-dinitrosalicylic acid to amine groups, and itself is oxidised to 1,3-dihydroxypropionic acid. The resultant derivatives of 3,5-dinitrosalicylic acid display an orange colour, the intensity of which depends on the concentration of the reducing compound being determined [Summer 1921].

The analytical sample (2 cm^3) was mixed with 3,5-dinitrosalicylic acid (i.e. solution of 3,5-dinitrosalicylic acid: 3,5-dinitrosalicylic acid 10 g/l, 2M NaOH 200 cm^3·l^{-1}; sodium potassium tartrate 300 g/l) at the ratio of 1:1 (v/v) and incubated at 100°C for 10 min. Afterwards, the samples were cooled and transferred quantitatively to 20 cm^3 of water. Absorbance was measured at a wavelength of 550 nm against the control sample containing appropriate production medium. DHA concentration was read out from a standard curve with regression equation: $A = 0.0005 \cdot c - 0.0539$.

Determination of glycerol concentration with the refractometric method

This method is based on the measurement of a refractive index, being a ratio of the speed of light in two media with different optical properties [Wong et al. 2009]. The index equals to the ratio of sinuses of the angles of incidence and refraction equivalent to the ratio of velocities in two media. A digital refractometer (ATAGO, Japa) was used in these assays. Glycerol concentration was read out from a standard curve with regression equation: $Brix° = 0.00132948 + 0.999711 \cdot c$.

Determination of the yield of glycerol biotransformation to dihydroxyacetone

The yield of biotransformation was computed based on the equation of a chemical reaction of glycerol oxidation to DHA, which indicates that 92 g·mol^{-1} glycerol enable producing 90 g·mol^{-1} dihydroxyacetone. Knowing the concentration of glycerol in the production media before and after the reactions, theoretical concentration of DHA was calculated assuming 100% process yield. Results of determinations of DHA concentration in the production media were then used to calculate the real yield of the biotransformation process.
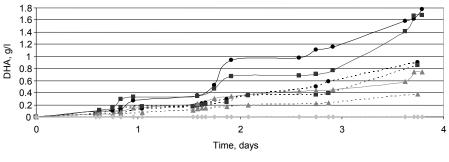
RESULTS AND DISCUSSION

Dihydroxyacetone synthesized as a result of glycerol oxidation by cells of acetic acid bacteria has been isolated for the first time by Bertrand in 1904. Its biosynthesis was then run with the strain *Acetobacter suboxydans* (*Gluconobacter oxydans*). The production medium applied contained 40-55 g of glycerol/l, active acidity reached 5.2 and the process spanned for 14 days [Mishra et al. 2008]. In later studies [Hekmat et al. 2003], scientists have postulated optimal conditions of glycerol biotransformation to DHA and paid special attention to the selection of the appropriate concentration of substrate and active acidity of the reaction medium. Research conducted in the past demonstrated that glycerol concentration assuring the apt course of biotransformation should range between 20 and 50 g/l [Nabe et al. 1979]. A higher concentration of the substrate may contribute to impaired growth of acetic acid bacteria as a result of hyperosmotic stress and to decreased biotransformation yield [Ma et al. 2010].

The reaction of glycerol biotransformation to DHA by bacteria of the genus *Gluconacetobacter*, is catalyzed by glycerol dehydrogenase (GlyDH, EC 1.1.1.6) [Mishra et al. 2008]. This enzyme belongs to groups of pyrroloquinoline quinine (PQQ)-dependent dehydrogenases that are bound with the cytoplasmic membrane of bacteria [Lapenaite et al. 2005]. PQQ serves a function of a co-factor for most of dehydrogenases that catalyze oxidation of saccharides in the periplasmic space of bacterial cells [Hölscher and Görisch 2006]. The active centre of GlyDH is located in the periplasma, which enables eliminating the energy-consuming process of substrate transportation to cells and that of products outside the cell [Gätgens et al. 2007, Bauer et al. 2005].

The objective of this study was, among other things, to facilitate glycerol transformation into DHA and to make this process independent of the presence of viable bacterial cells. After disintegration, the suspension of acetic acid bacteria cells was immobilized in calcium alginate and used immediately for biotransformation. The enzyme was

not extracted nor purified deliberately, for these treatments extend the duration of the entire process and considerably increase its costs. Procedures of isolation of this type of enzymes elaborated so far cannot be applied in the industrial scale [Lapanaite et al. 2005]. The isolated enzyme displays a lower activity as compared to the non-purified membraneous fraction in which it occurs. GlyDH displays the highest activity in pH range of 7.0-7.5 and in temperature range of 20-25°C [Lapanaite et al. 2005, Adach et al. 1988], for this reason in the reported study biotransformation with immobilized fractions of the cell preparation was conducted on media with pH 7.5 and at a temperature of 23°C. The arithmetic mean of measurements made for the concentration of protein present in the cell preparation from *G. xylinus* accounted for 4.9 mg·cm^{-3} of preparation.

In the reported experiment, the initial concentration of glycerol reached 30 g/l or 50 g/l, and biotransformation was terminated on day 4 (after 90 hours of the process; Fig. 1).

Fig. 1. Effect of glycerol content in the production medium and type of immobilizate on changes in DHA concentration during biotransformation

Analyses carried in the study demonstrated tangible differences in DHA content in particular production media (Fig. 1). Cells of *G. xylinus* immobilized in calcium alginate that were inserted into the production medium containing 30 g of glycerol/l produced nearly twice more DHA than these in the medium with a higher content of glycerol (50 g/l). After 24 h of biotransformation, the concentration of DHA accounted for 0.27 and 0.14 g/l, respectively, whereas after 90 h – for 1.77 and 0.90 g/l, respectively. A similar tendency was observed in the case of the immobilized cell suspension obtained after disintegration. The insertion of the immobilized suspension in the medium with the initial glycerol content of 30 g/l enabled producing 0.33 g DHA/l after 24 h, 0.67 g DHA/l after 48 h, and 1.68 g DHA/l after 90 h of the process. In turn, in the production medium with the initial concentration of glycerol reaching 50 g/l, the production of DHA accounted for 0.18, 0.35 and 0.86 g/l, after the respective times of biotrans-

formation. The application of the 4-day biotransformation of immobilized cell suspension allowed to finally produce 0.75 g DHA/l (in the medium with 30 g of glycerol/l) and 0.38 g DHA/l (in the medium with 50 g of glycerol/l). The immobilized supernatant did not display the catalytic activity of glycerol dehydrogense, which confirms earlier reported data [Matsushita et al. 2003] that this enzyme is bound with the cytoplasmic membrane of bacteria. As a result of centrifugation of the disintegrated cells, fragments of cytoplasmic membranes occurred in the precipitate, thus were no longer present in the supernatant. At this stage of the study, it was determined that in the process of glycerol biotransformation to DHA proceeding with both immobilized viable cells of *G. xylinus* and immobilized fractions of the cell preparation, the initial concentration of the substrate reaching 30 g/l enabled higher production of DHA as compared to the initial glycerol concentration of 50 g/l. Based on this finding, a decision was made that further analyses would be conducted in the production media containing 30 g of glycerol/l.

Determination of the initial concentration of glycerol in the production medium is of key significance to the planning of this type of experiments. Excessive concentration of substrate may greatly impair or even make the reaction impossible as a result of inhibiting the biological activity of cells of acetic acid bacteria [Ma et al. 2010]. In an earlier investigations, attempts have been undertaken to increase the reaction's yield by applying high doses of the substrate (90-120 g/l) [De Muynck et al. 2007, Charney and Montclair 1978]. However, it has soon turned out that with the high concentrations of glycerol, cell divisions of acetic acid bacteria are disturbed and synthesis of DHA is becoming impossible. The increasing of the initial concentration of glycerol in the production medium results in elongation of reaction time needed to achieve a high concentration of dihydroxyacetone. Furthermore, increasing glycerol concentration over 50 g/l has been reported to cause partial loss of acetic acid bacteria capability to oxidize that compound [Mishra et al. 2008].

The subsequent stage of the study involved 20-day biotransformation in the production medium with glycerol concentration of 30 g/l (Fig. 2). Prior to immobilization, the biological material was twofold condensed. The optical density of cell suspensions prepared for the first stage of the study (spanning for 4 days) was 1.2 ($\lambda = 600$ nm). In turn, the OD of suspensions used for the 20-day biotransformation accounted for 2.5 ($\lambda = 600$ nm). The condensation of the biological material was aimed at increasing the concentration of bacterial glycerol dehydrogenase and, thus, at accelerating glycerol oxidation to DHA. According to the Michaelis-Menten's model describing kinetic properties of enzymes, the rate of product synthesis depends on the concentration of enzyme and that of substrate (in the case when substrate concentration is low as compared to K_m) or exclusively on the concentration of enzyme (in the case when substrate concentration is high as compared to K_m) [Stryer 2000].

Despite the increased concentration of the product (Fig. 2), the reaction was terminated after 20 days. The major reason of process termination was the leaching of bacterial cells from structures of the carrier to the reaction medium. However, the number of *G. xylinus* cells released to the medium during DHA production was not determined in this study. At the Department of Food Biotechnology and Microbiology at the Warsaw University of Life Sciences, investigations are under way to improve glycerol biotransformation to DHA by shortening the reaction time, hence the reported study was preliminary in character. Future studies are, therefore, aimed at elaborating such parameters of the biotransformation process that would enable reaching the maximum content of product in possibly the shortest period of time.
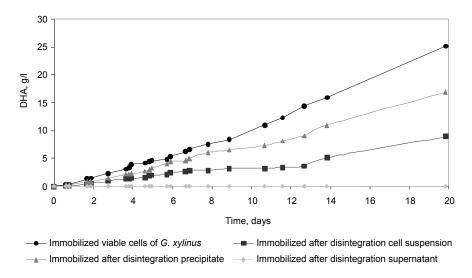
Fig. 2. Changes in DHA concentration in the production medium with the initial glycerol content of 30 g/l as affected by the time of biotransformation and type of immobilizate applied

For the first seven days of the experiment, samples for determination of glycerol and DHA concentrations were collected twice a day. Over the next week, one sample was collected a day. The last sample was collected on the twentieth day of biotransformation. Likewise in the first stage of the study (Fig. 1), the highest concentration of the product was achieved in the medium with immobilized viable cells of *G. xylinus* (Fig. 2). The reaction was run at pH optimal for acetic acid bacteria, i.e. pH 5.0, and at the temperature optimal for their growth, i.e. 28°C [Ahnert et al. 2000]. So adjusted parameters were supposed to assure a high metabolic activity of *G. xylinus* cells. In the case of the immobilizates, pH 7.4 and temperature of 23°C have been found optimal for preserving high activity and stability of glycerol dehydrogenase [Lapanaite et al. 2005]. The lack of nutrients in the production medium was aimed at facilitating determinations of the concentration of substrate and product, and – in the future – also DHA crystallization from the reaction medium.

Results of analyses depicted in Figure 2 show explicitly that the immobilized cells of *G. xylinus* were producing greater quantities of DHA than the immobilized fractions of the cell preparation. With the process progressing, the concentration of DHA in the production medium was observed to increase systematically. After ca. 70 hours of biotransformation, the concentration of DHA produced by the immobilized cells, cell suspension and precipitate accounted for: 2.34, 0.95 and 1.49 g/l. After 20 days of the process, the highest concentration of DHA (25 g/l) was determined in the production medium in which biotransformation proceeded with immobilized viable cells of acetic acid bacteria. In contrast, the lowest concentration of the product (16.9 g/l) was noted in the medium containing the precipitate immobilized after disintegration. Over the same period, in the production medium containing the suspension immobilized after disintegration the concentration of produced dihydroxyacetone reached 8.95 g/l (Fig. 2). In the first stage of the experiment, the yield of glycerol biotransformation to DHA was

higher for the immobilized suspension as compared to that noted for immobilized precipitate (Fig. 1). After twofold condensation of the biological material, the precipitate demonstrated better capability to oxidize glycerol than the suspension (Fig. 2). Immobilization of the suspension, containing the condensed quantity of proteins, organelles and other cellular constituents present in the cytozole, might to a great extent impair the access of the substrate (glycerol) to the active centers of the enzyme (GlyDH). Centrifugation of disintegrated cells and removal of supernatant enabled partial purification of the mixture from water-soluble proteins and from fine cellular organelles [Alberts et al. 1999]. The concentration of glycerol dehydrogenase in the condensed and immobilized cell precipitate was likely to be similar to that in the cell suspension. However, in that case the enzyme trapped in the structures of the carrier was more available to glycerol, which was reflected in the reaction yield (Fig. 2). Likewise in the first stage of the study (Fig. 1), the immobilized supernatant did not display the capability to oxidize glycerol (Fig. 2), which confirmed that glycerol dehydrogenase was strongly bound with the cytoplasmic membrane and after centrifugation remained in the precipitate [Lapanaite et al. 2005]. In addition, the increasing concentration of DHA produced was observed to be accompanied by a decreasing concentration of substrate (Fig. 3).
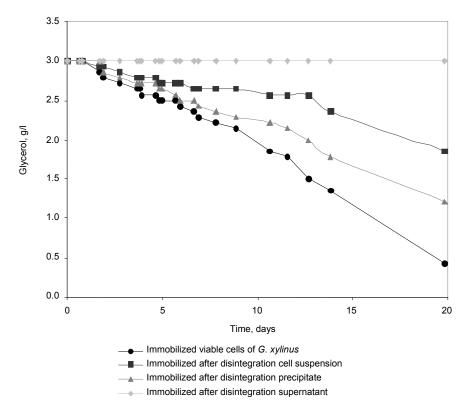

Fig. 3. Effect of the applied production medium and type of immobilizate on changes in glycerol concentration during biotransformation

The yield of the biotransformation reaction was calculated based on results of determination of the increasing DHA concentration and decreasing substrate content in the course of the reaction (Table 1).

Table 1. Yield of biotransformation proceeding

Time, days	Reaction yield, %			
	A	B	C	D
2	71	68	58	0
5	94	70	93	0
10	98	77	95	0
15	99	82	92	0
20	100	80	97	0

A – immobilized cells of *G. xylinus*.
B – cell suspension immobilized after disintegration.
C – cell precipitate immobilized after disintegration.
D – supernatant immobilized after disintegration.

After 10 days of biotransformation, the highest reaction yield, i.e. 98%, was determined for the immobilized viable cells of *G. xylinus* (Table 1). In the same period, the reaction yield noted for the immobilized precipitate accounted for 95%. After 20 days, the yield of the biotransformation reached 100% for the viable bacterial cells. No analyses were conducted in the study for the composition of the post-production medium, therefore it cannot be confirmed whether it contained other than DHA cell metabolites that could have affected the results obtained. Biotransformation yield noted in the same period for the immobilized precipitate accounted for 97%, whereas the immobilized cell suspension was observed to catalyze glycerol oxidation with 80% yield.

In the year 1926, Virtanen and Barlund conducted a research on the application of free cells of acetic acid bacteria of the *Acetobacter dioxyaceticum* species for the production of DHA. They have demonstrated that in a stationary culture run for 30 days, the acetic acid bacteria were oxidizing glycerol to dihydroxyacetone with 84% yield. The applied production medium contained 30 g/l of glycerol and its pH reached 5.0 [Rainbow 1961]. A few years later, Kluyver [1931] noticed that aeration of the incubated culture shortened remarkably the duration of the biotransformation process, and with initial glycerol concentration of 20-50 g/l, enabled reaching the reaction yield of 95%. This experiment was conducted with free cells of acetic acid bacteria. In turn, Nabe et al. [1979] run glycerol biotransformation to DHA with cells of acetic acid bacteria from *Acetobacter suboxydans*, *A. xylinum* A-9, *Gluconobacter melanogenus* IFO 3293 and IFO 3294 species immobilized in carrageenan gel. The highest reaction yield (80% after 40 h) was achieved with the use of the *A. xylinus* A-9 strain.

Although immobilization of cells may, in part, suppress their oxidative activity, it affords a great possibility of facilitating the process of DHA recovery from the post-reaction mixture [Tkač et al. 2001]. As reported by Holst et al. [1985], cells of *Gluconobacter suboxydans* immobilized in polyacrylamide gel or in calcium alginate may display even twice as low oxidative activity when compared to the free cells. It is most

likely due to the suppressed activity and stability of glycerol dehydrogenase immobilized in the carrier and to impaired diffusion of the substrate to structures of the carrier [DeMuynck et al. 2007]. In view of the above, it may be speculated that making the course of the process independent of the metabolic activity of acetic acid bacteria will facilitate the separation of biotransformation product from the post-reaction mixture. Inclusion of the stage of acetic acid bacteria cells disintegration to the entire process will afford such possibilities in the future.

SUMMARY

The conducted study indicates the feasibility of applying an immobilized preparation with the activity of glycerol dehydrogenase, obtained from cells of *G. xylinus*, for biotransformation of glycerol to dihydroxyacetone. The initial concentration of glycerol, reaching 30 g/l, enabled achieving higher (as compared to initial glycerol concentration of 50 g/l) content of DHA both in the case of immobilized viable bacterial cells and different fractions of the cell preparation obtained after enzymatic disintegration of cells. The quantity of the immobilized biological material was found to significantly affect the concentration of the final product. Double condensation of the material which was then immobilized enabled reaching nearly two times higher final concentration of DHA. The method of biotransformation applied in this study did not require the presence of viable bacterial cells, but only the presence of an active enzyme they had produced. The application of the immobilized cell preparation, containing glycerol dehydrogenase, may – to a great extent – reduce the problem posed by the presence of bacterial cells and their metabolites, other than dihydroxyacetone, in the reaction medium. The reaction catalysed by such a preparation may proceed faster than the traditional reaction which requires prior adaptation of cells to conditions of the reaction medium, multiplication of biomass and activation of the enzyme, which occurs in the final stage of the stationary growth. It is highly likely that the immobilized preparation will be re-applied several times in the subsequent reactions of glycerol oxidation to dihydroxyacetone, with the stage of acetic acid bacteria biomass multiplication and GlyDh activation omitted each time.

The results obtained in the study demonstrate that the application of the cell preparation needs adjusting the process parameters. The use of *G. xylinus* cells still yields better effect than the use of the preparation. Hence, researches on the optimization of parameters of glycerol biotransformation to DHA with the use of immobilized biocatalysts are underway. They involve the selection of acetic acid bacteria strains and addition of ions to the reaction medium that enhance the activity of the cell preparation. Of great significance is also the selection of an appropriate carrier for immobilization. Alginate gels applied in this study are characterised by a porous structure, which however poses the risk of penetration of the immobilized biocatalyst to the reaction medium as a result of diffusion or erosion of hydrogel. The conducted study was cognitive in character. Prospective research will allow to improve process yield by the application of alternative methods of disintegration of bacteria cells walls (e.g. sonification). Condensation of the immobilized preparation in a vacuum evaporator or ultracentrifugation of the mixture after disintegration, its purification and then immobilization of the membrane fraction containing GlyDH might additionally accelerate the process of glycerol oxidation to DHA.

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ZASTOSOWANIE IMMOBILIZOWANEGO PREPARATU KOMÓRKOWEGO POZYSKANEGO Z BIOMASY BAKTERII *GLUCONACETOBACTER XYLINUS* W BIOTRANSFORMACJI GLICEROLU DO DIHYDROKSYACETONU

Wstęp. Dihydroksyaceton (DHA), będący produktem utleniania glicerolu przez bakterie octowe, jest ważnym związkiem mającym zastosowanie w przemyśle kosmetycznym, spożywczym, farmaceutycznym oraz medycynie. Biotransformacja glicerolu do DHA jest katalizowana przez związaną z błoną cytoplazmatyczną bakterii dehydrogenazę glicerolową (GlyDH, EC 1.1.1.6). W pracy podjęto próbę przeprowadzenia biotransformacji glicerolu z udziałem immobilizowanych frakcji preparatu komórkowego o aktywności GlyDH. Zawartość dihydroksyacetonu otrzymanego z udziałem preparatu komórkowego porównano z zawartością otrzymaną w reakcji z wykorzystaniem immobilizowanych całych komórek *G. xylinus*.

Materiał i metody. Przeprowadzono enzymatyczną dezintegrację ścian komórkowych bakterii *Gluconacetobacter xylinus*. Uzyskany w ten sposób preparat immobilizowano w alginianie wapnia lub rozdzielano na dwie frakcje, poprzez wirowanie, a następnie unieruchamiano. Zawartość DHA oznaczono kolorymetrycznie po reakcji z kwasem 3,5--dinitrosalicylowym. Glicerol oznaczono refraktometrycznie.

Wyniki. Po 20 dniach procesu stężenie DHA, uzyskanego z udziałem całych unieruchomionych komórek, wynosiło 25 g/l. W tym samym czasie zawartość DHA otrzymana z udziałem immobilizowanych frakcji preparatu komórkowego wynosiła 16,9 g/l oraz 8,95 g/l (w zależności od użytej frakcji).

Wnioski. DHA można uzyskać w procesie niezależnym od aktywności życiowej *G. xylinus*, wykorzystując preparat o aktywności katalitycznej dehydrogenazy glicerolowej, otrzymany w wyniku dezintegracji żywych komórek bakterii. Wykorzystanie takiego preparatu w przyszłości może wyeliminować problemy technologiczne związane z obecnością w podłożu komórek bakterii oraz wytwarzanych przez nie metabolitów.

Słowa kluczowe: dihydroksyaceton, glicerol, *Gluconacetobacter xylinus*, biotransformacja, dehydrogenaza glicerolowa