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BIOCONVERSION OF GLYCEROL INTO DIHYDROXYACETONE DHA USING *ACETOBACTER XYLINUM*

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Key words: bioconversion, glycerol, dihydroxyacetone, *Acetobacter* xylinum.

The objective of this research was to compose a medium for microbiological conversion of glycerol into dihydroxyacetone (DHA). It was found that the selected strain, *Acetobacter xylinum*, ensures a ca. 80% yield in the process (i.e. it produces over 8% of DHA from 10% glycerol). It also displays a number of practical advantages, the most important of which is the facility of its extraction from postfermentation fluids, a factor of crucial importance in the production of pure preparations applicable in medicine or various branches of industry.

INTRODUCTION

Bacteria of the *Acetobacter genus*, widely used in the production of acetic acid, have the ability to oxidize a number of substrates, glycerol among their number, to dihydroxyacetone (DNA). The most important applications of this compound include its use in medicine and in cosmetics industry for the production of creams giving the skin a sun-tan colour as a result of the formation of a colour complex between DHA and arginine present in the epidermis protein [2, 3, 9].

DHA is obtained by oxidating glycerol, either chemically or microbiologically. The transformation of glycerol into DHA by means of chemical compounds is an inhomogeneous process leading to the formation of complex by-products that are hard to remove, and the DHA yield does not exceed a few per cent [5]. The use of glycerol dehydrogenase enzyme produced by some microorganism species enables oxidation of glycerol to DHA, with the yield of the process being 80-90% (and sometimes more) of the utilized substrate. Given this fact, microbiological synthesis of DHA is of primary interest in industrial production of this compound.

Recent years have witnessed increasingly frequent experiments with microbiological processes with the use of microorganisms immobilized on various carriers. In studies of glycerol bioconversion into DHA use was also made of Gluconobacter suboxydans cells immobilized on polyacryloamide gel. However,

as Machotkina and Pomorceva demonstrated [6], unlike in the traditional fermentation methods, the oxidation activity of the immobilized cells was almost twice lower.

In this research we strove to determine the conditions of glycerol bioconversion into DHA by acetobacters and to select medium components ensuring maximum yield of dihydroxyacetone.

EXPERIMENTAL

PREPARATION OF INOCULUM

The organism used in our experiments was the species *Acetobacter xylinum*, obtained from the collection of pure cultures of the Department of Technological Microbiology, Warsaw Agricultural University.

The bacteria were kept on wort slants (wort of 5°Blg density) with a 2% addition of ethanol. In order to maintain the strain's activity, the bacteria were passaged to fresh wort slants with ethanol every three-four weeks. After seven-day culture at ca. 28°C the slants were transferred to a refrigerator and stored at 4°C.

The *Acetobacter xylinum* strain was prepared for fermentation tests by inoculation on fresh wort slants with ethanol and thermostating at about 30°C until clear growth was observed (usually after 48 h of culture). The multiplied bacteria were then washed with sterile water from the wort slants into flat-bottom flasks containing a sterile liquid medium (1% water solution of yeast extract with pH of about 5.0, 2% ethanol addition). To prevent the formation during culture of a slimy surface skin hindering subsequent sampling, it was necessary to add ground glass beads to the medium (about ten per flask).

The bacteria were cultured at about 28°C for 48 h on a plane-motion shaker, and then used as inoculum in the further stage of research.

DETERMINATIONS

DHA was determined colorimetrically, making use of the fact that it reduces triphenyltetrazole chloride (TTC) to fermazone which in its turn lends the solution a raspberry red colour. Extinction was measured at wavelength $\lambda =$ $= 480$ nm [8].

Glycerol was also determined colorimetrically, making use of the colour reaction of glycerol with methylmorphine (codeine). The application of an ion-exchange column made possible determinations of glycerol in the presence of reducing trioses such as dihydroxyacetone or glyceric aldehyde. Solution extinction was measured at wavelength $\lambda = 654$ nm [8].

Dry mass of the bacteria was determined by the drier method at 110°C.

Active acidity (pH) was measured automatically with a N-517 pH meter.

COURSE AND RESULTS OF THE RESEARCH

It is pointed out in the literature [11] that carbon sources in the inoculum have a specific effect on the oxidation activity of acetobacters. It was thus necessary to select the carbon substrate ensuring maximally effective bioconversion into DHA. We tested the effect of 14 different carbon sources on the course and yield of this process (Table). Each series of determinations in our experiments featured two stages:

I. *Acetobacter xylinum* culture in spherical flat-bottomed flasks placed on plane-motion shakers; temperature about 28°C; liquid medium composed of 1% water solution of yeast extract with a 5% addition of the studied carbon source. After 24 h, 5-cm^3 portions of fluid were taken from the various cultures and centrifuged (3500 r.p.m. for 10 min) in centrifuge test-tubes having a capillary in their bottoms where the bacteria deposited forming a column. Different growth rates of the cultured bacteria resulted in different lengths of this column. Bacteria biomass yield was determined with a standard curve illustrating the dependence between column length in the capillary and bacteria dry mass content in 1 cm^3 of the medium.

II. In the second stage the oxidation activity of *Acetobacter xylinum* with respect to glycerol was studied by means of fermentation. To this end, 24-h old

Table. Effect of selected carbon sources in the culture medium on the oxidation activity of Acetobacter xylinum

Carbon source	Concentration (%)	Bacteria biomass yield (mg dry mass/cm^3)	Oxidation activity* of bacteria relative to the control test (%)
Control test	0	2.0	100.0
Glucose	5	3.0	60.2
Fructose	5	3.0	130.7
Galactose	5	1.5	133.0
Maltose	5	2.0	136.4
Saccharose	5	2.0	193.2
Lactose	5	2.0	152.3
Raffinose	5	1.5	55.7
Ethylene glycol	5	1.5	39.8
Glycerol	5	2.0	84.1
Sorbitol	5	5.0	44.3
Mannitol	5	2.0	92.0
Sodium lactate	5	1.0	19.3
Sodium acetate	5	1.0	13.6
Ethanol	5	13.0	40.0

* Oxidation activity of *Acetobacter xylinum* was determined by the fermentation method, taking as 100 the yield of glycerol bioconversion into DHA in the control sample lacking a carbon source other than that introduced into the culture medium together with the extract

acetobacter cultures maintained on the investigated carbon sources were transfered to media with glycerol composed of 10% water solution of glycerol, 1% yeast extract and 0.3% of $CaCO_3$. The $CaCO_3$ addition guaranteed a constant level of pH throughout the bioconversion process. The amount of bacteria inoculum amounted to 10% of the fermentation medium volume. The cultures were maintained at about 28°C in flat-bottomed flasks on plane-motion shakers. At 24 h intervals, 5-cm^3 samples were taken from each culture in a sterile manner, centrifuged at 3500 r.p.m. for 10 min, and in the obtained clear fluid the increase of dihydroxyacetone concentration and the drop of glycerol concentration were determined.

The course of glycerol bioconversion into DHA is illustrated in Fig. 1. The effect of carbon source in the inoculum on the oxidation activity of Acetobacter xylinum bacteria with respect to glycerol is presented in Table. The experiments demonstrate that the carbon source favouring glycerol bioconversion into DHA was saccharose.

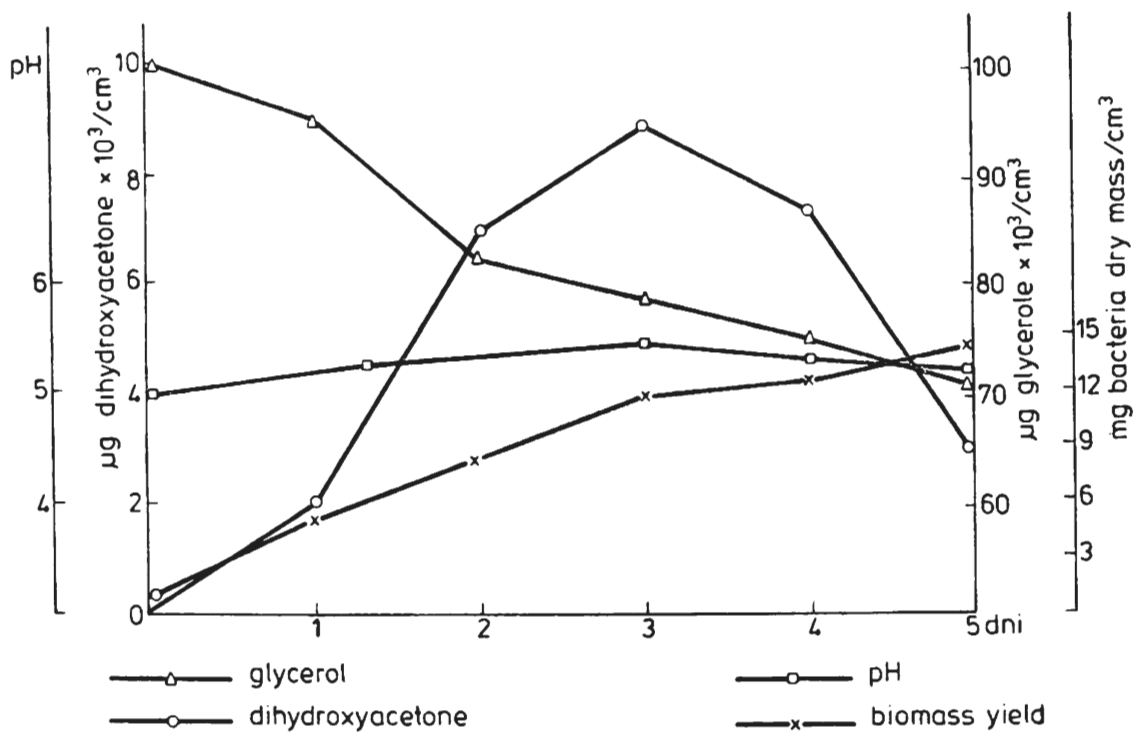

Fig. 1. Course of glycerol bioconversion into dihydroxyacetone

1. Effect of saccharose concentration in the inoculum on glycerol bioconversion into DHA

To determine the effect of sacchorose content in the inoculum on glycerol bioconversion into DHA, the acetobacters were cultured on media containing 0%, 5%, 10%, 15%, 20% or 25% saccharose with an addition of 1% yeast extract..After 24 h of culture, the biomass yield was determined by centrifugation, and inoculation was performed with bacteria from the various fermentation cultures (composition of the medium, culture conditions, and determinations as described above).

According to the results (Fig. 2) the optimum saccharose concentration at which bioconversion yield is highest is 10%.

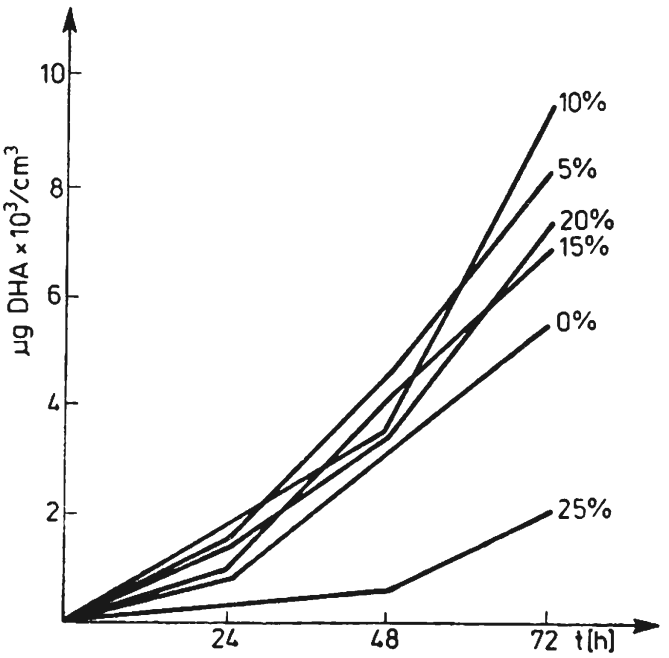

Fig. 2. Effect of saccharose concentration in the inoculum on the course and yield of glycerol bioconversion into DHA

2. Effect on glycerol bioconversion into DHA of organic and inorganic nitrogen sources and of their concentrations in the inoculum

A significant bearing of inorganic nitrogen in the inoculum on oxidation activity of acetobacters relative to glycerol is acknowledged by Japanese and Russian researchers [10, 11]. We thus decided to check which of the selected inorganic nitrogen sources favourably affects glycerol bioconversion into DHA.

We studied compounds containing ammonia nitrogen and compounds with anionic nitrogen (in the form of nitrate ion) as well as urea. The following sources of inorganic nitrogen (i.e. $NaNO_3$) were considered: NH_4NO_3, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, ammonia lactate, ammonia acetate, and urea. The bacteria were cultured on media containing 10% saccharose, an amount of the studied nitrogen source providing 1% pure nitrogen, and 1% yeast extract; pH was about 5.0. After 24 h of culture, the biomass yield was determined by centrifuging 5-cm^3 samples from each culture in test tubes for centrifugation equipped with a capillary. Approximately equal amounts of bacterial biomass were transferred to glycerol-containing media, and subsequent bioconversion was monitored as previously described.

The obtained results (Fig. 6) reveal that the inorganic nitrogen source with the best effect on oxidation activity of *Acetobacter xylinum* towards glycerol, viz. the one ensuring highest yield in all the experiments, was ammonium sulphate.

In studies of the effect of organic nitrogen we used five of its sources, most often used by microbiologists to prepare culture media, namely yeast extract, maltose extract, meat extract, casein hydrolysate, and peptone. As in the previous part of the research, the initial culture of the inoculum was followed by fermentation tests proper.

In the first stage, bacteria were cultured on media with 10% saccharose, 4.75% $(NH_4)_2SO_4$ (1% inorganic nitrogen) and 1% of the studied source of

organic nitrogen; pH was about 5.0. After 24 h biomass yield was determined, and a glycerol-containing medium inoculated with the bacteria. In the second stage, the increase of DHA concentration and the drop of glycerol concentration were checked at 24-h intervals.

As can be seen from the results (Fig. 3), the most advantageous course and yield of glycerol biococversion into DHA were obtained with a 1% addition of yeast extract.

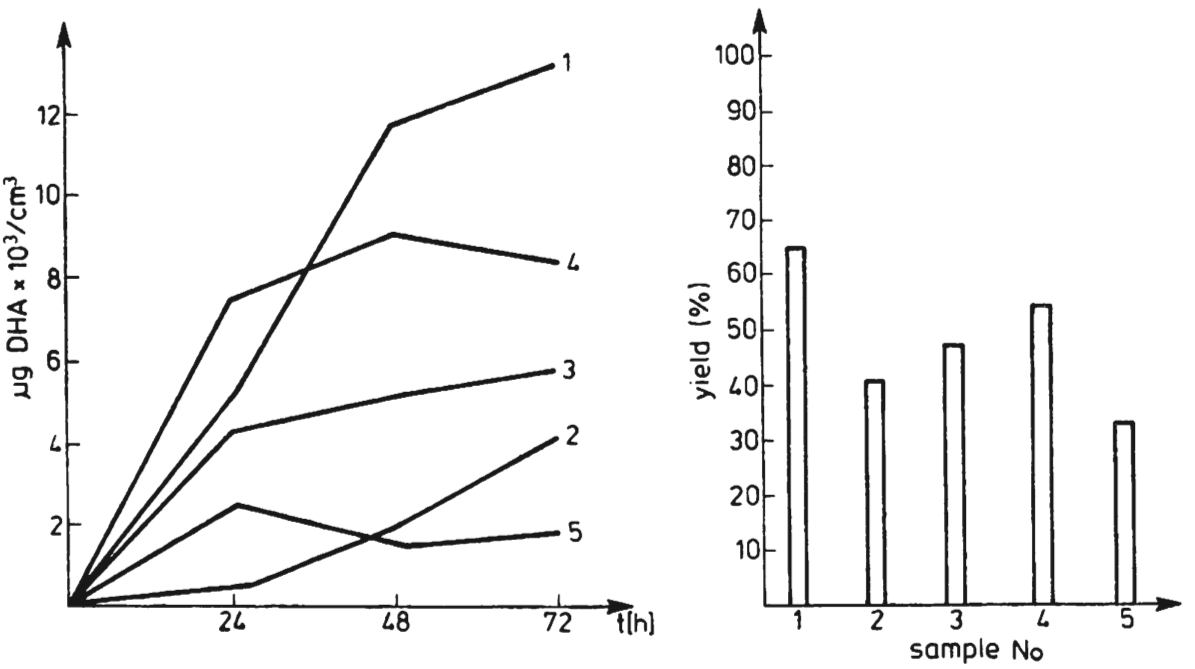

Fig. 3. Effect of selected sources of organic nitrogen on glycerol bioconversion into DHA; 1 — yeast extract, 2 — maltose extract, 3 — meat extract, 4 — casein hydrolysate, 5 — peptone

3. Effect of glycerol concentration in the fermentation medium on glycerol bioconversion into DHA in the presence of *Acetobacter xylinum*

To determine the optimum proportion of glycerol in the fermentation medium, the acetobacters were cultured on a medium composed of 10% saccharose solution with a 4.75% addition of $(NH_4)_2SO_4$, 1% yeast extract; pH was about 5.0. After 24 h of culture at about 30°C, the biological material was collected and used to inoculate media with glycerol (in doses amounting to 10% of glycerol volume).

The compositions of glycerol-containing media were as follows: 1% water solution of yeast extract, 0.3% $CaCO_3$ and glycerol in concentrations of 5%, 10%, 15%, 20% and 25% in the various experiments. 5-cm^3 portions of each medium were collected at 24-h intervals for determinations of DHA concentration increase and glycerol concentration decrease. The course of the experiments is illustrated in Fig. 4. It was found that maximum yield of the process took place when glycerol concentration in the fermentation medium was 10%.

4. Effect of pH on the course and yield of glycerol bioconversion into DHA

The pH optium for acetobacters development is about 5.0, and such was maintained during the preparation of inocula used in the experiments. However,

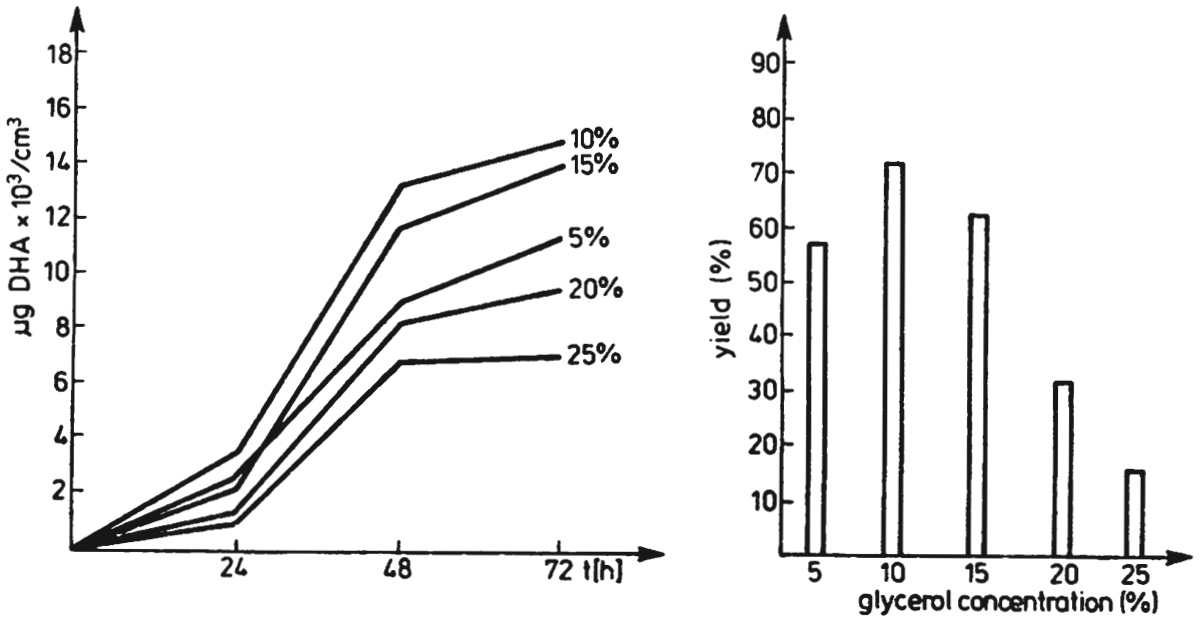

Fig. 4. Effect of glycerol concentration on the course and yield of glycerol bioconversion into DHA

biological oxidation of glycerol requires a slightly different level of active acidity of the medium. According to literature [4] glycerol bioconversion may occur in a twofold manner:

— at pH ca. 6.0 — direct conversion of glycerol into DHA,

— at pH ca. 8.0 — indirect conversion involving phosphorylation of glycerol to glycerol α-phosphate and subsequent transformation of this compound into DHA.

We thus decided to study the effect of pH on the yield of glycerol bioconversion in the pH range 5.0-8.0 (modifying acidity by full pH units in the various experiments).

Glycerol-containing media were inoculated with the cultured bacteria doses amounting to 10% of the medium volume. The composition of the media was given previously, and pH was 5.0, 6.0, 7.0 and 8.0. DHA and glycerol concentrations were determined at 24-h intervals. It was found that the highest yield of glycerol bioconversion into DHA in the investigated conditions (80%) occurred at pH around 6.0 (Fig. 5).

DISCUSSION AND CONCLUSIONS

The results of experiments reported here concern mainly the selection of substrate as carbon source, carbon content ensuring optimum production of glycerol dehydrogenase, and the selection of organic or inorganic nitrogen source. Also pointed out were fairly important factors which may affect glycerol bioconversion into dihydroxyacetone, namely glycerol concentration and active acidity of the media. In the experiments efforts were made to select medium parameters most advantageous for the process of glycerol bioconversion into DHA using *Acetobacter xylinum* bacteria.

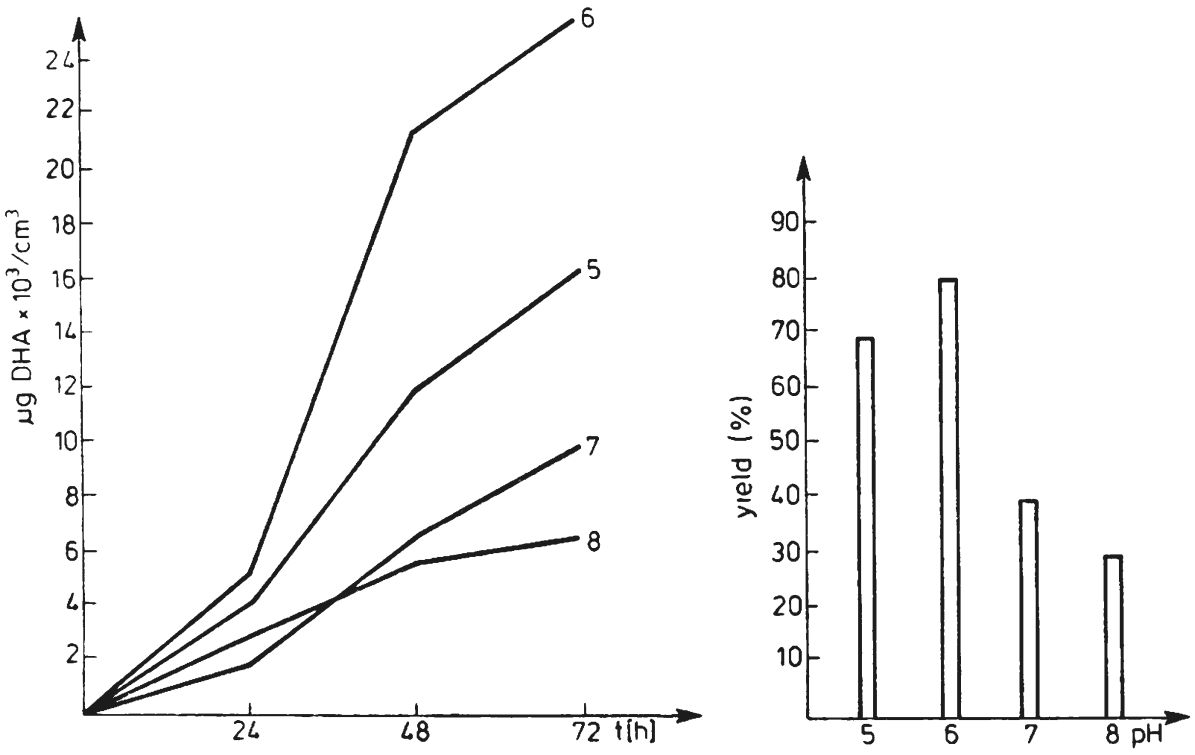

Fig. 5. Effect of pH on the course and yield of glycerol bioconversion into DHA

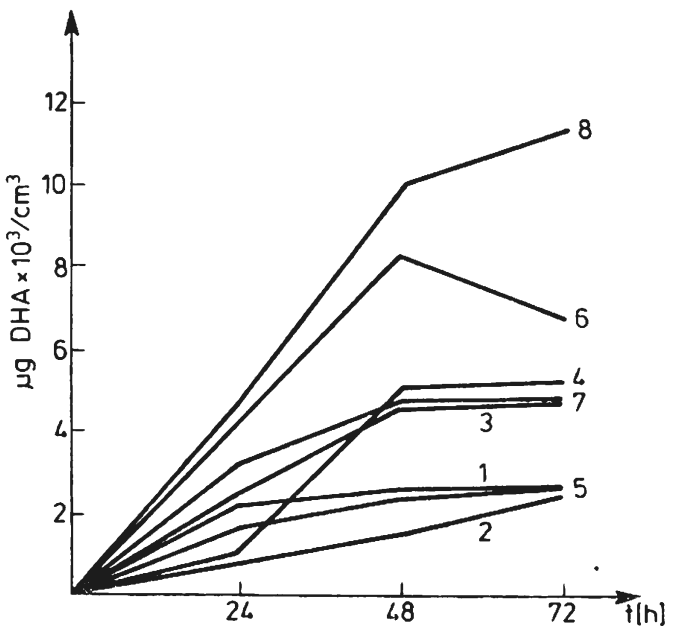

Fig. 6. Effect of selected sources of inorganic nitrogen on the course and yield of glycerol bioconversion into DHA; 1 — control, 2 — $NaNO_3$, 3 — NH_4NO_3, 4 — $(NH_4)_2HPO_4$, 5 — ammonia lactate, 6 — urea, 7 — ammonia acetate, 8 — $(NH_4)_2SO_4$

The following conclusions may be formulated against the relevant literature data [1, 4, 5, 7, 11]:

1. The carbon source in the inoculum ensuring the best yield of glycerol bioconversion into DHA with the use of Acetobacter xylinum bacteria may be saccharose introduced in a dose amounting to 10% of the medium volume.

2. It was found that the inorganic nitrogen source most favourably affecting the oxidation activity of the bacteria was $(NH_4)_2SO_4$. The concentration of this

compound in the medium was 4.75%, and this amount provided 1% pure nitrogen.

3. As regards the effect of selected organic nitrogen sources on the course and yield of glycerol bioconversion into DHA, the results indicate that the highest oxidation activity of acetobacters towards glycerol, compared to control, was obtained with a 1% addition of yeast extract to the inoculum.

4. Optimum glycerol concentration in the fermentation medium was 10%.

5. The level of active acidity ensuring highest yield in the process taking place in the studied conditions was ca. 6.0.

6. The time of glycerol bioconversion into DHA giving maximum efficiency was 72 h. Following this point, no DHA increase was observed, and there was even some decrease in the content of this compound.

7. Maximum yield of bioconversion was obtained during 72 h in a medium composed of 10% glycerol, 1% yeast extract, 0.3% $CaCO_3$, with pH around 6.0. This medium was inoculated with bacteria cultured on a medium containing 10% saccharose, 4.75% $(NH_4)_2SO_4$, and 1% yeast extract (pH was about 5.0). In these conditions we obtained 26 mg DHA/cm^3 of the fermentation medium; the yield was thus about 80%.

8. The experiments reported here leave aside many aspects of glycerol bioconversion into DHA. Work should continue, dealing with problems such as the effect of trace elements on the course and yield of the process, the elaboration of a method of isolating DHA, and partial utilization of glycerol in the process of its oxidation into dihydroxyacetone.

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BIOKONWERSJA GLICEROLU W DIHYDROKSYACETON (DHA) PRZY UŻYCIU *ACETOBACTER XYLINUM*

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Streszczenie

Celem pracy był dobór składu jakościowego i ilościowego podłoża do biokonwersji glicerolu w DHA pod kątem maksymalnej wydajności procesu przy użyciu szczepu bakterii *Acetobacter xylinum.*

Badania objęły wybór źródła węgla, azotu organicznego i nieorganicznego i ich stężeń oraz określenie stężenia glicerolu i poziomu kwasowości czynnej podłoża zapewniających maksymalną wydaność procesu.

W wyniku przeprowadzonych badań stwierdzono, że dobrym i sprzyjającym źródłem węgla w biokonwersji glicerolu do dihydroksyacetonu (DHA) była sacharoza, co można zaobserwować w danych tabeli oraz na rys. 1. Zaobserwować tutaj można wyraźny wpływ źródła węgla w inoculum na aktywność oksydacyjną bakterii *Acetobacter xylinum* w stosunku do glicerolu. Na rys. 2 przedstawiono wyniki wpływu stężenia sacharozy w inoculum na biokonwersję glicerolu w DHA, a uzyskane dane wskazują, że optymalne stężenie sacharozy, przy którym wydajność procesu biokonwersji osiąga najwyższą wartość jest 10%.

Do bardzo ważnych czynników można zaliczyć wpływ wybranych źródeł azotu nieorganicznego i organicznego oraz ich stężenie w inoculum na biokonwersję glicerolu w DHA. Z danych uzyskanych w toku badań wynika, jak to przedstawiono na rys. 6, że najlepszym źródłem azotu nieorganicznego wpływającym korzystnie na aktywność oksydacyjną *Acetobacter xylinum* w stosunku do glicerolu był siarczan amonu w stężeniu 4,75%, natomiast w przypadku azotu organicznego okazał się ekstrakt drożdży w stężeniu 1%, co można zaobserwować na rys. 3.

Do ważnych czynników można zaliczyć wpływ stężenia glicerolu w podłożu fermentacyjnym na jego biokonwersję w DHA przy udziale *Acetobacter xylinum.* Uzyskane dane z tej serii badań, jak to przedstawiono na rys. 4 wynika, że maksymalną wydajność procesu osiąga się przy 10% stężeniu glicerolu w podłożu fermentacyjnym. Ponadto na przebieg i wydajność biokonwersji glicerolu w DHA ma również wpływ pH środowiska. Z przeprowadzonych badań wynika, że proces biokonwersji glicerolu w DHA osiąga najwyższą wartość ok. 80% przy pH bliskim 6,0, co przedstawiono na rys. 5.

Podsumowując uzyskane dane można stwierdzić, że najwyższe stężenie DHA, tj. 26 mg/cm^3 uzyskano z 80% wydajnością w czasie 72 h z podłoża fermentacyjnego o następującym składzie: 10% glicerolu, 1% ekstraktu drożdżowego, 0,3% $CaCO_3$ i przy wartości pH bliskim 6,0. Bakterie użyte do szczepienia prób fermentacyjnych hodowano w celu osiągnięcia przez nie możliwie najwyższej aktywności oksydacyjnej w stosunku do glicerolu na podłożu zawierającym 10% sacharozy, 1% ekstraktu drożdży, 4,75% siarczanu amonu, pH = 6,0 w czasie 24 h.