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Production of 1,3-Dihydroxyacetone from Glycerol by *Gluconobacter oxydans* ZJB09112

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The culture variables were optimized to increase 1,3-dihydroxyacetone (DHA) production by *Gluconobacter oxydans* ZJB09112 in shake flasks and bubble column bioreactors. After fermentation in the optimized medium (g/l: yeast extract 5, glycerol 2.5, mannitol 22.5, K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.1, $CaCO_3$ 2.0, pH 5.0), when five times of glycerol feeding were applied, 161.9±5.9 g/l of DHA was attained at a 88.7±3.2% conversion rate of glycerol to DHA.

Keywords: 1,3-Dihydroxyacetone, *Gluconobacter oxydans*, glycerol, fed-batch fermentation, bubble column bioreactor

1,3-Dihydroxyacetone, known in abbreviated form as DHA, is the effective ingredient of sunless tanning products and has been widely used in cosmetic preparations for many years [16, 20]. DHA has also been proposed to be involved in weight augmentation and fat loss, antioxidant activity, and increasing endurance capacity [19, 26], and it offers a safe and effective therapeutic option for recalcitrant vitiligo [24]. Moreover, DHA serves as a versatile building block for the organic synthesis of a variety of fine chemicals [7, 11, 31].

DHA could be produced with bioconversion of glycerol. Some microbial strains have been isolated and used in our laboratory [13, 17], one of which belongs to *Gluconobacter oxydans*. *G. oxydans* is a Gram-negative bacterium belonging to the family of acetic acid bacteria, and is well known for its efficient oxidation of sugars, sugar acids, and sugar alcohols [23]. The biosynthesis of DHA in the *G. oxydans* cell is complicated and there are three possible pathways involved in DHA production. In particular, glycerol can be directly oxidized into DHA by membrane-bound glycerol dehydrogenase using oxygen as the final acceptor of the reduced equivalent without NADH mediation [11, 19, 27].

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Obviously, the microbial production of DHA has a high oxygen requirement [8, 11]. Moreover, the main challenge of DHA production is the inhibitory effect of high concentrations of both glycerol and DHA, and high DHA concentration may cause irreversible cell damage [4, 5]. Thus, a fed-batch bioprocess was developed for DHA production [1, 11]. Furthermore, numerous dehydrogenases exist in *G. oxydans*, and some of them can be induced by polyols, such as galactitol, sorbitol, and ribitol [25, 30]. Thus, optimization of the production conditions for microbial production of DHA by *G. oxydans* is essential.

There are several types of bioreactors designed for microbial bioprocessing, such as the stirred tank bioreactor, membrane bioreactor, shaking bioreactor, packed-bed bioreactor, bubble column bioreactor, and external/internal-loop airlift bioreactor [2, 3, 15, 18]. The bubble column bioreactor has been extensively used as multiphase contactors and reactors in the chemical, biochemical, and petrochemical industries, usually in low-viscosity systems [9, 14, 22]. It is basically a cylindrical vessel with a gas distributor at the bottom, and the gas flow from the bottom is dispersed in the form of bubbles in the liquid phase. Bubble column bioreactors are characterized by their simple design and the complete absence of mechanically moving internals. In addition, it provides several advantages during operation and maintenance, such as high heat and mass transfer rates, high oxygen transfer rate, compactness, and low operating and maintenance costs. An important application area of bubble columns is as bioreactors in which microorganisms are utilized in order to produce industrial products such as enzymes, proteins, organic acids, antibiotics, *etc.* [14]. The applicability and performance of the bubble column bioreactor in DHA production from glycerol were investigated in this work.

The bioprocess can be separated into two stages: cell growth of *G. oxydans* and microbial production of DHA from glycerol by fed-batch fermentation.

In this study, the necessity for cells preparation was first evaluated by studying the effect of nutrient sources and production conditions on cell growth and its ability for

production of DHA from glycerol in shake flasks and bubble column bioreactors. Moreover, the effects of glycerol feeding strategy on DHA fermentation and the performances of fed-batch fermentation of DHA in a bubble column bioreactor were also reported.

MATERIALS AND METHODS

Microorganism and Medium

Gluconobacter oxydans ZJB09112, a mutant strain from *G. oxydans* ZJB-605 (CCTCC No. M 208069) by mutagenesis of ultraviolet (UV) irradiation, was employed in this work, and stored at 4°C on medium GY agar slants and transferred monthly. The medium GY contained (in g/l) glucose 25, yeast extract 5, and agar 20. The medium BM was a carbon-free and nitrogen-free medium (in g/l: K_2HPO_4, 0.5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCO_3$, 2.). The medium G (G1-G7 series) contained (in g/l) glycerol, mannitol, yeast extract 5, peptone 3, K_2HPO_4 0.5, KH_2PO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.1, and $CaCO_3$ 2, and the concentrations of glycerol and mannitol were varied in G1–G7 series (Table 2). All media were autoclaved for 20 min at 121°C.

Fed-Batch Fermentation by Shake Flask

Fed-batch fermentation for DHA production could be considered as a 2-stage bioprocess: the first stage at 0–24 h and the second stage after 24 h. At the first-stage bioprocess, inocula were prepared by transferring a loop of cells from 24-h-old slant cultures into 500-ml flasks containing 30 ml of liquid medium GY (without agar). The flasks were kept on a rotatory shaker (XWW 25/450, orbital diameter of 25 mm; Leshan Changzheng Pharmaceutical Machine Co., Ltd) at 150, 30°C, for 24 h. Then, 1.0 ml of seed culture was transferred into another 500-ml flask containing sterile 30-ml liquid medium, and the flasks were incubated at 30°C for 24 h on the rotatory shaker at 150 rpm. At the second stage, 1.2 g of sterile glycerol was added to the flasks after 24-h fermentation, which resulted in 40 g/l of glycerol concentration. Glycerol and DHA were tested during fed-batch fermentation.

Analytical Methods

Glycerol and DHA were analyzed by gas chromatography according to Wang *et al.* [28]. Cell dry weight was determined gravimetrically and gave a linear relationship to the optical density at 660 nm in a 1.0-cm cuvette (DU800 Spectrophotometer; Beckman Coulter, U.S.A.).

Experimental Apparatus of Bubble Column Bioreactor and Fed-Batch Procedure

A vertical bubble column bioreactor made of glass was used in this work. The working volume of the column was 500 ml, with a ratio of height-to-diameter of 5.0. The gas diffuser at the bottom of the column was constructed of sintered glass and had pore sizes of 80–120 μm with an average of 100 μm.

The sterile liquid medium was added to the aseptic bubble column bioreactor, and 5.0 ml of seed culture was inoculated to the bubble column, and then the feeding glycerol was added after 24 h fermentation. The air flow rate was measured in units of vvm (volumes of air/effective volume of reactor/minute). The gas flow rate of the bubble column was controlled manually in the range of 0.5–2.5 vvm using a flow-meter with a needle valve. The operation temperature was controlled at 30°C by water circulation in the outer jacket on the column. pH control was evaluated by studying the performance of shake flasks and bubble column bioreactors with addition of 1.0 M HCl/NaOH. The foam was controlled by the addition of polyethylene glycol as an antifoam agent. To compensate for the volume reduction due to evaporation, a water-cooled condenser was fitted on the top of the bioreactor, and an appropriate volume of sterile water was added to the bioreactor during the experiments.

Samples were withdrawn from bubble column at every 2 h for testing pH, DHA, glycerol, and biomass.

Three batches were repeated for each experiment. The DPS software (Version 6.55; Refine Information Tech. Co., Ltd) was used for statistical analysis. The conversion rate of glycerol to DHA and DHA productivity were defined as follows, respectively.

$$\text{Conversion rate of glycerol to DHA (\%)} = \frac{\text{DHA (g/l)}}{\text{Total glycerol (g/l)}} \times 100\% \quad (1)$$

$$\text{DHA productivity (g/l/h)} = \frac{\text{DHA concentration (g/l)}}{\text{Fermentation time (h)}} \quad (2)$$

RESULTS AND DISCUSSION

Effects of Carbon and Nitrogen Sources

Glycerol is not only the substrate of DHA production, but also the carbon source for cell growth. Except for glycerol, the carbon sources in the experiment are not the substrate for DHA production, which can affect the cell growth, cell activity, and the ability of bioconversion of glycerol to DHA. It is necessary to select the optimal carbon source for DHA production in this work.

Several different carbon sources, including mannitol, glycerol, glucose, sucrose, galactose, maltose, and fructose, were employed, and medium BM with added carbon source and peptone (in g/l: carbon source, 25; peptone, 5; pH 5.0) was used as the initial medium at fed-batch fermentation by shake flask. The results showed that glycerol was the favorable carbon source for cell growth. However, mannitol, galactose, maltose, and glycerol were optimal carbon sources for DHA production (Fig. 1). Of all the carbon sources employed, glycerol gave the maximum DHA concentration, 34.1±1.3 g/l of DHA, in which 6.2±0.3 g/l and 27.9±1.2 g/l of DHA were produced during the first and the second stage of bioprocess, respectively. Furthermore, the conversion rate of glycerol to DHA was only 52.5±2.2% in the whole bioprocess. Glycerol was used as the only carbon source for cell growth coupling with some DHA formation at the first-stage bioprocess (0–24 h), and as the substrate of biosynthesis of DHA at the second stage of the bioprocess (after 24 h). Except for the experiment of glycerol as carbon source, the maximum DHA concentration (30.2±1.5 g/l) with 75.5±3.8% of the conversion rate of glycerol to DHA was obtained in the experiment of mannitol as carbon source (column 2 in

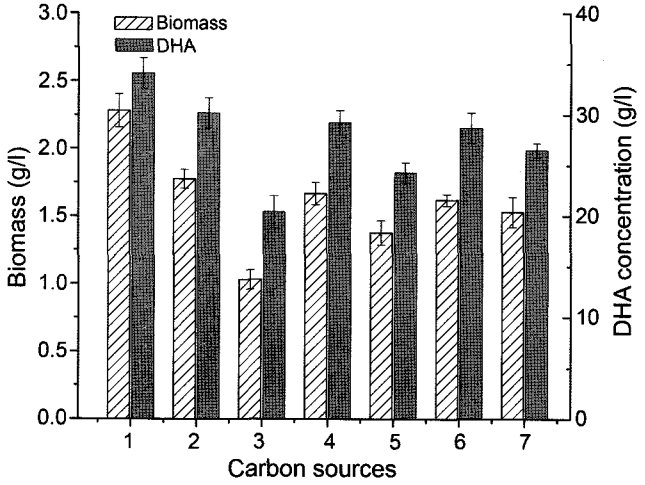

Fig. 1. Effects of carbon sources on DHA production.

Fig. 1). As comparison with the experiments of glycerol and mannitol, the conversion rate of glycerol to DHA in the experiment of mannitol was much higher than that of glycerol, by 43.8%. It was obvious that mannitol was the optimal carbon source for the cell's ability of conversion of glycerol to DHA among the experiments.

Some polyols have been reported as inducers of dehydrogenase activity in *G. oxydans*, and the cell's ability of bioconversion of polyols to corresponding ketone was improved [25, 30]. In our preliminary work, it was found that a mixture of glycerol and mannitol affected dramatically the conversion rate of glycerol to DHA and DHA production. Accordingly, the glycerol content in the medium G was replaced gradually with mannitol (Table 1), which resulted in gradually changing the rates of glycerol to mannitol.

No significant differences were observed in DHA concentration among the experiments of the G1–G5 series. However, with the increase of mannitol content in the medium, the conversion rate of glycerol to DHA increased gradually, and reached the maximum of 79.8±3.3% in the medium G5 containing glycerol and mannitol at a 1:9 mixture (Fig. 2), which was higher than that of the medium containing only mannitol by an average of 5.7%. It was suggested that a low glycerol content was beneficial for a cell's ability to convert of glycerol to DHA.

Table 1. Glycerol and mannitol concentrations in the G media (G1–G7 series).

Medium	Substrate (g/l)	
	Glycerol	Mannitol
G1	20.0	5.0
G2	15.0	10.0
G3	10.0	15.0
G4	5.0	20.0
G5	2.5	22.5
G6	1.0	24.0
G7	0.5	24.5

Glycerol dehydrogenase is a membrane-bound enzyme, located at the outer surface of the cytoplasmic membrane; some of them are inducible enzymes. A low content of glycerol can induce the activity of glycerol dehydrogenase. In addition, mannitol is the optimal carbon source for the formation of the intracytoplasmic membrane of *G. oxydans* cells, which results in increasing the catalytic activity of glycerol dehydrogenase.

The effects of nitrogen sources on DHA production were also investigated in this study, which included peptone, yeast extract, beef extract, corn steep, NH_4Cl, $(NH_4)_2SO_4$, and NH_4NO_3. Medium BM with added nitrogen source, mannitol, and glycerol (in g/l: nitrogen source, 5; mannitol, 22.5; glycerol, 2.5; pH 5.0) was used in this experiment. As shown in Fig. 3, yeast extract, peptone, and corn steep were favorable nitrogen sources for DHA production. Although the maximum DHA concentration of 34.1±2.0 g/l was achieved in the medium containing yeast extract as the nitrogen source, there were little differences in DHA production using these three nitrogen sources. However, the inorganic compounds tested in this study were not favorable nitrogen sources for DHA production.

Effect of Initial pH

The initial pH of the medium was adjusted to set point by adding 1.0 M HCl/NaOH, and then $CaCO_3$ was added to the medium. Results of the shake-flask fermentation with various initial pHs ranging from 4.0 to 7.0 are shown in Fig. 4. The medium contained $CaCO_3$ that can neutralize acid to keep the pH at a certain range, and some phosphates that

Table 2. Performances of fed-batch fermentation by pulse feeding.

Experiment	Initial glycerol concentration (g/l)	Feeding glycerol (g)	DHA concentration (g/l)	DHA productivity (g/l/h)	Conversion rate of glycerol to DHA (%)	Fermentation time (h)
F1	2.5	15+15+15+15	111.3±4.6	1.99	90.9±3.7	56
F2	2.5	20+20+15+15	129.9±3.6	2.16	91.1±2.5	60
F3	2.5	20+20+20+20	147.5±5.0	2.24	90.8±3.1	66
F4	2.5	18+18+18+18+18	161.9±5.9	2.38	88.7±3.2	68
F5	2.5	20+20+20+20+20	165.5±5.4	2.30	81.7±2.9	72

±: Standard deviation.

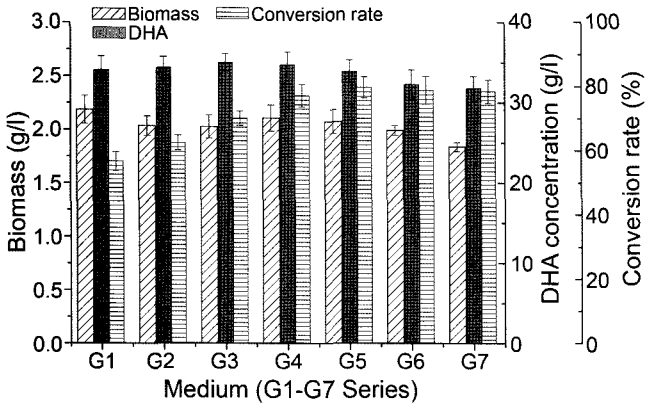

Fig. 2. Effects of carbon sources (mixture of glycerol and mannitol, G1-G7 series) on DHA production.

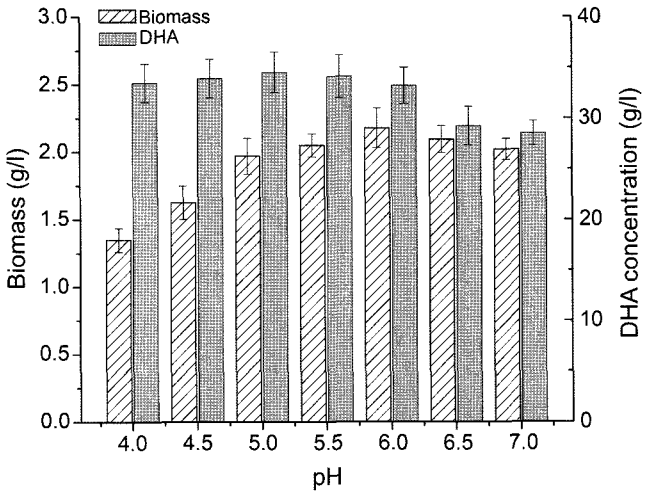

Fig. 4. Effect of initial pH on DHA production under different initial pHs at 30°C.

have buffer action, so the pH nature of the flask cultures was partially buffered. Although the maximum DHA concentration occurred at pH 5.0, there were no significant differences in DHA production among the results of pH 4.0–6.0. The results indicated that a low pH range might favor DHA production.

Effect of Gas Flow Rate on DHA Production

The aeration in a bubble column bioreactor provides the required oxygen for an aerobic microorganism, and also mixing. Proper aeration provides suitable gas holdup, a higher residence time of the gas in the liquid, and a high gas–liquid interaction area available for mass transfer [12, 21].

Based on the results of fed-batch fermentation in shake flask, an optimized initial medium (in g/l: yeast extract, 5.0; glycerol, 2.5; mannitol, 22.5; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCO_3$, 2) was used in DHA production by fed-batch fermentation in a bubble column bioreactor, and after 24-h fermentation, 20 g of sterile glycerol was added to the bubble column. The cell growth and DHA concentration were affected dramatically by air flow, where the biomass content increased with increase of the airflow; however, a maximum DHA concentration of 38.9±2.0 g/l was achieved at an aeration rate of 1.5 vvm, with 91.5±4.8% of the conversion rate of glycerol to DHA at 30°C, pH 5.0 (Fig. 5).

Effect of Glycerol Feeding Strategies on DHA Production

A high glycerol content exerts an inhibitory effect on DHA production by *G. oxydans* [4, 6]. Owing to the inhibitory effect of DHA, glycerol feeding strategies were introduced for DHA production. The feeding strategy, keeping glycerol concentration constant, is a method in which the time-interval glycerol consumption is forecasted by the former

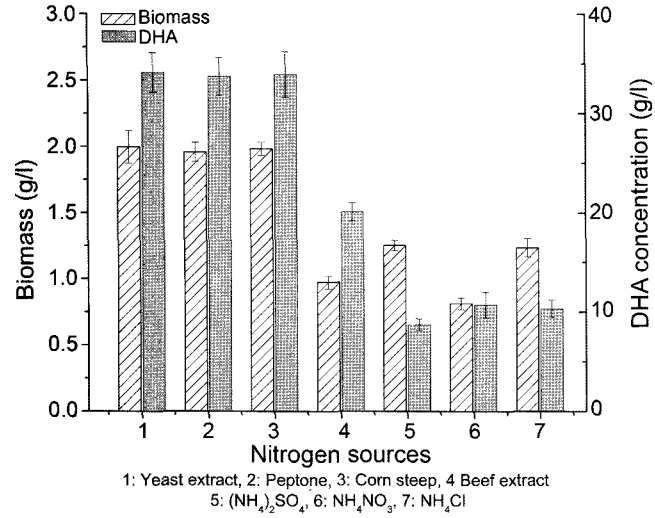

Fig. 3. Effects of nitrogen sources on DHA production.

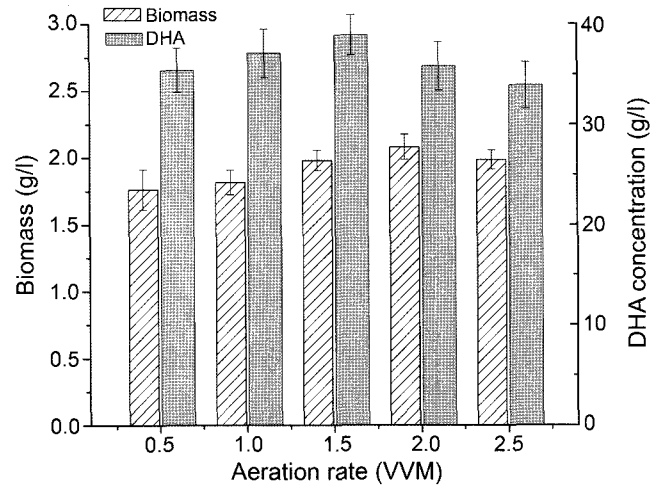

Fig. 5. Effect of aeration rate on DHA production at 30°C, pH 5.0 in a bubble column bioreactor.

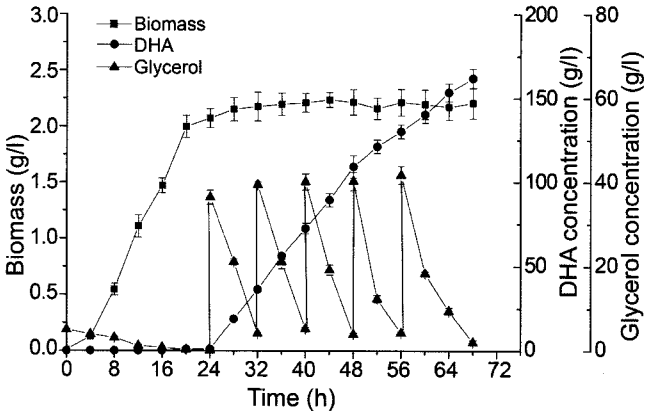

Fig. 6. Time-course of fed-batch DHA fermentation in a bubble column bioreactor at 30°C, pH 5.0, 1.5 vvm of aeration rate, with pulse glycerol feeding strategy.

time-interval consumption, and thus the addition rate can be adjusted to an appropriated value. However, it is difficult to operate and to keep the glycerol concentration constant in the experiment. Thus, a pulse-feeding strategy was applied in this work, where glycerol as the substrate was added instantaneously to the bioreactor at various discrete times, and four or five times of pulse feedings were made. The system behaved as batch fermentation in each interval, since the volume remained constant during this period. The aeration rate and pH were controlled at 1.5 vvm and 5.0, respectively. The first time of feeding was carried out at 24 h of fermentation. The residual glycerol and the time of feeding were used as criteria for feeding of glycerol. When the residual glycerol dropped to 5 g/l, the feeding glycerol was added to the bioreactor. Different feeding times and total glycerol concentration were employed in the experiments. The results of fed-batch fermentation with pulse-feeding glycerol are listed in Table 2. The DHA concentration increased with increase of total glycerol concentration, and reached the maximum DHA concentration of 165.5±5.9 g/l at experiment F5, which was slightly higher than that at experiment F4 by 2.2%. However, the conversion rate of glycerol to DHA was only 81.7±2.9% in experiment F5, which was lower than that of experiment F4 by 8.6%, and the fermentation time in experiment F5 was much more than 4 h compared with that of experiment F4, whereas the DHA productivity in experiment F5 decreased by 3.4% in comparison with F4.

Considering the cost of bioprocess, the appropriate conversion rate of glycerol to DHA, and the DHA productivity, the experiment F4 was recommended in this study. When 182.5 g/l of total glycerol concentration and four times of feeding were employed in experiment F4, the maximal DHA concentration reached 161.9±5.9 g/l at 68-h fermentation with an 88.7±3.2% conversion rate of glycerol to DHA and 2.38 g/l/h of productivity (Fig. 6). The DHA concentration and the conversion rate of glycerol to DHA reported in this work are higher than those of previous reports [10, 11, 29]. Furthermore, the cost of DHA produced by fed-batch fermentation in the bubble column reactor will decrease dramatically because of the high DHA productivity and low operating and maintenance costs of the bubble column bioreactor.

By fine tuning the mutagenesis programme and process optimization, there is further scope for enhancing DHA production.

Acknowledgments

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