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Enhancement of 1,3-Dihydroxyacetone Production by a UV-induced Mutant of *Gluconobacter oxydans* with DO Control Strategy

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Abstract The 1,3-dihydroxyacetone (DHA)-overproducing mutant of *Gluconobacter oxydans* was screened via UV mutagenesis to enhance the DHA production, and the DHA fermentation condition was optimized using the dissolved oxygen (DO) control strategy. The stable mutant *G. oxydans* ZJB11001 exhibits high DHA productivity and can tolerate high DHA concentrations. The optimal condition for DHA production by *G. oxydans* ZJB11001 in a 15-L fermentor required an initial medium containing 5 g/L yeast extract, 20 g/L glycerol, 0.5 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$. The glycerol feeding rate was manually controlled to maintain the glycerol concentration at 5–10 g/L range. The culture pH was maintained at 6.0 within the first 20 h, and then adjusted to 5.0 until the end of the fermentation. The DO concentration increased from 20% to 30% after 24 h of fermentation, and then to 40% after 60 h of fermentation. The maximum DHA concentration of 209.6±6.8 g/L was achieved after 72 h of fed-batch fermentation at 30 °C.

Keywords 1,3-Dihydroxyacetone · *Gluconobacter oxydans* · UV irradiation · DO control strategy · Glycerol

Introduction

1,3-Dihydroxyacetone (DHA) has been extensively used in the cosmetic and chemical industries [1, 2]. DHA can be produced from glycerol by *Gluconobacter oxydans* [3, 4], which is one of the most frequently used microorganisms in industrial biotechnology for the production of valuable chemicals [5–7].

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The biosynthesis of DHA in a *G. oxydans* cell is complicated and involves three possible pathways. Glycerol is the substrate of DHA biosynthesis, and is the only carbon source for cell growth. It can be directly oxidized into DHA by membrane-bound glycerol dehydrogenase [8–10]. A high concentration of both glycerol and DHA can inhibit cell growth and DHA biosynthesis, resulting in low DHA yield and productivity [11, 12]. Thus, in order to overcome the inhibitory effects of a high glycerol concentration, a fed-batch bioprocess in which glycerol concentration can be controlled at certain range by feeding of glycerol, was introduced for DHA production. The fed-batch bioprocess has been the major method of DHA production [13–16]. However, preventing the inhibitory effects of a high DHA concentration through bioprocess optimization is very difficult. A high DHA concentration may cause irreversible cell damage [14]. Moreover, DHA can be used by cells as a carbon source, especially in a glycerol-limited medium, resulting in a decrease of DHA production [17]. Thus, one possible way to increase DHA production is to screen for a mutant that can resist high DHA concentrations.

Mutagenesis is highly essential in industrial biotechnology, and most microorganisms used in industrial production are mutant strains. However, studies on mutant cultivation and mutagenesis to screen for DHA-overproducing mutants are limited [18]. Ultraviolet (UV) irradiation is one of the most widely used mutagens, particularly in screening for mutants for application in industrial biotechnology [19, 20]. A rapid and precise identification of highly productive mutants and verification of their productivity after UV irradiation will therefore play vital roles in mutagenesis. Our previous study had established a high throughput screening method for DHA-overproducing mutant by cultivation in a 96-well microtiter plate, and batch fermentation to verify their productivity [21]. However, batch fermentation cannot screen for DHA-resistant mutants and their ability to produce DHA. In the present study, a two-stage method, consisting of batch fermentation and fed-batch fermentation, was developed to screen for DHA-overproducing and DHA-resistant mutants.

The optimization of fermentation bioprocess for mutants is also very important in DHA production. Dissolved oxygen (DO) concentration is a very important parameter in DHA fermentation, because of the high DO requirement of *G. oxydans* for DHA production. A number of studies have focused on the effects of DO on DHA fermentation, and the application of a DO control strategy [15, 22]. Moreover, the feedback control of glycerol feeding with varying DO concentration has been established in our previous work [15]. The primary factors affecting DO concentration during fermentation are the degree of agitation, gas flow rate, and oxygen partial pressure in the supplied gas, especially the degree of agitation [23, 24]. DHA fermentation bioprocess can be separated into three stages. The first stage is characterized by cell growth coupling with some DHA production, in which glycerol is used both as a carbon source and as the substrate in DHA biosynthesis. At the second stage, cell growth almost ceases, and glycerol is used primarily as a substrate for DHA biosynthesis. At the last stage, when the DHA concentration is above 150 g/L, the rate of DHA biosynthesis decreases, possibly because of the high DHA concentration [15]. Every stage of the fermentation bioprocess has a different DO concentration requirement. Thus, investigating the effects of the DO control strategy on DHA fermentation is very necessary.

The objective of the current work is to screen for DHA-overproducing and DHA-resistant mutants from *G. oxydans* after UV irradiation using a high throughput screening method, batch fermentation and fed-batch fermentation. The study also aims to optimize the DHA fermentation conditions using a DO control strategy.

Materials and Methods

Microorganism and Cultivation Conditions

G. oxydans ZJB-605 (CCTCC No. M208069), used for UV mutagenesis, was stored at 4 °C on a GYA agar slant consisting of 25 g/L glucose, 5 g/L yeast extract, and 20 g/L agar, and transferred monthly.

The strain was cultivated on a GYA agar slant at 30 °C for 24 h. The cells from slant were then inoculated into 500-mL flasks containing 50 mL sterile GYC medium, which contained 5 g/L yeast extract, 2 g/L $CaCO_3$, 0.5 g/L K_2HPO_4, 0.1 g/L $MgSO_4{\cdot}7H_2O$, 25 mL/L glycerol (pH 6.5). The flasks were kept on a rotary shaker (ZHWY-211B, Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd., China) at 150 rpm for 20 h at 30 °C. The broth was used for the subsequent UV mutagenesis.

The standard DHA was obtained from Fluka Chemie GmbH (Buchs, Switzerland), and all other chemicals were commercially available and of analytical grade.

Mutagenesis Program by UV Irradiation

The *G. oxydans* ZJB-605 broth was diluted appropriately, and 0.1 mL dilution was spread on GYA plates. Afterward, the plates were placed under a UV lamp (15 W, 253 nm) at a distance of 15 cm and were irradiated for 60 s. After UV mutagenesis, the cells were separated in plates and cultivated in 96-well microtiter plates [21]. The original strain was selected as the control, and verification of positive mutants was conducted in subsequent experiments.

Screening for DHA-overproducing Mutants

Shake flask fermentation was carried out to verify the positive DHA-producing mutant isolates after UV irradiation. The 24-h-old slant was inoculated in 500-mL flasks containing 50 mL GYC medium. The shake flasks were then kept on a rotary shaker at 150 rpm for 24 h at 30 °C.

Fed-batch fermentation in shake flasks was also introduced for testing the positive mutant strains. Following the batch fermentation process mentioned as above, then about 1.25 mL sterile glycerol was added to flasks, and fermentation was then continued under the same conditions.

Screening for DHA-resistant Mutants

The DHA-resistant isolates were obtained after treating strains with UV irradiation. Preliminary screening was then conducted using the high throughput screening method. The positive isolates were examined via fed-batch fermentation in shake flasks. The 24-h-old slant was inoculated in 500-mL flasks containing 50 mL GYC medium. The flasks were kept on a rotary shaker at 150 rpm for 24 h at 30 °C. Afterward, approximately 1.25 mL sterile glycerol and specific amounts of pure DHA were added to the flasks to obtain DHA concentrations of 110, 130, and 150 g/L, respectively, at different cycles of the mutagenesis program. The fermentation was then continued under the same conditions.

Mutant Strain Stability

The stability of the mutant strains was investigated as follows: the 24-h-old slant of mutant isolate was transferred to the new GYA slant and into 500-mL flasks containing 50 mL CYC

medium, respectively. The GYA slants were kept at 30 °C for 24 h, and the flasks were kept on a rotary shaker at 150 rpm for 24 h at 30 °C. Approximately 1.25 mL sterile glycerol and 7 g DHA were added to the flasks to obtain a 150 g/L DHA concentration, and the fermentation was continued under the same conditions. This procedure was repeated at least ten times.

Optimization of DHA Fermentation Bioprocess Using the DO Control Strategy in a 15-L Fermentor

The fed-batch fermentation for DHA production was performed in a 15-L fermentor (Biostat C, B. Braun Biotech International, Germany) using an initial medium consisting of 5 g/L yeast extract, 20 g/L glycerol, 0.5 g/L K_2HPO_4, 0.1 g/L $MgSO_4 \cdot 7H_2O$. Glycerol feeding commenced when the residual glycerol concentration dropped to approximately 5–10 g/L. The glycerol feeding rate was manually controlled to keep the glycerol concentration within this range. The time-interval glycerol consumption is forecasted by the former time-interval consumption; thus, the feeding rate can be adjusted to an appropriated value, which is approximately 0.50–0.70 g/min. The fermentation temperature was maintained automatically at 30 °C. The DO concentration was controlled following a cascaded model by automatically adjusting the agitation speed (200–700 rpm) and aeration rate (0.5–1.5 vvm, volumes of air/effective volumes of the bioreactor per minute). The culture pH was automatically adjusted to 6.0 within the first 20 h and then to 5.0 until the end of the fermentation.

Analytical Methods

The samples were withdrawn from the shake flasks and centrifuged at 10,000×*g* for 10 min. The supernatant was then collected and used for DHA quantification. DHA was analyzed by gas chromatography according to the Wang et al. reported [25]. The cell dry weight was determined gravimetrically and gave a linear relationship to optical density at 660 nm in a 1.0-cm cuvette (Du800, spectrophotometer, Beckman Coulter, USA),

Each experiment was repeated individually for three times, and two parallel assays were performed for each experiment.

Results

Screening for DHA-overproducing Mutants

Batch fermentation in shake flasks was carried out to screen for DHA-overproducing mutants. More than 1,000 mutants were screened after repeated UV irradiation. Some of the results are shown in Fig. 1. UV irradiation was effective in breeding DHA-overproducing mutant strains from the original strain, *G. oxydans* ZJB605. Six mutants (8D3, 8D74, 8F13, 8F19, 8F6, and 8C6) showed a much higher DHA yield compared with the original strain, indicating that these isolates have potential applications in DHA production.

Distinguishing the mutants' characteristics during DHA batch fermentation is very difficult. Thus, the mutants were then checked continuously using fed-batch fermentation. Although the DHA concentration was no significantly different among the 8F13, 8F19, 8F6, 8D74, and 8C6 mutants, the DHA productivity and conversion rate of glycerol to DHA of mutant 8C6 were much higher than those of the others (Table 1). This result suggests that mutant 8C6 performed better in DHA production compared with the other mutants.

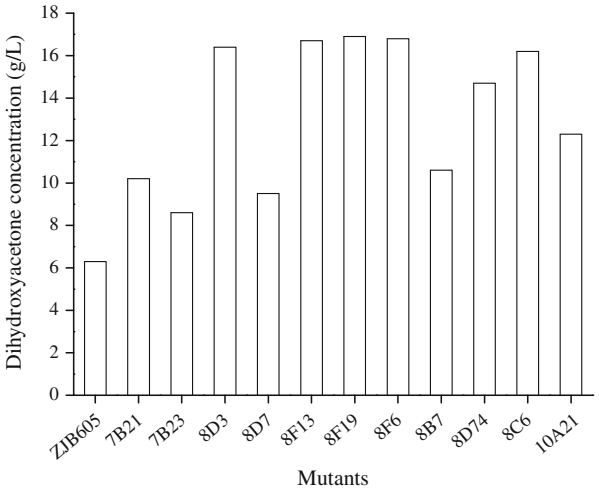

Fig. 1 DHA batch fermentation of mutants screened from *G. oxydans* ZJB605

Screening for DHA-resistant Mutants

Mutant 8C6 was selected for the subsequent UV mutagenesis experiment and fed-batch fermentation to screen for DHA-resistant mutants. In the present work, pure DHA was added to the broth after 24 h of fermentation. The amount of added DHA was gradually increased at different cycles of UV mutagenesis, resulting in a high DHA concentration. More than 1,000 mutants were screened after several mutagenesis cycles. Table 2 shows the results of the fed-batch fermentation of some mutants in the presence of approximately 150 g/L DHA. Compared with the results in Table 1, the decreases of DHA productivity and conversion rate of glycerol to DHA were common phenomena, because the fermentation was carried out at a high DHA concentration. Therefore, the mutants, which can tolerate high DHA concentration, and exhibit high conversion rate of glycerol to DHA as well as high DHA productivity, were selected. Based on these criteria, mutant ZJB11001 tolerated the highest DHA concentration, and showed the excellent performance of DHA production compared with the other mutants.

Table 1 Fed-batch fermentation of mutants screened from *G. oxydans* ZJB605

Mutant	Biomass content (g/L)	Final DHA concentration (g/L)	DHA concentration[a] (g/L)	Fermentation time (h)	DHA productivity (g/L/h)	Conversion rate of glycerol to DHA[b] (%)
8D3	1.75±0.11	37.1±1.2	20.4±0.8	60	0.62±0.02	81.6±1.9
8F13	2.01±0.09	40.1±1.4	23.2±0.9	60	0.67±0.02	77.7±4.3
8F19	1.88±0.12	40.3±1.5	23.2±1.0	72	0.56±0.02	79.5±2.0
8F6	2.18±0.04	40.2±1.4	23.3±1.0	60	0.67±0.02	77.7±4.7
8D74	2.21±0.09	40.1±1.3	25.2±1.4	60	0.67±0.02	71.8±2.2
8C6	1.87±0.11	41.6±0.8	25.7±1.7	60	0.69±0.01	86.8±2.7

[a] $\text{DHA concentration} = \frac{\text{DHA formation after 24 h fermentation(g)}}{\text{total volume(L)}}$

[b] $\text{Conversion rate of glycerol to DHA} = \frac{\text{DHA formation after 24 h fermentation}}{\text{glycerol consumption after 24 h fermentation}} \times 100\%$

Table 2 Fed-batch fermentation of mutants screened from *G. oxydans* 8C6

Mutant	Biomass content (g/L)	Final DHA concentration[a] (g/L)	DHA concentration[b] (g/L)	Fermentation time (h)	DHA productivity (g/L/h)	Conversion rate of glycerol to DHA[c] (%)
HD109	2.01±0.11	36.8±1.40	23.1±1.31	64	0.36±0.02	74.5±2.5
HD136	1.68±0.12	37.2±1.50	24.1±1.54	72	0.34±0.02	75.3±2.1
HD221	2.52±0.10	38.5±0.90	23.6±0.90	68	0.35±0.01	73.7±2.5
HD251	1.89±0.08	37.4±0.78	24.4±0.91	72	0.34±0.01	73.6±2.7
HD258	1.89±0.08	36.5±1.28	23.5±1.29	76	0.31±0.02	75.5±3.3
HD3261	1.68±0.10	38.7±1.01	22.9±1.32	72	0.32±0.02	67.7±2.6
HD3721	2.01±0.15	37.4±1.51	23.9±0.92	68	0.35±0.01	72.7±2.5
ZJB11001	2.06±0.18	39.4±1.41	25.1±0.96	64	0.39±0.02	78.4±2.6

[a] Final DHA concentration $= \frac{\text{final DHA(g)} - \text{added DHA(g)}}{\text{total volume(L)}}$

[b] DHA concentration $= \frac{\text{DHA formation after 24 h fermentation(g)}}{\text{total volume(L)}}$

[c] Conversion rate of glycerol to DHA $= \frac{\text{DHA formation after 24 h fermentation}}{\text{glycerol consumption after 24 h fermentation}} \times 100\%$

Mutant Stability

The results show that mutant ZJB11001 is a highly stable isolate with a 39.1±1.5 g/L average DHA concentration and 79.0±1.8% conversion rate of glycerol to DHA (Fig. 2). This suggests that the mutant ZJB11001 can be used in the industrial production of DHA by fermentation, because the stability of mutant is very important for industrial fermentation.

Effects of the DO Control Strategy on DHA Production in a 15-L Fermentor

The DHA production by fed-batch fermentation over mutant ZJB11001 using different DO control strategies was investigated in a 15-L fermentor. As shown in Table 3, increasing the DO concentration resulted in the increase in the DHA concentration and productivity (D1, D2, and D3 experiments). Meanwhile, the conversion rate of glycerol to DHA in D3 experiment was lower than in D1 and D2 experiments, indicating that a higher amount of

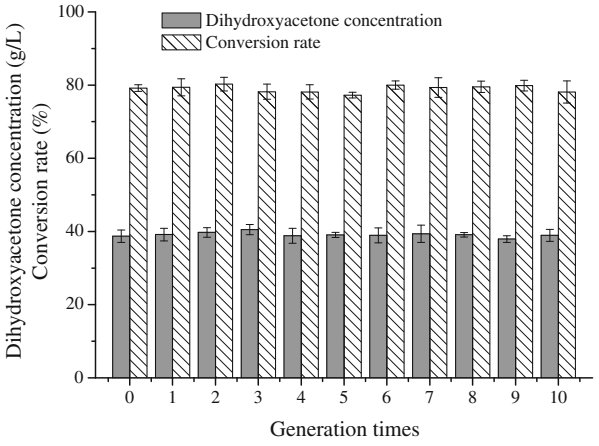

Fig. 2 Stability of the mutant strain ZJB11001

Table 3 Performance of DHA fed-batch fermentation using DO control strategy

Experiment	DO control	Biomass content (g/L)	DHA concentration (g/L)	DHA productivity (g/L/h)	Conversion rate of glycerol to DHA (%)
D1	20%	2.53±0.09	161.6±5.6	2.24±0.08	87.8±0.9
D2	30%	2.75±0.13	181.4±7.7	2.52±0.11	88.0±1.4
D3	40%	3.11±0.10	186.7±5.1	2.59±0.07	85.1±0.8
D4	DO-shift controlled[a]	2.58±0.04	197.0±8.2	2.74±0.11	89.0±0.4
D5	DO-shift controlled[b]	2.55±0.04	209.6±6.8	2.91±0.10	90.1±1.3

[a] DO was controlled at 20% air saturation within the first 24 h and then increased to 30% until the end of the fermentation

[b] DO was controlled at 20% air saturation within the first 24 h, increased to 30% until 60 h, and then readjusted to 40% until the end of the fermentation

glycerol converted to by-products at high DO concentration (D3 experiment). This resulted in a low conversion rate of glycerol to DHA; the bioprocess of D3 experiment is not economical because of the high substrate cost and high energy consumption for agitation and airflow. The DHA concentrations and productivities in D4 and D5 experiments were much higher than those in D1, D2, and D3 experiments. It was clear that DHA production by fed-batch fermentation using DO control at step-wise model was a suitable method. The maximum DHA concentration of 209.6±6.8 g/L was achieved in the D5 experiment, in which the DO concentration was maintained at 20% air saturation within the first 24 h, and increased to 30% until 60 h, and then adjusted to 40% until the end of the fermentation (Fig. 3). Therefore, the mutant ZJB11001 showed an excellent performance in the DHA fed-batch fermentation, and can be used at an industrial scale. The DHA concentration reported in the present work is slightly higher than those in our previous studies [13, 15], and much higher than in previous reports by other laboratories [2, 26, 27].

Discussion

The screening method for positive mutants during UV mutagenesis is very important in selecting usable isolates. In the present study, several screening methods were employed at different stages of the breeding program, including the high throughput screening method

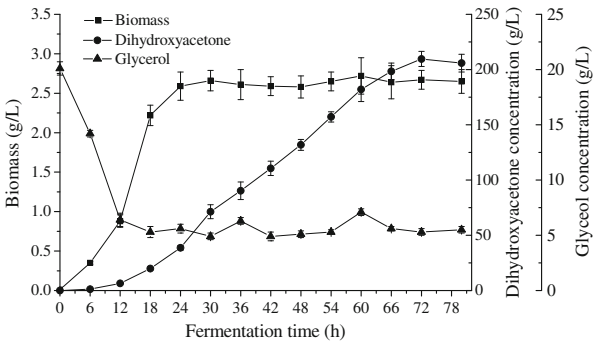

Fig. 3 Time course of the DHA fed-batch fermentation at 30 °C, with the DO concentration increased from 20% to 30% after 24 h of fermentation, and then readjusted to 40% after 60 h of fermentation

by cultivation in 96-well microtiter plate, the batch fermentation in shake flasks, the fed-batch fermentation in shake flasks, and the fed-batch fermentation at high DHA concentrations. These are typical step-by-step methods of screening mutants to meet the requirements for industrial DHA production. First, the high throughput screening method by cultivation in 96-well microtiter plate was used as a rapid, preliminary screen for positive isolates by visual inspection, which is a useful method for preliminary screening [21]. Second, the batch fermentation in shake flasks was introduced to verify the positive mutants. Third, when DHA production of mutants was increased to a certain degree, distinguishing the different mutant performances in DHA batch fermentation became very difficult. Therefore, fed-batch fermentation, a major method for DHA production, was developed to identify potent mutants with high productivity, high conversion rate of glycerol to DHA, and excellent performance for DHA production, because much more properties of mutants were checked by fed-batch fermentation than by batch fermentation. Fourth, a high DHA concentration can inhibit cell growth and DHA biosynthesis [2, 12]. Therefore, screening for DHA-resistant mutants is necessary. At the start of DHA production by fed-batch fermentation, no inhibition on cell growth and DHA biosynthesis occurred because of the low DHA concentration. However, cell growth almost ceased after 24 h of fermentation. Thus, the inhibitory of high DHA concentration on cell growth can be ignored during the first 24 h of fermentation. Meanwhile, a high DHA concentration can inhibit DHA production. Bories et al. reported that glycerol conversion ceased and no more DHA could be produced when the DHA concentration in the medium reached 108 g/L [28]. When the DHA concentration exceeded 160 g/L, the DHA production was irreversibly inhibited [14]. Therefore, inhibition is the key factor influencing on DHA biosynthesis, especially during the later stages of fermentation. The present study designed experiments that screen for mutants resistant to high DHA concentrations. Pure DHA was added into the fermentation broth to create an artificial environment with a high DHA concentration, and the DHA concentration was increased step by step from approximately 110 to 130, and then to 150 g/L. After several cycles of mutagenesis, some DHA-resistant mutants were obtained. The mutant ZJB11001 showed potential for DHA production at an industrial scale. This method is clearly useful and feasible for application in the mutagenesis program.

DO control at stepwise model is suitable for DHA production because the DO requirement varies at different stages of fermentation. At the first stage of fermentation, a suitable DO concentration for cell growth is very important for DHA fermentation [13, 15]. At the second stage, DO is used mainly as the final acceptor of the reduced equivalent from the biocatalysis of glycerol dehydrogenase, which plays a vital role in DHA biosynthesis [9]. At the last stage, the high DHA concentration exhibits inhibitory effects on DHA production. Therefore, controlling the DO concentration may reduce the inhibition and achieve a high DHA production.

Conclusion

In the current work, a stable DHA-overproducing mutant, *G. oxydans* ZJB11001, was screened after several cycles UV mutagenesis via the high throughput screening method, batch fermentation, fed-batch fermentation, and fed-batch fermentation in a medium containing a high DHA concentration. The results of the fed-batch fermentation for DHA production by *G. oxydans* ZJB11001 in a 15-L fermentor with a DO control strategy shows that the mutant performed excellently in DHA production. Moreover, the maximum DHA

concentration of 209.6±6.8 g/L was achieved at 72 h of fed-batch fermentation using the DO-shift strategy at 30 °C.

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