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Disruption of the Membrane-Bound Alcohol Dehydrogenase-Encoding Gene Improved Glycerol Use and Dihydroxyacetone Productivity in *Gluconobacter oxydans*

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Dihydroxyacetone (DHA) production from glycerol by *Gluconobacter oxydans* is an industrial form of fermentation, but some problems exist related to microbial DHA production. For example, glycerol inhibits DHA production and affects its biological activity. *G. oxydans* produces both DHA and glyceric acid (GA) from glycerol simultaneously, and membrane-bound glycerol dehydrogenase and membrane-bound alcohol dehydrogenases are involved in the two reactions, respectively. We discovered that the *G. oxydans* mutant Δ*adhA*, in which the membrane-bound alcohol dehydrogenase-encoding gene (*adhA*) was disrupted, significantly improved its ability to grow in a higher concentration of glycerol and to produce DHA compared to a wild-type strain. Δ*adhA* grew on 220 g/l of initial glycerol and produced 125 g/l of DHA during a 3-d incubation, whereas the wild-type did not. Resting Δ*adhA* cells converted 230 g/l of glycerol aqueous solution to 139.7 g/l of DHA during a 3-d incubation. The inhibitory effect of glycerate sodium salt on Δ*adhA* was investigated. An increase in the glycerate concentration at the beginning of growth resulted in decreases in both growth and DHA production.

Key words: glycerol use; dihydroxyacetone; glyceric acid; *Gluconobacter oxydans*; membrane-bound alcohol dehydrogenase

Dihydroxyacetone (DHA) is frequently used in the cosmetics industry as the main active ingredient in all sunless tanning skincare preparations, and it has also become a pharmaceutical precursor and a building block for chemical synthesis. DHA, which has a global market of approximately 2,000 tons per year,[1)] is produced by biotechnological conversion of glycerol, a biomass renewable resource that can be obtained in about 10% weight as a by-product of biodiesel fuel production through transesterification of vegetable oils and animal fats.[2)] Many studies and patents involve microbial DHA production (see review, reference 3).

The acetic acid bacterium *Gluconobacter oxydans* is currently used for DHA production, and its membrane-bound, pyrroloquinoline quinone (PQQ)-dependent glycerol dehydrogenase (GLDH) involves the oxidative reaction. Without NADH, GLDH reduces membranous ubiquinone, which is re-oxidized by a terminal oxidase using oxygen. Because its reactive center is oriented to the periplasmic space,[4)] DHA is directly released into and accumulated in the culture medium. Considering that no energy-consuming substrate transport into the cell or products out of the cell are required, the use of GLDH from *G. oxydans* appears to be an efficient method for DHA production.

However, problems exist concerning microbial DHA production. Not only glycerol (feedstock) but also DHA (product) inhibits *G. oxydans* growth and DHA production. Claret *et al.* (1992) demonstrated that higher initial concentrations of glycerol (31 to 129 g/l) resulted in an inhibitory effect on cell growth and DHA production.[5)] Also, the presence of higher concentrations of DHA (0–100 g/l) inhibit the oxidative fermentation process.[6,7)] Therefore, further improvements in microbial DHA production yield and efficiency should be achieved.

From glycerol, *G. oxydans* simultaneously produces DHA by GLDH and glyceraldehyde by membrane-bound alcohol dehydrogenase (mADH), and the latter is subsequently converted to glyceric acid (GA; Fig. 1).[8,9)] Membrane-bound multimeric quinoprotein GLDH (2 subunits) and membrane-bound quinohemoprotein mADH (3 subunits) have been found to donate electrons to ubiquinone, which is embedded in the membrane phospholipids, and then to the terminal oxidase.[10)] Hence, as we intended to prevent GA by-production, DHA production was examined using the *G. oxydans* NBRC12528 mutant, in which the mADH-encoding gene (*adhA*) was disrupted. We discovered that the *adhA* disruptant significantly improved growth and DHA productivity in high-concentration glycerol as compared to the wild-type strain.

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Abbreviations: DHA, dihydroxyacetone; PQQ, pyrroloquinoline quinone; GLDH, glycerol dehydrogenase; GA, glyceric acid; mADH, membrane-bound alcohol dehydrogenase; HPLC, high-performance liquid chromatography; vvm, volume of air per volume of medium per min; OD, optical density

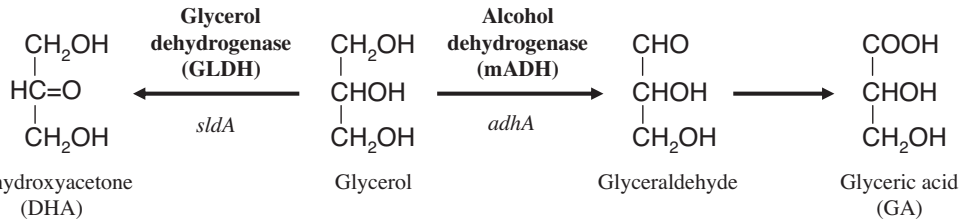

Fig. 1. Pathway for the Conversion of Glycerol to Dihydroxyacetone and Glyceric Acid by *Gluconobacter oxydans*. *sldA* and *adhA* are the genes encoding the catalytic subunits of GLDH and mADH respectively.

Materials and Methods

Bacterial strains. G. oxydans NBRC12528 (formerly IFO12528, obtained from the Institution for Fermentation, Osaka, Japan) and *G. oxydans* Δ*adhA*[9] were precultivated in 5 ml of glucose medium containing 30 g/l of glucose, 2 g/l of polypepton (Nihon Pharmaceutical, Tokyo), and 5 g/l of yeast extract (Difco Laboratories, Detroit, MI) at 30 °C for 48 h in test tubes (200 mm × Φ18 mm). The seed cultures (1.5 ml) were transferred to 300-ml Erlenmeyer flasks containing 30 ml of glycerol medium (pH 6.5), consisting of 150 g/l of glycerol, 10 g/l of polypepton, 1 g/l of yeast extract, 0.9 g/l of KH_2PO_4, 0.1 g/l of K_2HPO_4, and 1 g/l of $MgSO_4 \cdot 7H_2O$. The cultures were incubated at 30 °C on a BR-23FP rotary shaker (200 rpm; taitec, Saitama, Japan) for 4 d. After removal of the cells by centrifugation, the supernatant was filtered with a 0.45-μm cellulose filter. A 20-μl aliquot of the supernatant was analyzed by high-performance liquid chromatography (HPLC) to quantify DHA.

Jar fermentor experiments. DHA production by *G. oxydans* NBRC12528 and Δ*adhA* was conducted in a 1-liter jar fermentor (Model MDL; B.E. Marubishi, Tokyo). Jar fermentor experiments were performed as follows: Strains were cultivated in five test tubes, each containing 5 ml of glucose medium (total, 25 ml of culture) for 2 d (30 °C, 200 rpm). All seed cultures were transferred to a 1-liter jar fermentor containing 500 ml of glycerol medium (pH 6.5), consisting of 100–250 g/l of glycerol, 10 g/l of polypepton, 1 g/l of yeast extract, 0.9 g/l of KH_2PO_4, 0.1 g/l of K_2HPO_4, and 1 g/l of $MgSO_4 \cdot 7H_2O$, and incubated for 3 d. During the jar fermentor experiments, the aeration rate and agitation speed were set to 0.5 volumes of air per volume of medium per min (vvm) and 500 rpm. The temperature was maintained at 30 ± 1 °C. pH was controlled with 10 M NaOH to keep it above 5.

In the glycerol-feed experiment, Δ*adhA* were precultivated as described above, and the seed cultures were transferred to a 1-liter jar fermentor containing 500 ml of medium (pH 6.5), consisting of 50 g/l of glycerol, 10 g/l of polypepton, 1 g/l of yeast extract, 0.9 g/l of KH_2PO_4, 0.1 g/l of K_2HPO_4, and 1 g/l of $MgSO_4 \cdot 7H_2O$, and incubated for 1 d at 30 °C, 0.5 vvm, and 500 rpm. After removal of the cells by centrifugation, the cells (2.1 g/l of dry weight) were resuspended in 500 ml of fresh glycerol medium (pH 6.5), consisting of 150 g/l of glycerol, 10 g/l of polypepton, 1 g/l of yeast extract, 0.9 g/l of KH_2PO_4, 0.1 g/l of K_2HPO_4, and 1 g/l of $MgSO_4 \cdot 7H_2O$, and incubated again in a 1-liter jar fermentor for 4 d at 30 °C, 0.5 vvm, and 500 rpm. After a 2-d cultivation, an additional 75 g of glycerol was added to the fermentor. pH was controlled with 10 M NaOH to keep it above 5.

In the resting cell reaction experiment, Δ*adhA* cells were prepared as described above, and were resuspended in 500 ml of glycerol aqueous solution (230 g/l). The reactions were conducted for 4 d at 30 °C, 2 vvm, and 700 rpm. pH was controlled with 10 M NaOH to keep it above 5.

Inhibition of dihydroxyacetone production and growth of G. oxydans ΔadhA *due to glycerate.* To investigate the inhibitory effects of glycerate on *G. oxydans* Δ*adhA*, seed cultures of Δ*adhA* were transferred to 30 ml of medium (pH 6.5), consisting of 0, 0.2, 0.4, 0.6, 0.8, 1.0, or 2.5% w/v DL-glycerate sodium salt (stock solution: about 5.2 mol/l DL-GA in water (Tokyo Chemical Industry, Tokyo)), 10 g/l of polypepton, 1 g/l of yeast extract, 0.9 g/l of KH_2PO_4, 0.1 g/l of K_2HPO_4, and 1 g/l of $MgSO_4 \cdot 7H_2O$. Cultures were incubated at 30 °C on a rotary shaker (200 rpm) for 4 d. After removal of the cells by centrifugation, the various supernatants were analyzed by HPLC.

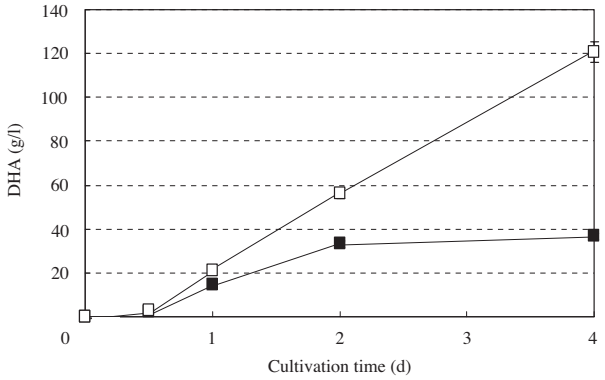

Fig. 2. Dihydroxyacetone Production by *Gluconobacter oxydans* NBRC12528 and the Δ*adhA* Mutant.

Symbols: Black and white squares represent wild-type and Δ*adhA* respectively. Error bars represent the standard deviation calculated from three independent experiments.

Quantification of glycerol, DHA, and GA. The glycerol, DHA, and GA concentrations in the culture broth were analyzed by HPLC with an LC-20AD HPLC pump (flow rate, 1.0 ml/min) and an RID-10A detector (Shimadzu) equipped with a Shodex® SC1011 column (Showa Denko, Tokyo) for glycerol and DHA and a Shodex® SH1011 column (Showa Denko) for GA. A mobile phase of pure water and 5 mM H_2SO_4 solution was chosen as the eluent for the columns. During analysis, the column temperature was maintained at 80 °C and 60 °C for the two columns, respectively. DL-GA calcium salt dihydrate (Wako Pure Chemicals, Osaka, Japan) or DL-GA (40% in water; Tokyo Chemical Industry) and DHA (MP Biomedical, Santa Ana, CA) was used to determine the standard curve to quantify GA and DHA, respectively.

Chemicals. All chemicals were the purest commercially available (98–100%; Sigma-Aldrich, Kanto Chemical, Wako Pure Chemicals, Nacalai Tesque, Tokyo Chemical Industry, MP Biomedical).

Results

Differences in DHA productivity between G. oxydans *and its mutant*

Gluconobacter oxydans Δ*adhA* was examined for its ability to produce DHA from 150 g/l of initial glycerol, and DHA productivity was compared to that of the wild-type strain (Fig. 2). The wild-type strain produced 33.2 g/l of DHA after a 2-d incubation, and further incubation had little effect on DHA production. The wild-type strain accumulated 17.8 and 19.3 g/l of GA in the culture after 2- and 4-d incubations respectively. In contrast, Δ*adhA* appeared to be a more efficient DHA producer, as DHA production continued to increase and

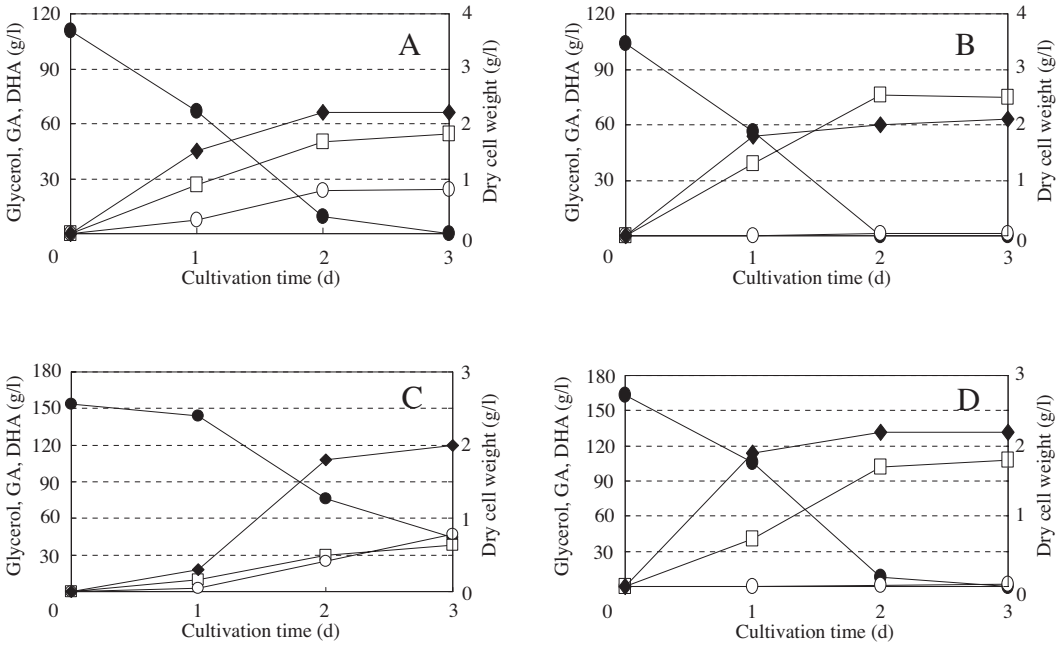

Fig. 3. Comparison of Dihydroxyacetone (DHA) and Glyceric Acid (GA) Production between *Gluconobacter oxydans* NBRC12528 and the Δ*adhA* Mutant.

A, initial glycerol (100 g/l), wild type; B, initial glycerol (100 g/l), Δ*adhA*; C, initial glycerol (150 g/l), wild type; D, initial glycerol (150 g/l), Δ*adhA*. In all the experiments, the reactions were conducted at 30 °C, 0.5 volumes of air per volume of medium per min, and 500 rpm. pH was controlled with 10 M NaOH to keep it above 5. Symbols: Black circles, glycerol concentration; white circles, GA concentration; black diamonds, dry cell weight; white squares, DHA concentration.

was 3.3 times (120.5 g/l) greater than that of the wild-type strain (36.6 g/l) after a 4-d incubation (Fig. 2). Δ*adhA* produced only 0.9 g/l of GA during this period.

Comparison of DHA productivity between G. oxydans *and its mutant using several initial glycerol concentrations*

We examined growth, DHA production, and GA by-production in both *G. oxydans* and Δ*adhA* using 100 g/l of initial glycerol in a jar fermentor (Fig. 3A, B). The *G. oxydans* wild-type strain consumed most of the glycerol within 2 d, and 54.3 g/l of DHA and 24.3 g/l of GA was produced during a 3-d incubation (Fig. 3A). A DHA yield of 0.59 mol/mol glycerol consumed was obtained. In contrast, Δ*adhA* accumulated only 1.0 g/l of GA. Thus 74.8 g/l of DHA was produced during the same period (0.74 mol/mol glycerol consumed; Fig. 3B).

More differences between the mutant and the wild type appeared when we used an initial glycerol concentration of 150 g/l (Fig. 3C, D). The wild-type strain accumulated 38.1 g/l of DHA (0.36 mol/mol glycerol consumed) and 47 g/l of GA, indicating that a higher concentration of initial glycerol decreased DHA production but increased GA production (Fig. 3C). This tendency for higher concentrations of initial glycerol to accumulate more GA corresponded to our recent results on the effect of initial glycerol concentration on GA production.[9,11,12] Besides scarcely producing GA (2.2 g/l), DHA production of Δ*adhA* reached 108 g/l (0.68 mol/mol glycerol consumed) within the same period (Fig. 3D). Poor growth of the wild-type strain was observed after a 1-d incubation, whereas Δ*adhA* reached the mid-log phase (Fig. 3D) within 1 d.

A further big change was observed when a concentration of more than 200 g/l of initial glycerol was used. Δ*adhA* grew on 220 g/l of initial glycerol and produced 125 g/l of DHA, whereas the wild-type did not grow on such a high glycerol concentration during a 3-d incubation. GA by-production was just 1.7 g/l in Δ*adhA*, and the remaining glycerol was 63.6 g/l, and therefore the yield of DHA was 0.81 mol/mol glycerol consumed. These results indicate that disrupting the *adhA* gene significantly improved growth and DHA productivity in *G. oxydans* in a high concentration of initial glycerol.

Next, we investigated the DHA productivity of Δ*adhA* with higher cell density and additional glycerol-feed. Approximately 2 g/l of Δ*adhA* cells (dry weight) were resuspended in glycerol medium and incubated in a 1-liter jar fermentor. After a 2-d cultivation, results of 3.5 g/l of dry cell weight, no remaining glycerol, and 116.5 g/l of DHA (0.68 mol/mol glycerol consumed) in the culture were obtained. At this point, an additional 75 g of glycerol was added to the fermentor (132.9 g/l of glycerol), but this had no significant effect on the increase in DHA production during a further 2-d incubation (data not shown).

Time course of DHA production by resting cell reactions using the G. oxydans *mutant*

Since Δ*adhA* scarcely formed the GA by-product, it was considered advantageous for further purification. However, to make DHA purification easier, it is better not to use medium constituents other than glycerol, such as polypepton or yeast extract. Hence, we conducted resting cell reactions using Δ*adhA* and 230 g/l of glycerol aqueous solution. The Δ*adhA* preculture was incubated on 50 g/l of glycerol for 1 d, resulting in approximately 2 g/l of cells (dry cell weight). After centrifugation, the cells were resuspended in a glycerol-water solution and incubated for 3 d. The time course of

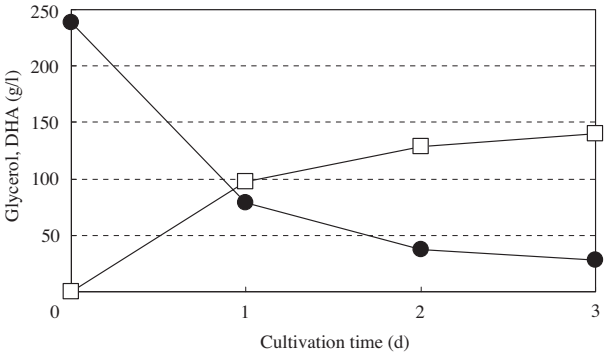

Fig. 4. Time Course of Dihydroxyacetone (DHA) Production in Resting *Gluconobacter oxydans* Δ*adhA* Cells Using 230 g/l of Glycerol Solution.

The reaction was conducted at 30 °C, 2 volumes of air per volume of medium per min, and 700 rpm. pH was controlled with 10 M NaOH to keep it above 5. Symbols: Black circles, glycerol concentration; white squares, DHA concentration.

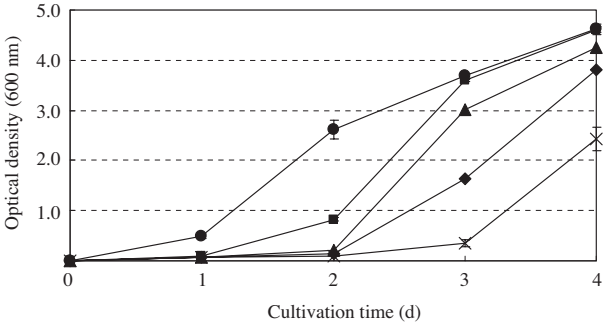

Fig. 5. Changes in Growth in Cultures of *Gluconobacter oxydans* Δ*adhA* on 150 g/l of Initial Glycerol with 0 (●), 0.2 (■), 0.4 (▲), 0.6 (◆), and 0.8% (x; w/v) Sodium Glycerate Concentrations.

DHA production at a 2 vvm aeration rate and 700 rpm agitation speed showed that approximately 139.7 g/l of DHA was produced during the 3-d incubation (Fig. 4). The remaining glycerol and GA accumulated were 28.4 and 2.3 g/l respectively.

Inhibitory effect of glycerate on dihydroxyacetone production and the growth of G. oxydans ΔadhA

The inhibitory effect of glycerate on Δ*adhA* was tested in a preliminary way in the presence of 0, 1.0, and 2.5% w/v DL-glycerate sodium salt. Similarly to Fig. 2, in no-glycerate conditions, Δ*adhA* grew well on glycerol (OD_{600}, 4.66) and produced 116.5 g/l of DHA within 4 d. By contrast, in the presence of only 1% w/v glycerate, very poor growth of the strain was observed during a 4-d cultivation (OD_{600}, 0.75), and it accumulated DHA to only about one tenth (11.5 g/l) as compared to the no-glycerate condition. Further addition of initial glycerate to the medium (2.5% w/v) resulted in both no growth and no DHA accumulation. We further investigated Δ*adhA* inhibition due to glycerate in detail with 0, 0.2, 0.4, 0.6, and 0.8% w/v DL-glycerate sodium salt. As shown in Fig. 5, an increase in the initial glycerate concentration resulted in a decrease in the strain's growth velocity. After a 4-d incubation, 104.7, 79.9, 62.9, 48.0, and 29.0 g/l of DHA accumulated in the respective cultures. These results indicate that glycerate has an inhibitory effect on the cells of *G. oxydans*, especially at the beginning of its growth.

Discussion

We found that the *G. oxydans adhA* disruptant had the ability to grow on a higher concentration of initial glycerol and to produce more DHA than the wild type. It has been found that higher concentrations of initial glycerol and accumulated DHA inhibit cell growth and DHA production,[3,5] and these problems are of great concern in DHA fermentation. Several process changes have been made to solve these problems. For example, a higher amount of DHA was obtained in a fed-batch culture than in a batch culture,[13] and a repeated fed-batch process was more useful.[14] Besides these processes, a two-stage repeated fed-batch process[7] and immobilizing the cells in a polymeric matrix[15] also solved these problems. However, until now, the desired yield has not been achieved. Therefore, our finding that the use of an *adhA* disruptant in the above developed processes for solving such problems in DHA production suggests a possibly useful technique. Also, the mutant strain may be advantageous for applying recent excess glycerol and excluding the GA by-product.

As shown in Fig. 3A and B, when we used 100 g/l of initial glycerol, few differences in the bacterial growth profile or glycerol consumption were found, but a higher amount of DHA was produced in Δ*adhA* (yields, 0.59 and 0.74 mol/mol glycerol consumed for the wild type and Δ*adhA* respectively). Considering that the amounts of total glycerol derivatives (DHA plus GA) in the culture were almost the same in the wild type and Δ*adhA* (0.83 and 0.84 mol/l respectively), the amount of glycerol consumed in GA by-production in the wild type was used for DHA production in Δ*adhA*.

Also, the *adhA* disruptant was better than the wild type under 150 g/l of initial glycerol (Fig. 3C and D). Yields of 0.36 and 0.68 mol/mol glycerol consumed for the wild type and Δ*adhA* respectively were obtained. The yield of the wild type at 150 g/l of glycerol was about 60% of that at 100 g/l. The reason is that part of the glycerol for DHA production is consumed in GA by-production, because GA accumulation in the culture at 150 g/l of initial glycerol (47 g/l; Fig. 3C) was much more than that at 100 g/l of initial glycerol (24.3 g/l; Fig. 3A).

Concerning bacterial growth, some delay was observed in the wild type at 150 g/l of initial glycerol (Fig. 3C). Furthermore, only the wild-type strain was not able to grow on 220 g/l of initial glycerol. Probably, these phenomena were not due to the inhibitory effect of initial glycerol on the cells, as described previously,[5] because Δ*adhA* exhibited faster growth than the wild type under the same initial glycerol concentrations (Fig. 3D). The difference between two strains lies in whether GA can be produced or not. Hence, we investigated the inhibitory effect of glycerate sodium salt on Δ*adhA*, and it was found that an increase in the initial glycerate concentration (0 to 0.8% w/v) resulted in poorer growth (Fig. 5).

As described above, *G. oxydans* produces both DHA and GA from glycerol at the same time (Fig. 1), and the production ratio is determined mainly by aeration conditions[8,9] and the initial glycerol concentration.[9] We have found that at initial glycerol concentrations

higher than 100 g/l (up to 220 g/l), an increase in the initial glycerol concentration increased the concentration of GA accumulated in the culture, and that mADH showed stronger activities oxidizing glycerol to glyceraldehyde with a higher concentration of glycerol (substrate).[9] These results suggest that the higher amounts of GA produced in very early growth play stronger inhibitory roles in cell activities. Hence Δ*adhA*, which do not produce GA, grow well on such higher concentrations of glycerol, and this better growth of Δ*adhA* than the wild type results in better DHA productivity as well as no competition for glycerol between DHA and GA production.

Gätgens *et al.* (2007) have reported that the *G. oxydans* DSM 2343 mutant strain (designated strain MF1), in which the gene encoding gluconate-2-dehydrogenase was disrupted, produced more DHA than the corresponding wild-type strain, because disruption of the gene led to a slight increase in the *sldAB* transcription level when cells were grown on glucose.[16] Gätgens *et al.* (2007) also found that *sldAB* overexpression using the *G. oxydans* MF1 recombinant (the gluconate-2-dehydrogenase disruptant) had a significant effect on growth and DHA production.[16] Hence the use of recombinant *G. oxydans* Δ*adhA* carrying *sldAB* can achieve more efficient DHA production than Δ*adhA*. Experiments are now underway.

Acknowledgments

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