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Production of Glyceric Acid by *Gluconobacter* sp. NBRC3259 Using Raw Glycerol

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***Gluconobacter* sp. NBRC3259 converted glycerol to glyceric acid (GA). The enantiomeric composition of the GA produced was a mixture of DL-forms with a 77% enantiomeric excess of D-GA. After culture conditions, such as initial glycerol concentration, types and amounts of nitrogen sources, and initial pH, were optimized, *Gluconobacter* sp. NBRC3259 produced 54.7 g/l of GA as well as 33.7 g/l of dihydroxyacetone (DHA) from 167 g/l of glycerol during 4 d of incubation in a jar fermentor with pH control. GA production from raw glycerol samples, the main by-product of the transesterification process in the biodiesel production and oleochemical industries, was also evaluated after proper pretreatment of the samples. Using a raw glycerol sample with activated charcoal pretreatment, 45.9 g/l of GA and 28.2 g/l of DHA were produced from 174 g/l of glycerol.**

Key words: glycerol use; glyceric acid; dihydroxyacetone; acetic acid bacteria; *Gluconobacter* sp.

Due to recent concern about renewable resources other than petroleum, much attention has been paid to biorefineries, which enable the production of biofuels as well as building-block chemicals from biomass.[1,2)] One of the renewable resources from biomass, glycerol (1,2,3-propanetriol), is thought to be a promising and abundant carbon source for industrial microbiology.[3)] Glycerol can be obtained up to 14% weight as a by-product of biodiesel fuel (BDF) production through transesterification of vegetable oils and animal fats. In several European countries, the production of glycerol has increased significantly due to BDF uptake. As a result, the price has fallen, and chemical synthesis of glycerol from petrochemical feedstock has shut down owing to a surplus.[4)] This indicates that glycerol is an attractive feedstock for the production of various chemicals.

Research has been carried out to find new applications of glycerol in both chemical processes[5–7)] and bioprocesses.[3)] In this study, we focused on the production of a promising glycerol derivative, glyceric acid (GA), *via* a bioprocess that has the potential to be a building block for several chemical compounds. In the chemical synthesis of GA, a racemic mixture of DL-GA can be obtained by metal-catalytic oxidation of the primary hydroxyl groups of glycerol.[8–10)] On the other hand, little is known about GA production by microorganisms (Fig. 1), although GA is known as a by-product during the production of dihydroxyacetone (DHA), the main active ingredient in all sunless tanning skincare preparations, by *Gluconobacter oxydans*.[11)] According to a Japanese patent application in 1987 (JP0751069, Daicel Chemical Industries), four *Gluconobacter* strains produced 38.3 g/l of D-GA (on average) from 100 g/l of glycerol in a fermentor, but detailed investigation has not been done. Also, in our recent report, *Acetobacter tropicalis* NBRC16470 produced 22.7 g/l of D-GA from 200 g/l of glycerol in a fermentor.[12)]

Gluconobacter strains oxidize a broad range of substrates, including alcohols, sugars, sugar acids, and sugar alcohols, and the corresponding oxidative products are accumulated in the culture. These bacteria have numerous membrane-bound dehydrogenases and oxidoreductases, and thus, energy-consuming transport of substrates into the cell and of products out of the cell is not required.[13)] Hence, we searched for an efficient producer of GA among various *Gluconobacter* strains (including type strains), and investigated the production of GA and the by-production of DHA by the strains selected. Also, we tried to use raw glycerol as a feedstock for GA production.

Materials and Methods

Bacterial strains. Seven *Gluconobacter* strains, *G. albidus* NBRC3250^T (type strain), *G. frateurii* NBRC3262, *G. condonii* NBRC3266^T, *G. cerinus* NBRC3267^T, *G. thalilandicus* NBRC100600^T, *G. oxydans* NBRC14819^T, and *Gluconobacter* sp. NBRC3259 were obtained from the National Institute of Technology and Evaluation (NITE), Japan. Stock cultures were cultivated at 30 °C on an agar medium containing 5 g/l of polypepton (Nihon Pharmaceutical, Tokyo), 5 g/l of yeast extract (Difco Laboratories, Detroit, MI), 5 g/l of glucose, and 1 g/l of $MgSO_4 \cdot 7H_2O$ (pH 6.5). Bacterial strains were precultivated in 5 ml of the above glucose medium at 30 °C for 48 h in test tubes (200 mm × Φ18 mm), and the seed cultures (1.5 ml) were transferred to 300-ml Erlenmeyer flasks containing 30 ml of the medium (pH 6.5) consisting of 10% (v/v) glycerol, 5 g/l of

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Abbreviations: BDF, biodiesel fuel; DHA, dihydroxyacetone; GA, glyceric acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; RT, retention time

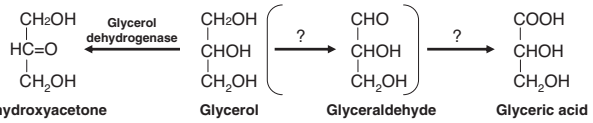

Fig. 1. Proposed Pathway for the Conversion of Glycerol to Glyceric Acid by Acetic Acid Bacteria.

Bioconversion of glycerol to dihydroxyacetone is also represented. The "?" indicates that no enzymes have been reported yet. Parentheses represent a compound that has not been found yet as an intermediate during GA production.

polypepton (Nihon Pharmaceutical), 5 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$, and were incubated at 30 °C on a rotary shaker BR-23FP (200 rpm; TAITEC, Saitama) for 4 d. After removal of the cells by centrifugation, the supernatant was filtrated with a 0.45-μm cellulose filter. To quantify both glycerol and GA, a 20-μl sample of the supernatant was analyzed by high-performance liquid chromatography (HPLC).

Structural analysis of GA produced by Gluconobacter *sp. NBRC3259.* Seed culture (1.5 ml) of *Gluconobacter* sp. NBRC3259 precultivated with the above glucose medium was transferred to a flask containing 30 ml of a medium (pH 6.5) consisting of 10% (v/v) glycerol, 5 g/l of polypepton, 5 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$, and was incubated at 30 °C on a rotary shaker for 4 d. After removal of the cells by centrifugation, 25 ml of the supernatant was filtrated and adjusted to pH 5 with 10 M NaOH. GA was separated from glycerol by ion exchange chromatography with DOWEX 1-X8 (Dow Chemical, Midland, MI) in a 20 × 300 mm column. The supernatant (25 ml) was applied to the column, and chromatography was carried out with 0.5 M HCl. The eluate was fractionated and checked for GA amounts by HPLC. An appropriate amount of $CaCl_2 \cdot 2H_2O$ (half moles of GA) was added to the fractions containing GA (concentration, about 30 g/l; pH adjusted to pH 5), and GA calcium salt was precipitated by adding ethanol to the crude GA solution. The purified GA calcium salt was dissolved in deuterium oxide (D_2O), and ^1H and ^{13}C NMR analysis was performed using a Varian INOVA 400 (400 MHz). The enantiomeric composition of the GA calcium salt was analyzed by HPLC consisting of an LC-20AD HPLC pump (flow rate, 1.0 ml/min) and an SPD-20AV UV/VIS detector (detection, 254 nm; Shimadzu, Kyoto) equipped with two tandemly-linked CHIRALPAK® MA(+) Columns (Daicel Chemical Industries, Osaka). A mobile phase of 0.45 mM $CuSO_4$ solution was chosen as the eluent. During analysis, the column temperature was kept at 21 °C. DL-GA calcium salt dihydrate (Wako Pure Chemicals, Osaka), D-GA calcium salt dihydrate (Sigma-Aldrich, St. Louis, MO), and L-GA calcium salt dihydrate (Sigma-Aldrich) were used as standard samples.

Growth characteristics of Gluconobacter *sp. NBRC3259 on glycerol.* The seed culture of *Gluconobacter* sp. NBRC3259 was transferred to 30 ml of a medium (pH 6.5) consisting of 1% (v/v) glycerol, 5 g/l of polypepton, 5 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$ (nutrient-rich condition) or 1% (v/v) glycerol, 1 g/l of yeast extract, 2 g/l of $(NH_4)_2SO_4$, and 1 g/l of $MgSO_4 \cdot 7H_2O$ (nutrient-poor condition), and was incubated at 30 °C on a rotary shaker (200 rpm) for 4 d. Samples (0.5 ml) were taken from the cultures at regular intervals. After removal of the cells by centrifugation, the respective supernatants were analyzed by HPLC.

Optimization of culture conditions for GA production. All cultures of *Gluconobacter* sp. NBRC3259 were incubated at 30 °C on a rotary shaker (200 rpm) for 4 d. After removal of the cells by centrifugation, the respective supernatants were analyzed by HPLC.

(i) Effect of initial glycerol concentration. Seed cultures were transferred to 30 ml of a medium (pH 6.5) consisting of 5, 10, or 15% (v/v) glycerol, 5 g/l of polypepton, 5 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$.

(ii) Effect of nitrogen sources. Seed cultures were transferred to 30 ml of medium (pH 6.5) consisting of 10% (v/v) glycerol, 1 g/l of yeast extract, 1 g/l of $MgSO_4 \cdot 7H_2O$, and 9 g/l of nitrogen compounds (polypepton, peptone, yeast extract $(NH_4)_2SO_4$, and $NaNO_3$).

(iii) Effect of initial polypepton concentration. Seed cultures were transferred to 30 ml of the medium (pH 6.5) consisting of 10% (v/v) glycerol, 5, 10, 15, 20, 25, or 30 g/l of polypepton, 1 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$.

(iv) Effect of pH. Seed cultures were transferred to 30 ml of medium (pH 3, 4, 5, 6, 7, 8, or 9) consisting of 10% (v/v) glycerol, 25 g/l of polypepton, 1 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$.

(v) Effect of methanol. Seed cultures were transferred to 30 ml of medium (pH 6.5) consisting of 10% (v/v) glycerol, 0.2, 0.4, 0.6, 0.8, or 1% (v/v) methanol, 25 g/l of polypepton, 1 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$.

(vi) Effect of NaCl or Na_2SO_4. Seed cultures were transferred to 30 ml of medium (pH 6.5) consisting of 10% (v/v) glycerol, 1, 3, 5, 10, or 15 g/l of NaCl or Na_2SO_4, 25 g/l of polypepton, 1 g/l of yeast extract, and 1 g/l of $MgSO_4 \cdot 7H_2O$.

Jar fermentor experiments with pure glycerol. GA production by *Gluconobacter* sp. NBRC3259 was carried out in a 1-liter jar fermentor (Model MDL; B.E. Marubishi). Jar fermentor experiments were performed as follows: *Gluconobacter* sp. NBRC3259 was 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 medium (pH 6.5) consisting of 150–170 g/l of glycerol, 25 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 4 d. During the jar fermentor experiments, the aeration rate and agitation speed were set to 1.0 vvm and 500 or 700 rpm. Temperature was maintained at 30 ± 1 °C. When necessary, pH was controlled with 5 M NaOH so as not to be under pH 5.

Pretreatment of raw glycerol samples. Raw glycerol sample A, obtained from the transesterification of tryglyceride (kindly provided by Sun Care Fuels Corporation),[14] contained the following components (analyzed by our group): glycerol, 66.4% (w/v); methanol, 30.9% (w/v); and sodium salt, 0.54% (w/v); pH 12. A second type of raw glycerol sample B (kindly provided by LION Cooperation) of a higher grade contained the following components (analyzed by our group): glycerol, 88.4% (w/v); and sodium salt, 0.19% (w/v); pH 8. The methanol was not detected in raw glycerol sample B. Methanol in raw glycerol sample A was removed by evaporation *in vacuo*. The pH of glycerol sample A was adjusted to pH 7 to 8 with HCl or H_2SO_4. When necessary, raw glycerol samples were treated with granular, activated charcoal (Wako Pure Chemicals) after the samples were diluted with the same volume of water. Approximately 20% (w/v) activated charcoal was added directly to the raw glycerol samples, and the samples were incubated statically at room temperature for more than 8 h. The charcoal was removed from the treated samples by filtration, and the resulting raw glycerol samples were used in further experiments.

Jar fermentor experiments with raw glycerol samples. Jar fermentor experiments with raw glycerol were carried out similarly to the jar fermentor experiments with pure glycerol described above. Seed cultures were transferred to a 1-liter jar fermentor containing 500 ml of medium (pH 6.5) consisting of raw glycerol samples (150–170 g/l of glycerol at final concentration), 25 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 4 d. During the jar fermentor experiments, the pH was controlled with 5 M NaOH so as not to be under pH 5.

Quantification of glycerol, DHA, and GA. The concentrations of glycerol, DHA, and GA in 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 for the respective columns as the eluent. During analysis, the column temperature was kept at 80 °C and 60 °C for the respective columns. DL-GA calcium salt dihydrate (Wako Pure Chemicals) or DL-GA (40% in water; Tokyo Chemical Industry, Tokyo) and DHA (MP Biomedicals, Santa Ana, CA) was used to determine the standard curve for GA and DHA quantification, respectively.

Table 1. Production of Glyceric Acid and Dihydroxyacetone from Glycerol by *Gluconobacter* Strains

Strain	Glyceric acid (g/l)	Dihydroxyacetone (g/l)
Gluconobacter albidus NBRC3250^T	9.1	48.2
Gluconobacter frateurii NBRC3262	10.4	18.9
Gluconobacter condonii NBRC3266^T	4.1	74.4
Gluconobacter cerinus NBRC3267^T	18.0	44.6
Gluconobacter thalilandicus NBRC100600^T	2.3	37.9
Gluconobacter oxydans NBRC14819^T	1.4	108.2
Gluconobacter sp. NBRC3259	18.2	41.0

^{T}Type strain

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

Results

Structural characterization of GA produced by Gluconobacter *sp. NBRC3259*

Seven *Gluconobacter* strains including type strains were examined for their ability to produce GA and DHA from 10% (v/v) glycerol (Table 1). Preliminary examination to determine culture periods revealed that GA production reached a maximum within 4 d (data not shown). Among the strains tested, *Gluconobacter* sp. NBRC3259 was the most efficient GA producer at 10% (v/v) glycerol concentration, and hence was used in further studies as the representative strain.

The culture supernatant of *Gluconobacter* sp. NBRC3259 was collected and roughly purified by ion exchange chromatography with DOWEX 1-X8. Using the resulting sample, GA calcium salt was precipitated and further purified. By ^{1}H and ^{13}C NMR analyses, we confirmed that the ^{1}H and ^{13}C spectra of the purified GA calcium salt were identical to those of three authentic samples, DL-GA calcium salt dihydrate (Wako Pure Chemical), D-GA calcium salt dihydrate (Sigma-Aldrich), and L-GA calcium salt dihydrate (Sigma-Aldrich).[12)]

The enantiomeric composition of the GA produced by *Gluconobacter* sp. NBRC3259 was investigated. As shown in Fig. 2, two peaks in retention time (RT) occurred at 13.2 and 15.8 min in the authentic DL-GA (L- and D-GA respectively; Fig. 2B), and the purified GA calcium salt also had two peaks corresponding to L- and D-GA (Fig. 2A). This result indicates that *Gluconobacter* sp. NBRC3259 produced both D- and L-GA, and yielded D-GA with 77% enantiomeric excess.

Optimization of culture conditions for GA production

We first tested the growth of *Gluconobacter* sp. NBRC3259 on 1% (v/v) glycerol under both nutrient-poor and -rich conditions. In nutrient-poor conditions, *Gluconobacter* sp. NBRC3259 grew on glycerol and reached stationary phase within 2 d. Approximately 91% of glycerol was degraded in 2 d of cultivation; however, not even a trace amount of GA was detected during this period (data not shown). In contrast, the strain reached stationary phase within 24 h in nutrient-rich conditions, and approximately 0.6 g/l GA was detected within this period. In both cases, no DHA production was observed (data not shown). Based on these results, *Gluconobacter* sp. NBRC3259 can use glycerol as a carbon source under both culture conditions, but produces GA when cultivated under nutrient-rich conditions. We then investigated the effect of the initial glycerol concentration on GA production under nutrient-rich conditions, and found that the GA concentration in the culture reached a maximum (20.3 g/l) with the addition of 10% (v/v) glycerol. Therefore, both nutrient-rich conditions and 10% (v/v) glycerol were used as glycerol media in the following experiments.

The effects of types and amounts of nitrogen sources on GA production were investigated. Figure 3A illustrates GA production according to nitrogen source. The addition of polypepton exhibited the highest productivity among several nitrogen sources. In addition, the effect of polypepton concentration on GA production was examined. Maximum yield of 27.9 g/l was attained with the addition of 25 g/l polypepton (Fig. 3B). Then GA production was investigated over a range of initial pH values. Approximately 25 g/l of GA was produced at the same level from pH 4 to pH 7 (maximum at pH 5), but decreased at pH 3 and pH 8 (Fig. 3C). In addition, the effect of NaCl or Na_2SO_4 on GA production was investigated. It was found that an increase in the amount of Cl^- affected GA productivity as well as the strain's growth (Fig. 3D).

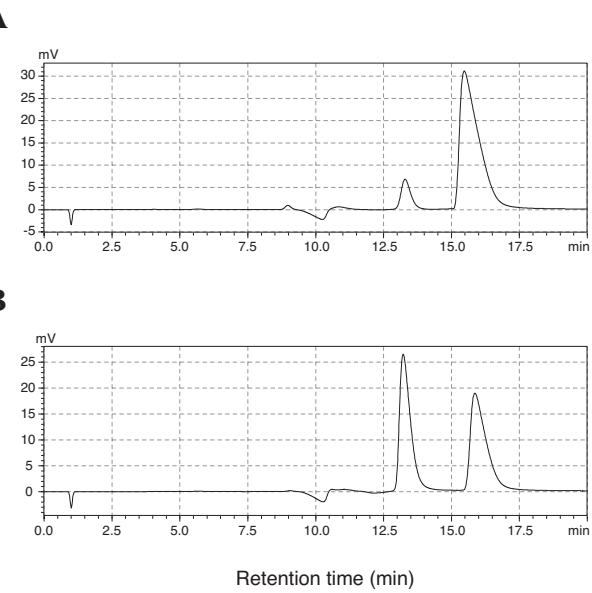

Fig. 2. Enantiomeric Separation of DL-GA.

The first peak (13.2 min) and the second (15.8 min) represent L- and D-GA, respectively. Two tandemly-linked CHIRALPAK® MA(+) Columns (Daicel Chemical Industries, Osaka) were used with a mobile phase 0.45 mM $CuSO_4$ solution as the eluent. A, GA sample produced by *Gluconobacter* sp. NBRC3259 (approximately 0.5 g/l); B, Authentic sample, DL-GA calcium salt (0.5 g/l).

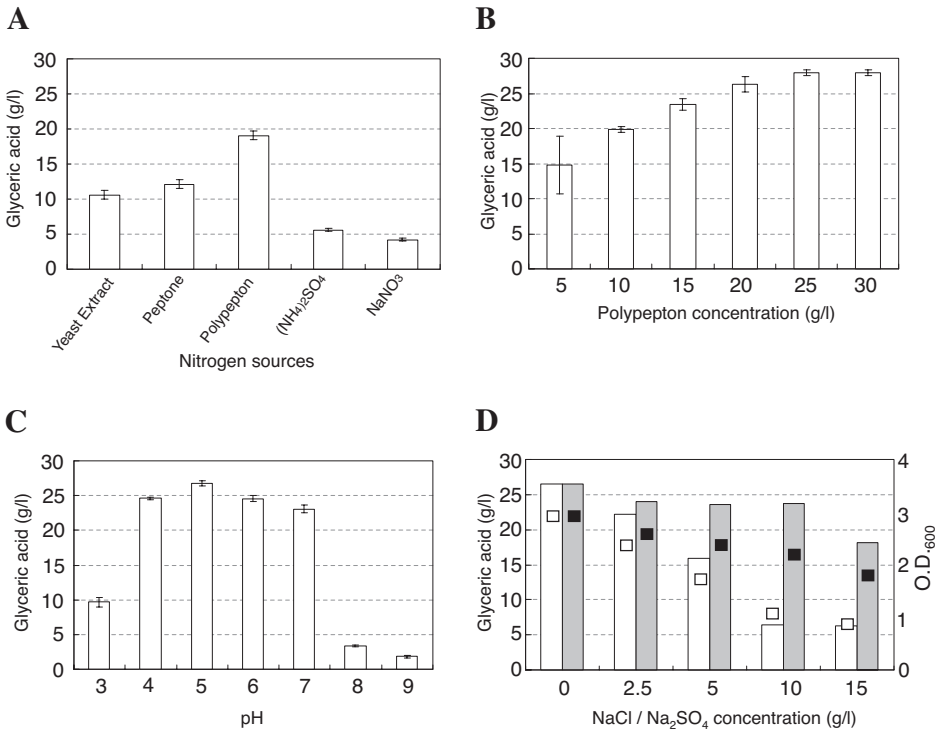

Fig. 3. Effects of Nitrogen Sources (A), Polypepton Concentration (B), Initial pH (C), and NaCl or Na_2SO_4 (D) on GA Production by *Gluconobacter* sp. NBRC3259.

A, B, and C, Error bars represent the standard deviation calculated from three independent experiments. D, The data represent the average of two independent experiments. White and gray bars represent GA production with the addition of NaCl and Na_2SO_4 respectively and □ and ■ represent the OD_{600} with NaCl and Na_2SO_4 respectively.

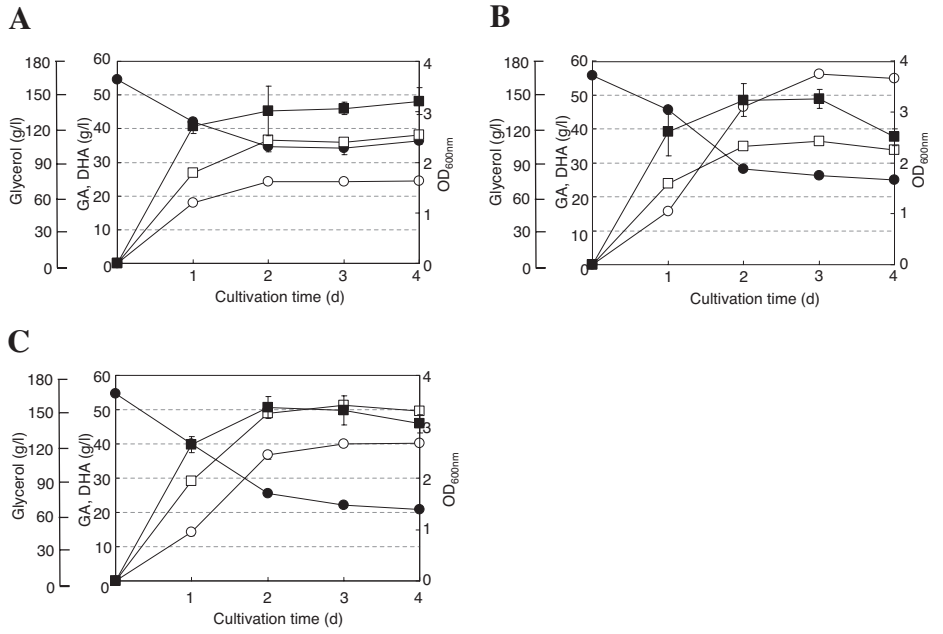

Fig. 4. Typical Time Course of GA Production from Pure Glycerol by *Gluconobacter* sp. NBRC3259.

A, pH was not controlled, 500 rpm. B, pH was controlled with 5 M NaOH so as not to be under pH 5, 500 rpm. C, pH was controlled so as not to be under pH 5, 700 rpm. Symbols: solid circles, glycerol concentration; open circles, GA concentration; solid squares, optical density; open squares, DHA concentration.

Time course of GA production

Under the optimized conditions (approximately 150 g/l of initial glycerol and the addition of 25 g/l of polypepton), *Gluconobacter* sp. NBRC3259 was cultivated using a jar fermentor. The time course of GA production with 150–170 g/l of glycerol, pH uncontrolled, and 500 rpm of agitation speed showed that approximately 24.6 g/l of GA was produced during 4 d of incubation (Fig. 4A). The pH of the medium (initially 6.3) decreased to 3.2. By contrast, Fig. 4B illustrates the typical time course of GA production under pH control (pH 5) and 500 rpm of agitation speed. GA at 54.9 g/l

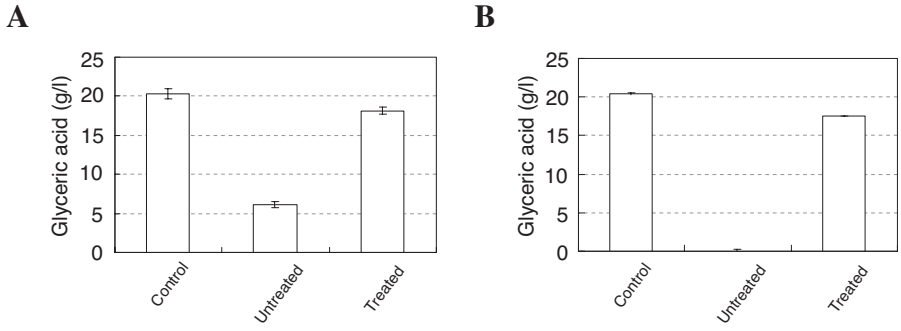

Fig. 5. Effects of Activated Charcoal Pretreatment of Raw Glycerol Samples on GA Production.
A, Raw glycerol sample A neutralized with H_2SO_4. B, Raw glycerol sample B. Error bars represent the standard deviation calculated from three independent experiments.

was obtained after 4 d of incubation, and productivity was enhanced approximately 2-fold with pH control. At the same time, the amount of DHA in the culture was analyzed by HPLC, resulting in 33.7 g/l of DHA production after 4 d of incubation (Fig. 4B). Considering that glycerol consumption was 92.3 g/l, GA and DHA yields of 0.52 and 0.37 mol/mol-glycerol respectively were obtained. Further cultivation of *Gluconobacter* sp. NBRC3259 did not increase GA production (data not shown). In the case of an agitation speed of 700 rpm with pH control (pH 5), the productivity of GA by the strain decreased to 40.2 g/l after 4 d in culture (Fig. 4C). Instead, the amount of DHA increased to 49.6 g/l. These results indicate that agitation speed is also an important factor for efficient GA production.

Effect of raw glycerol pretreatment on GA production

With the use of raw glycerol sample A as a feedstock, the effects of methanol on GA production were investigated. As compared to GA productivity in no methanol culture (24.4 g/l), the addition of 0.4% (v/v) methanol drastically decreased to 1.9 g/l of production, although the optical density (OD_{600}) decreased slightly to 2.1 from 2.9. When 1% (v/v) methanol was added, GA production and OD_{600} were 0.2 g/l and 0.5 respectively. According to the above results, methanol must be removed from raw glycerol samples. Therefore, methanol was removed from raw glycerol sample A to less than 0.1 g/l by evaporation.

Figure 5 illustrates the effect of activated charcoal pretreatment of raw glycerol samples on GA production. In the case of raw glycerol sample A (Fig. 5A), 35% of GA productivity was obtained without activated charcoal treatment as compared to the control samples using pure glycerol, while 90% of GA productivity was obtained with activated charcoal treatment. The OD_{600} of the cultures using intact and pretreated raw glycerol samples were 1.5, and 2.1 respectively. In contrast, in the case of raw glycerol sample B (Fig. 5B), no GA was produced without activated charcoal treatment. With activated charcoal treatment, 86% of GA productivity was obtained as compared to the control. At this time, the OD_{600} of the cultures using intact and pretreated raw glycerol samples were 0 and 2.4, respectively. This result indicates that pretreatment by activated charcoal is very effective for GA production with raw glycerol samples as the feedstock.

Time course of GA production with raw glycerol

Using charcoal-pretreated raw glycerol samples, jar fermentor experiments were performed. Figure 6 illustrates the typical time course of GA production using raw glycerol sample A neutralized with HCl (Fig. 6A) or H_2SO_4 (Fig. 6B). The agitation speed was set to 500 rpm and the pH was kept at pH 5. When neutralized with HCl (final Cl^- concentration, 5.2 g/l), GA was produced, but the amount was only 15.1 g/l after 4 d of incubation. In contrast, when neutralized with H_2SO_4 (final SO_4^{2-} concentration, 4.5 g/l), the maximal amount of GA (45.9 g/l) was reached after 4 d of incubation, and productivity was 84% of the results with pure glycerol. The amount of DHA was 28.2 g/l after 4 d of incubation (Fig. 6B). Since glycerol consumption was 87.1 g/l, GA and DHA yields of 0.46 and 0.33 mol/mol-glycerol respectively were obtained.

Figure 7A represents the typical time course of GA production with raw glycerol sample B, pH control (pH 5), and 500 rpm. The maximum amount of GA (39.6 g/l) was reached after 4 d of incubation, and productivity was 72% of the results with pure glycerol. The amount of DHA was 22.7 g/l after 4 d of incubation (Fig. 7A). As glycerol consumption was 63.3 g/l, GA and DHA yields of 0.54 and 0.36 mol/mol-glycerol respectively were obtained. At 700 rpm (Fig. 7B), GA production decreased to 28.0 g/l, but the amount of DHA increased to 31.1 g/l after 4 d of incubation.

Discussion

We found that *Gluconobacter* sp. NBRC3259 produced 45.9 g/l of GA and 28.2 g/l of DHA from raw glycerol sample A (containing 174 g/l of glycerol in culture) during 4 d of incubation. This is the first report describing the production of GA with raw glycerol as a feedstock. According to this and previous studies,[14–16] the impurities of raw glycerol were mainly composed of methanol, sodium (or/and potassium) salts, heavy metals-lignin, other organic materials, and so on. However, their compositions depended on respective transesterification processes for the BDF and oleochemical industries as well as the differences in the raw materials themselves (various vegetable oils, animal fats, and so on). In the use of both raw glycerol samples, pretreatment with activated charcoal was effective for GA production, but in raw glycerol sample A, 35% GA productivity was observed without activated charcoal

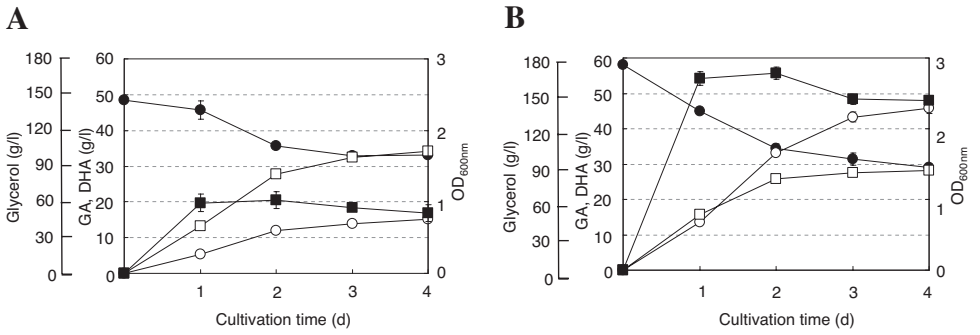

Fig. 6. Time Course of GA Production by *Gluconobacter* sp. NBRC3259 Using Raw Glycerol Sample A Neutralized with HCl (A) and H_2SO_4 (B). Symbols: solid circles, glycerol concentration; open circles, D-GA concentration; solid squares, optical density; open squares, DHA concentration.

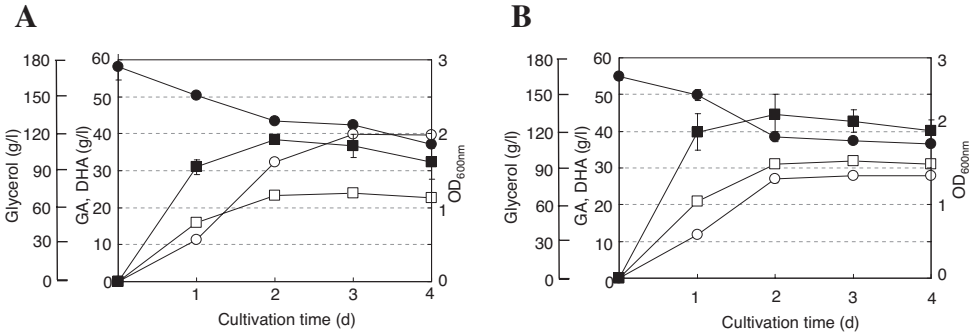

Fig. 7. Time Course of GA Production from Raw Glycerol Sample B by *Gluconobacter* sp. NBRC3259. A, 500 rpm. B, 700 rpm. Symbols: solid circles, glycerol concentration; open circles, GA concentration; solid squares, optical density; open squares, DHA concentration.

pretreatment (Fig. 5). This result suggests that raw glycerol sample B contained higher amounts of unknown impurities, with a negative effect on the growth of the strain. Also, we found that the type of acid for neutralization is important in GA production by the strain. As shown in Figs. 3D and Fig. 6, Cl^- showed a more negative effect on the enzymatic conversion of glycerol to GA than SO_4^{2-}. It is necessary to do prior purification properly, depending on the raw glycerol sample.

As shown in Figs. 4, 6, and 7, most of the glycerol consumed by *Gluconobacter* sp. NBRC3259 was converted to GA and DHA, and both GA and DHA production increased concomitantly with growth of the strain. When the strain's growth reached a stationary state after 2 d, GA and DHA production appeared to have ceased. In this stationary state, the pH of the culture became pH 3.2 (initial pH 6.3). When pH was controlled so as not to fall under pH5 during cultivation (initial pH 6.3), GA productivity was enhanced approximately 2-fold (Fig. 4). Also, an agitation speed of 700 rpm (higher aeration conditions) resulted in lower GA production than 500 rpm (lower aeration conditions), whereas it led to increasing DHA production. In contrast, an agitation speed of 250 rpm resulted in very poor growth of the strain over 4 d (data not shown). These results indicate that both pH control and agitation speed are important factors in efficient GA production. During all the experiments, a possible intermediate, glyceraldehyde (Fig. 1), was not found.

The enantiomeric composition of the GA produced by *Gluconobacter* sp. NBRC3259 was analyzed. The strain was able to produce not only D-GA (88.4%) but also L-enantiomer (11.6%, Fig. 1). In 1987, a Japanese patent application (JP0751069, Daicel Chemical Industries) described that the GA produced by four *Gluconobacter* strains was D-GA. This result indicates the possibility that the enantiomeric composition of GA produced by *Gluconobacter* strains is different in different strains. Until now, concerning the production mechanisms of GA from glycerol by acetic acid bacteria at the molecular level, no reports have been published. Adachi *et al.*[17,18] reported that purified membrane-bound alcohol dehydrogenase and membrane-bound aldehyde dehydrogenase from *G. suboxydans* did not exhibit specific activities toward glycerol and DL-glyceraldehyde respectively. Therefore, to elucidate the production mechanisms of D-GA and of L-GA, it is important to purify and characterize the enzymes involved in GA production.

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