Microbial Production of Glyceric Acid, an Organic Acid That Can Be Mass Produced from Glycerol[†]

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Glyceric acid (GA), an unfamiliar biotechnological product, is currently produced as a small by-product of dihydroxyacetone production from glycerol by *Gluconobacter oxydans*. We developed a method for the efficient biotechnological production of GA as a target compound for new surplus glycerol applications in the biodiesel and oleochemical industries. We investigated the ability of 162 acetic acid bacterial strains to produce GA from glycerol and found that the patterns of productivity and enantiomeric GA compositions obtained from several strains differed significantly. The growth parameters of two different strain types, *Gluconobacter frateurii* NBRC103465 and *Acetobacter tropicalis* NBRC16470, were optimized using a jar fermentor. *G. frateurii* accumulated 136.5 g/liter of GA with a 72% D-GA enantiomeric excess (ee) in the culture broth, whereas *A. tropicalis* produced 101.8 g/liter of D-GA with a 99% ee. The 136.5 g/liter of glycerate in the culture broth was concentrated to 236.5 g/liter by desalting electrodialysis during the 140-min operating time, and then, from 50 ml of the concentrated solution, 9.35 g of GA calcium salt was obtained by crystallization. Gene disruption analysis using *G. oxydans* IFO12528 revealed that the membrane-bound alcohol dehydrogenase (mADH)-encoding gene (*adhA*) is required for GA production, and purified mADH from *G. oxydans* IFO12528 catalyzed the oxidation of glycerol. These results strongly suggest that mADH is involved in GA production by acetic acid bacteria. We propose that GA is potentially mass producible from glycerol feedstock by a biotechnological process.

A shift from petroleum to bio-based feedstocks will be necessary for a sustainable industrial society and effective management of greenhouse gas emissions (2, 20). Biodiesel fuel (BDF) is produced from vegetable oils and animal fats and can replace the diesel in diesel engine motors. Although the European Union currently produces 82% of the BDF produced in the world (7), the use of BDF will probably continue to grow worldwide, because petroleum is a limited resource. Massive amounts of glycerol can be obtained as a by-product of BDF production (approximately 10% by weight) through transesterification with alcoholysis generally catalyzed by NaOH or KOH. As the use of this glycerol is an important component of the economics of the BDF industry, there is a worldwide demand for the efficient use of glycerol (24).

Among the recent developments in the conversion of glycerol into valuable chemicals, epichlorohydrin (ECH) and 1,2-propanediol (propylene glycol) are now commercially synthesized from glycerol by chemical processes, and 1,3-propanediol (1,3-PDO) and dihydroxyacetone (DHA) are produced from glycerol by biotechnological processes (4, 5, 17, 18, 24, 25).

ECH, propylene glycol, and 1,3-PDO are used mainly as intermediates for resins and polymers. However, an increase in the price of glycerol, such as that which occurred due to the collapse of the BDF market (its price increased nearly threefold in Germany by the end of 2007 [24]), can have a large negative effect on the production of such low-price commodity chemicals. Hence, research on the production of more value-added and functional chemicals from (raw) glycerol is important.

Recently, we have focused on the production of a glycerol derivative, glyceric acid (GA), using a bioprocess (Fig. 1) (9, 10). GA from an extract of *Cynara scolymus* leaves has liver stimulant and cholesterolytic activity in dogs (11), and D-GA calcium salt accelerates ethanol and acetaldehyde oxidation in rats (8). GA-based esters also exhibit antitrypsin activity (12), and novel oligoesters based on GA derivatives may be useful for pharmaceutical purposes, such as drug delivery systems (23). These reports suggest that GA is a promising chemical, but it is very expensive as a reagent for investigational use.

Before we began our research, little was known about GA as a biotechnological product, except for one Japanese patent from 25 December 1987 (Daicel Chemical Industries, Japanese patent application 51069) and a report on its by-production during DHA production by *Gluconobacter oxydans* (3, 21). According to the patent, resting cells of *Gluconobacter cerinus* IFO3262 (later *Gluconobacter frateurii* NBRC3262) converted 100 g/liter of glycerol to 57 g/liter of D-GA in a fermentor over a 2-day incubation. Recently, we revealed that *Acetobacter*

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FIG. 1. Proposed pathway for the conversion of glycerol to GA (glyceric acid) by acetic acid bacteria. The bioconversion of glycerol to DHA (dihydroxyacetone) is also represented.

tropicalis NBRC16470 produced 22.7 g/liter of optically pure D-GA from 200 g/liter of glycerol in a fermentor over a 4-day incubation (9). However, because this method of GA production is far from practical, we are attempting to develop a GA manufacturing bioprocess based on strain, fermentation, and process development.

In this study, we searched for a GA producer among 162 acetic acid bacterial strains and investigated the GA productivity and enantiomeric composition of 88 selected strains. We also investigated oxidative fermentation conditions in a 5-liter jar fermentor and applied electrodialysis (ED) to recover glycerate from culture broth. Furthermore, we clarified the gene and enzyme involved in GA production from glycerol for the first time.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. One hundred sixty-two acetic acid bacterial strains belonging to *Gluconobacter* spp. (105 strains), *Acetobacter* spp. (35 strains), and *Gluconacetobacter* spp. (22 strains) were obtained from the National Institute of Technology and Evaluation (NITE) of Japan. The *Gluconobacter* strains consisted of 8 *G. albidus*, 6 *G. cerinus*, 1 *G. condonii*, 67 *G. frateurii*, 11 *G. oxydans*, 10 *G. thailandicus*, and 2 other *Gluconobacter* sp. strains. The *Acetobacter* strains consisted of 4 *A. aceti*, 1 *A. cibinongensis*, 1 *A. estuensis*, 1 *A. indonesiensis*, 2 *A. lovaniensis*, 4 *A. orientalis*, 4 *A. orleanensis*, 11 *A. pasteurianus*, 1 *A. peroxydans*, 1 *A. syzygii*, 1 *A. tropicalis* isolates, and 4 other *Acetobacter* sp. strains. The *Gluconacetobacter* strains consisted of 1 *G. europaeus*, 3 *G. hanseni*, 6 *G. liquefaciens*, 1 *G. oboediens*, 10 *G. xylinus*, and 1 other *Gluconacetobacter* sp. strain. Stock cultures were cultivated using media and temperatures recommended by the NITE. Most strains were successfully cultivated on a glucose medium containing 5 g/liter of polypeptone (Nihon Pharmaceutical), 5 g/liter of yeast extract (Difco), 5 g/liter of glucose, and 1 g/liter of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 6.5). All chemicals were of the highest purity commercially available (98 to 100%; Sigma-Aldrich, Kanto Chemical, Wako Pure Chemical, and Nacalai Tesque).

Screen for GA-producing strains. The 162 strains were precultivated for 2 days in 1 ml of the glucose medium described above in 96-well deep-well plates (Greiner Bio-One GmbH) using a deep-well maximizer shaker (1,500 rpm; Bio Shaker M-BR-022UP; Taitec), and seed cultures (50 μl) were transferred to respective experimental media (1 ml). After a 4-day cultivation and removal of the cells by centrifugation, the supernatant was filtered with a 0.45- μm cellulose filter, and the final pHs of the culture broths were measured. A 20- μl sample of the supernatant was analyzed by high-performance liquid chromatography (HPLC) to quantify GA or DHA.

(i) **Effect of the initial glycerol concentration on GA production.** After the strains were precultivated for 2 days, seed cultures were transferred to a medium (pH 6.5) consisting of 50, 100, 150, or 200 g/liter of glycerol, 0.9 g/liter of KH_2PO_4 , 0.1 g/liter of K_2HPO_4 , 5 g/liter of polypeptone, 5 g/liter of yeast extract, and 1 g/liter of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and cultured for 4 days at 30°C. DHA production was also investigated with 100 g/liter of glycerol (partly 150 g/liter of glycerol).

(ii) **Effect of the initial medium pH on GA production.** After the strains were precultivated for 2 days, seed cultures were transferred to a medium consisting of 100 g/liter of glycerol, 0.9 g/liter of KH_2PO_4 , 0.1 g/liter of K_2HPO_4 , 5 g/liter of polypeptone, 5 g/liter of yeast extract, and 1 g/liter of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 9 and cultured for 4 days at 30°C.

(iii) **Effect of added DL-glycerate on strain growth.** Strains were cultured for 4 days at 30°C in a medium consisting of 100 g/liter of glycerol, 30 g/liter of sodium DL-glycerate, 0.9 g/liter of KH_2PO_4 , 0.1 g/liter of K_2HPO_4 , 5 g/liter of polypeptone, 5 g/liter of yeast extract, and 1 g/liter of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at pH 7.

Enantiomeric composition of GA produced by selected strains. The acetic acid bacteria were centrifuged, and the glycerate in each supernatant was recovered and concentrated with a desalting ED unit (Micro Acilyzer S1; Astom) equipped with a commercial membrane cartridge for anion recovery (AC-122-10; effective membrane area, 10 cm^2 ; Astom) according to the methods described in our previous report (10). The concentrated glycerate samples were diluted to approximately 1 g/liter, with a mobile phase of 0.45 mM CuSO_4 , and the respective enantiomeric compositions were analyzed by HPLC on a system consisting of an LC-20AD HPLC pump (1.0-ml/min flow rate), an SPD-20AV UV/VIS detector (254 nm detection; Shimadzu), and two tandemly linked Chiralpak MA(+) columns (Daicel Chemical Industries). A mobile phase of 0.45 mM CuSO_4 was used as the eluent. During the analysis, the column temperature was maintained at 21°C. D-GA and L-GA calcium salt dihydrates (Sigma-Aldrich) were used as standard samples.

Optimization of nitrogen sources for GA production using *Gluconobacter frateurii* NBRC103465. *G. frateurii* NBRC103465 was precultivated in 5 ml of glucose medium at 30°C for 2 days, and the seed cultures (1.5 ml) were transferred to 300-ml Erlenmeyer flasks containing 30 ml of media consisting of 150 or 200 g/liter of glycerol, 0.9 g/liter of KH_2PO_4 , 0.1 g/liter of K_2HPO_4 , 1 g/liter of yeast extract, 1 g/liter of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 9 g/liter of nitrogen compounds [polypeptone (Nihon Pharmaceutical), peptone (Difco), yeast extract, $(\text{NH}_4)_2\text{SO}_4$, and NaNO_3], each at pH 6.5. The flasks were incubated for 4 days at 30°C on a rotary shaker (200 rpm). After the cells were removed by centrifugation, the respective supernatants were analyzed by HPLC.

Jar fermentor experiments. GA production by *G. frateurii* NBRC103465 and *A. tropicalis* NBRC16470 was evaluated in a 5-liter jar fermentor (model MDL; B. E. Marubishi). Jar fermentor experiments were performed as follows. Both strains were precultivated for 1 day in test tubes containing 5 ml of each glucose medium (30°C, 200 rpm). The *G. frateurii* seed culture (1.5 ml) was transferred to three 300-ml Erlenmeyer flasks containing 30 ml of glucose medium (pH 6.5; total 90 ml) and incubated for 2 days at 30°C on a rotary shaker (200 rpm). In contrast, an *A. tropicalis* seed culture (0.75 ml) was transferred to six 300-ml Erlenmeyer flasks containing 15 ml of glucose medium (pH 6.5; total 90 ml), and incubated for 1 day at 30°C on a rotary shaker (200 rpm). The seed cultures were transferred to a 5-liter jar fermentor containing 2.5 liter of media (pH 6.5) consisting of 150 to 250 g/liter of glycerol, 9 g/liter of polypeptone, 1 g/liter of yeast extract, 0.9 g/liter of KH_2PO_4 , 0.1 g/liter of K_2HPO_4 , and 1 g/liter of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for *G. frateurii* and 150 to 250 g/liter of glycerol, 20 g/liter of yeast extract, 0.9 g/liter of KH_2PO_4 , 0.1 g/liter of K_2HPO_4 , and 1 g/liter of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ for *A. tropicalis*. The aeration rates were set to 0.5, 1.0, 1.5, 2.5, or 5.0 volumes of air per volumes of medium per minute (vvm), and the agitation speed was set to 250 or 500 rpm. The temperature was maintained at $30 \pm 1^\circ\text{C}$. If necessary, the pH was controlled with 5 M NaOH containing 50% (vol/vol) glycerol or 10 M NaOH to ensure at least a pH 5 or 6. Dissolved oxygen (DO) and pH were monitored using DO electrode OX-2500 and pH combination electrode DZP-220 (B. E. Marubishi), respectively.

Glycerate concentration and glycerol removal by ED. An ED unit (Acilyzer EX3B; Astom) equipped with a commercial membrane cartridge for anion recovery (AC-120-550; Astom) was used. The effective membrane area was 550 cm^2 . The electrode solution (500 ml of 0.5 M Na_2SO_4), the feed glycerate solution (1 liter of culture broth after removal of the cells by centrifugation), and the permeate solution (200 ml of pure water) were circulated through the corresponding compartments of the membrane stack at a flow rate of 1.4 liter/min. Desalting ED was automatically performed using the following steps: initially, the equipment was maintained at a constant voltage of 12 V; it was switched to a constant voltage of 10 V when the conductivity fell below 20 mS/cm; and it was switched to a constant voltage of 7 V when the conductivity fell below 8 mS/cm. The experiments were automatically terminated when the conductivity of the solution dropped to 2 mS/cm (which corresponds to approximately 3 g/liter of glycerate, when using a pure glycerate solution).

Crystallization of the GA calcium salt. After determination of the GA concentration, an amount of CaCl_2 corresponding to 0.5 mol of GA was added to the GA solution, and then 0.8 volumes of ethanol was added to the CaCl_2 -containing GA solution to precipitate GA calcium salt. The GA calcium salt in ethanol was filtered and dried, and the crystallization procedure was repeated at least twice. The GA calcium salt impurities were analyzed by HPLC with a Shodex SH1011 column (Showa Denko) and an RID-10A detector (Shimadzu) for organic compounds and with a Shim-pack IC-SA2 column and a CDD-10Asp conductivity detector (Shimadzu). Mobile phases of 5 mM H_2SO_4 or 1.8 mM Na_2CO_3 plus 1.7 mM NaHCO_3 were chosen for the respective columns. During the analysis, the column temperatures were maintained at 60°C and 25°C.

FIG. 2. An example showing the relationship between GA productivity (g/liter) and the final pH in the culture broth.

Disruption of membrane-bound alcohol dehydrogenase-encoding gene (*adhA*) in *Gluconobacter oxydans* and production of GA by the mutants. The *G. oxydans* strain carrying $\Delta adhA$ was constructed by the insertion of a kanamycin (Km)-resistant cassette into the *adhA* gene as follows. A 3.6-kb fragment partially containing the *adhAB* genes was amplified from *G. oxydans* IFO12528 genomic DNA by PCR using the primers adhD-5'-BgII (5'-GAAGATCTTACAGCCC GCTCGACCAG-3') and adhD-3'-SalI (5'-ACGCGTCGACACAGGGGTGG GGACGCTT-3') and then ligated to the pGEM T-easy vector (Promega). A 1.2-kb BamHI fragment containing the Km-resistant gene of pUC4K was inserted into the corresponding site in *adhA* to yield pGEM- $\Delta adhA$. The circular pGEM- $\Delta adhA$ was directly introduced into *G. oxydans* IFO12528 cells by electroporation to perform the homologous recombination. Inactivation of *adhA* in Km-resistant clones was confirmed by PCR with genomic DNA, immunoblotting, and membrane-bound alcohol dehydrogenase (mADH) activity as described elsewhere (16) (data not shown).

G. oxydans IFO12528 (wild type) and its $\Delta adhA$ mutant were precultivated in 5 ml of YPG medium (22) at 30°C for 2 days, and the seed cultures (1.5 ml) were transferred to 300-ml Erlenmeyer flasks containing 30 ml of media consisting of 150 g/liter of glycerol, 0.9 g/liter of KH_2PO_4 , 0.1 g/liter of K_2HPO_4 , 1 g/liter of yeast extract, 9 g/liter of polypeptone (Nihon Pharmaceutical), and 1 g/liter of $MgSO_4 \cdot 7H_2O$, each at pH 6.5. The flasks were incubated for 4 days at 30°C on a rotary shaker (200 rpm). After the cells were removed by centrifugation, the respective supernatants were analyzed by HPLC.

Enzyme assay. The dehydrogenase activity of the purified mADH from *G. oxydans* IFO12528 (1) with glycerol as a substrate was measured spectrophotometrically at 25°C by reducing 2,6-dichlorophenol indophenol (DCIP) coupled with phenazine methosulfate (PMS) (13) and by reducing potassium ferricyanide, as described previously (16). The activity measurement system (1 ml) contained McIlvaine buffer (pH 5.0), 0.1 mM DCIP, 0.2 mM PMS, 1.2 mM sodium azide, 6 to 20% (wt/vol) glycerol or 100 μ M of ethanol, and 2.22 μ g of purified enzyme for PMS-DCIP; for ferricyanide reductase activity, the system contained McIlvaine buffer (pH 5.0), 1 mM potassium ferricyanide, 6 to 20% (wt/vol) glycerol or 100 μ M of ethanol, and 2.22 μ g of purified enzyme. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μ M of substrate per minute, which was calculated using the millimolar extinction coefficient of DCIP of 3.43 mM^{-1} at pH 5, 14.7 mM^{-1} at pH 7, and 15.6 mM^{-1} at pH 9 (14) or calculated using the millimolar extinction coefficient of potassium ferricyanide of 1 mM^{-1} (16).

Quantification of glycerol, glyceraldehyde, DHA, and GA. The concentrations of glycerol, glyceraldehyde, DHA, and GA in culture broth and enzyme reaction mixtures were analyzed by HPLC with an LC-20AD HPLC pump (1.0-ml/min flow rate) and an RID-10A detector (Shimadzu) equipped with a Shodex SC1011 column (Showa Denko) for glycerol and DHA and a Shodex SH1011 column

(Showa Denko) for glyceraldehyde and GA. Mobile phases of pure water or 5 mM H_2SO_4 were chosen for the respective columns. During the analysis, the column temperatures were maintained at 80 and 60°C.

RESULTS

Investigation of GA production by acetic acid bacteria. In total, 105 *Gluconobacter* strains, 35 *Acetobacter* strains, and 22 *Gluconacetobacter* strains were examined for their ability to produce GA from glycerol, including previously reported strains, such as *G. frateurii* NBRC3262 (Daicel Chemical Industries, 1987, Japanese patent application 51069) and *A. tropicalis* NBRC16470 (9). Because the initial glycerol concentration is an important factor in GA production (9), we used media (pH 6.5) containing 50, 100, 150, or 200 g/liter of glycerol for all of the tested strains. Preliminary examinations using 24 strains revealed that a larger amount of GA was produced if there was a decrease in the final culture broth pH (Fig. 2). Hence, for the first screening, we measured the final pH of the culture broths, and the amounts of GA in the low-pH cultures were quantified by HPLC (see below). In addition, we investigated the effect of an initially alkaline pH of the medium (pH 9) on GA production, because raw glycerol samples often show alkaline pH during the transesterification of oils and fats with NaOH or KOH (25). The glycerate tolerance of the strains was also investigated.

(i) *Gluconobacter* strains. The lowest pH values among the 105 culture broths were 3.0, 2.8, 2.7, and 2.7 when 50, 100, 150, and 200 g/liter of glycerol were used, respectively. As for cultures exhibiting a pH below 3 (72 strains), we quantified the GA concentration, and HPLC analysis revealed that the cultures contained up to 35 g/liter of GA (Table 1; see also Table S1 in the supplemental material). The reproducibility of 14 *Gluconobacter* strains showing a productivity of more than 29 g/liter of GA was confirmed. *G. frateurii* strains NBRC103465 and NBRC103502 showed 35.1 and 34.8 g/liter of GA production from 200 and 150 g/liter of glycerol, respectively. *G. frateurii* NBRC103465 produced reproducibly larger amounts of GA with 200 g/liter glycerol than with 150 g/liter, and the strain could grow in medium at pH 9 or in medium containing 30 g/liter of sodium DL-GA (see Table S1 in the supplemental material), indicating that *G. frateurii* NBRC103465 can produce large amounts of GA from higher concentrations of glycerol. All of the tested *Gluconobacter* strains produced about 30 to 65 g/liter of DHA.

(ii) *Acetobacter* and *Gluconacetobacter* strains. The lowest pH values in the 57 culture broths were 3.3, 3.1, 3.2, and 3.5 when 50, 100, 150, and 200 g/liter of glycerol, respectively, were used. As for cultures exhibiting a pH below 4 (12 strains), we quantified the GA concentration, and HPLC analysis revealed that the cultures contained up to 17 g/liter of GA (Table 1; see also

TABLE 1. Summary of screening for GA-producing acetic acid bacteria

Species (total no. of strains analyzed)	No. of strains with D-GA productivity (g/liter) of:				No. of strains with D-GA ee (%) of:				
	<10	10–20	20–30	30–40	<70	70–80	80–90	90–99	>99
<i>Gluconobacter</i> spp. (72)	2	9	52	9	1	62	8	1	0
<i>Acetobacter</i> spp. (12)	8	4	0	0	2	4	3	2	1
<i>Gluconacetobacter</i> spp. (4)	3	1	0	0	0	2	1	1	0

Table S1 in the supplemental material). Among the tested strains, *A. syzygii* NBRC16604 was the best GA producer (16.6 g/liter from 150 g/liter of glycerol), and the second best was *A. orleanensis* NBRC13752 (16.3 g/liter GA from 100 g/liter of glycerol). However, their productivity was much lower than that of the *Gluconobacter* strains. *Acetobacter* and *Gluconacetobacter* strains yielded less than 22 g/liter of DHA, except for the *G. hansenii* NBRC14817 strain (48.5 g/liter).

Enantiomeric composition of GA produced by acetic acid bacteria. The enantiomeric compositions of the GA produced by the 88 strains are summarized in Table 1.

(i) ***Gluconobacter* strains.** All of the tested *Gluconobacter* spp. produced not only D-GA but also a small amount of L-GA. Most *Gluconobacter* strains (62 strains) produced D-GA with 71 to 79% enantiomeric excess (ee), and 8 strains yielded D-GA with 80 to 89% ee. The D-GA produced by *G. frateurii* NBRC103471 showed the highest ee, at 90%. However, none of the strains produced D-GA with more than a 91% ee (see Table S1 in the supplemental material).

(ii) ***Acetobacter* and *Gluconacetobacter* strains.** The enantiomeric compositions of the GA produced by the *Acetobacter* and *Gluconacetobacter* strains varied, depending on the strain, although the number of *Acetobacter* strains that were investigated was fewer than that of *Gluconobacter* strains. *G. liquefaciens* NBRC12257, *A. orleanensis* NBRC13752, and *A. syzygii* NBRC16604 produced D-GA with 93, 91, and 91% ee, respectively (see Table S1 in the supplemental material), and only *A. tropicalis* NBRC16470 produced D-GA with more than 99% ee, which is consistent with our previous result (9).

Optimization of culture conditions for GA production by *G. frateurii* NBRC103465. Among the 88 tested strains, we selected one strain, *G. frateurii* NBRC103465, which showed the highest GA productivity regardless of its optical purity (D-GA, 72% ee; see also Table S1 in the supplemental material). We have not yet investigated the *G. frateurii* culture conditions for GA production. Therefore, the effects of the types and amounts of nitrogen sources on GA production were investigated. The addition of 10 g/liter polypeptone resulted in the highest productivity among the tested nitrogen sources and was thus used for further 5-liter jar fermentor experiments.

Fig. S1A in the supplemental material shows the time course of GA production with 220 g/liter of initial glycerol, pH controlled at 5 with 10 M NaOH, a 1-vvm aeration rate, and a 500-rpm agitation speed. Under this condition, 53.4 g/liter of GA was produced over a 6-day incubation. When the pH was changed from 5 to 6 and controlled, the GA productivity was enhanced to 63.5 g/liter during the same period (see Fig. S1B in the supplemental material). Furthermore, when the aeration rate was shifted from 1 to 0.5 vvm at a pH controlled at 6, the GA productivity drastically increased, to 92.3 g/liter (see Fig. S1C in the supplemental material). Decreasing the agitation speed from 500 to 250 rpm resulted in poor growth (data not shown). Through all of the experiments, the enantiomeric composition of GA was 71 to 75% ee of D-GA (data not shown), and there was 25 to 35 g/liter of DHA by-production.

Fig. S1D in the supplemental material shows the time course of GA production by the same strain with 90 g/liter of initial glycerol, control of the pH to 6 with 10 M NaOH, a 1-vvm aeration rate, and a 500-rpm agitation speed. Compared to the results represented in Fig. S1B in the supplemental material,

FIG. 3. Time course of GA production by *Gluconobacter frateurii* NBRC103465 with 170 g/liter of initial glycerol, pH controlled to 6, a 0.5-vvm aeration rate, and a 500-rpm agitation speed. The pH was controlled with 5 M NaOH containing 50% (vol/vol) glycerol. Black circles, glycerol concentration; white circles, GA concentration; black squares, dry cell weight; white squares, DHA concentration.

the decrease in initial glycerol concentration resulted in low GA production but better growth. According to results depicted in Fig. S1A to C, GA was produced mostly during the log phase of growth. From these findings, we developed a method to feed glycerol to the culture during the log phase, instead of using an initially high glycerol concentration. In this method, 5 M NaOH containing 50% (vol/vol) glycerol, but not 10 M NaOH, was used as a pH control reagent. Figure 3 illustrates the typical time course of GA production with 170 g/liter of initial glycerol, control of the pH to 6 with 5 M NaOH containing 50% (vol/vol) glycerol, a 0.5-vvm aeration rate, and a 500-rpm agitation speed. This method produced 136.5 g/liter of GA after a 7-day incubation (130.7 g/liter after 6 days). During the experiments, approximately 180 g of glycerol was fed into the culture broth with the pH control reagent. These results demonstrate that GA is an organic acid that is highly producible from glycerol feedstock, accumulating to more than 100 g/liter.

Optimizing D-GA production culture conditions for *A. tropicalis* NBRC16470. We selected another strain, *A. tropicalis* NBRC16470, which showed the highest optical D-GA purity (>99%; Table 1), regardless of its GA productivity. Previously, we investigated some of the *A. tropicalis* NBRC16470 culture conditions for GA production with flasks and performed 1-liter jar fermentor experiments without pH control (9). However, further optimization of culture conditions with a 5-liter jar fermentor was necessary to compare the GA productivity of this strain with that of *G. frateurii* NBRC103465. Therefore, we optimized the *A. tropicalis* culture conditions for GA production and chose conditions of 2.5-vvm aeration and control of the pH to 6.

Figure 4A shows the time course of GA production with 220 g/liter of initial glycerol, control of pH to 6 with 10 M NaOH, a 2.5-vvm aeration rate, and a 500-rpm agitation speed. Under these conditions, 81.6 g/liter of D-GA was produced over a 6-day incubation. When using the method of feeding 5 M NaOH containing 50% (vol/vol) glycerol as a pH control reagent, 101.8 g/liter of D-GA was produced after a 6-day incubation (Fig. 4B). During the experiments, approximately 220 g of glycerol was fed to the culture broth with the pH control

FIG. 4. Time course of GA production by *Acetobacter tropicalis* NBRC16470 with a 5-liter jar fermentor. Conditions were as follows: 220 g/liter of initial glycerol, pH controlled to 6 with 10 M NaOH, 2.5-vvm aeration rate, and 500-rpm agitation speed (A); 220 g/liter of initial glycerol, pH controlled to 6 with 5 M NaOH containing 50% (vol/vol) glycerol, 2.5-vvm aeration rate, and 500-rpm agitation speed (B). Black circles, glycerol concentration; white circles, GA concentration; black squares, dry cell weight; white squares, DHA concentration; d, days.

reagent. This experiment demonstrates that more than 100 g/liter D-GA can be produced from glycerol using *A. tropicalis*.

Glycerate recovery from *G. frateurii* NBRC103465 and *A. tropicalis* NBRC16470 culture broths by ED. Figure 5A shows an example of desalting ED of the *G. frateurii* NBRC103465 culture broth (pH 6), which contained approximately 137 g/liter of glycerate. During the 140-min operating time, the initial glycerate concentration in the feed solution (137 g/liter) was concentrated to 236.5 g/liter in the permeate solution, and the sum of glycerol and DHA transferred to the permeate solution was 16.9 g/liter (Fig. 5A). In addition, 92 g/liter of initial glycerate in the feed solution derived from the *A. tropicalis* NBRC16470 culture broth (pH 6) was concentrated to 181.7 g/liter during 120 min of operating time; the sum of glycerol and DHA in the final permeate solution was 27.8 g/liter (Fig. 5B).

During ED, the yellowish color of the culture broth derived from medium constituents, such as polypeptone and yeast extract, almost disappeared (data not shown), indicating that such impurities other than glycerol and DHA were excluded. This finding was suitable for the crystallization process.

Crystallization of the GA calcium salt and analysis of impurities. Using the most concentrated GA solution described above (approximately 236 g/liter GA, 520 ml) from the *G. frateurii* culture broth, we investigated the recovery rate of GA calcium salt by crystallization and the purity of the resulting salt. From 50 ml of the solution, 9.35 g of GA calcium salt was obtained during 12 h of crystallization. Although the recovery rate was rather low (58.8%), it should improve if the crystallization procedure is repeated or further optimized. An un-

known organic impurity and phosphate ions in the aqueous solution of the GA calcium salt obtained by HPLC had retention times of 5.4 min using a Shodex SH1011 column and 12 min with a Shim-pack IC-SA2 column, respectively (data not shown). Hence, the GA calcium salt was dissolved in an appropriate volume of water, recrystallized with ethanol, and dried. The purity of the resulting GA calcium salt as analyzed by HPLC was the same as that of the authentic DL-GA calcium salt dihydrate (Wako Pure Chemicals).

The ability of the *G. oxydans* $\Delta adhA$ mutant to produce GA. As a preliminary step toward understanding the mechanism of GA production by *G. frateurii* NBRC103465, we investigated the enzyme activity of its membrane and soluble fractions with glycerol or glyceraldehyde. The results showed that dehydrogenase activities with both substrates were detected with the *G. frateurii* membrane fractions, as well as with the soluble fractions (data not shown). Then, in order to investigate the involvement of mADH in the GA production of *Gluconobacter* spp., we constructed the *adhA* disruptant of *G. oxydans* IFO12528, whose complete genome sequence has already been published (19), and examined the ability of the mutant to produce GA. As shown in Fig. S2 in the supplemental material, the wild-type strain produced 17.1 g/liter of GA, whereas the *adhA* disruptant produced only 0.64 g/liter of GA after a 4-day incubation, although the growth profiles of both strains were similar.

Dehydrogenase activity of purified mADH toward glycerol. Table 2 summarizes the results of enzymatic activities of purified mADH from *G. oxydans* IFO12528 with potassium ferri-cyanide. Glycerol was revealed to be oxidized by mADH. The

FIG. 5. Time course of desalting ED of culture broth from *Gluconobacter frateurii* NBRC103465 (A) and *Acetobacter tropicalis* NBRC16470 (B). White and black circles, glycerate concentrations (g/liter) in feed and permeate solutions, respectively; white and black triangles, the sum of the glycerol and DHA concentrations (g/liter) in the feed and permeate solutions, respectively.

TABLE 2. Dehydrogenase activity of purified mADH toward various concentrations of glycerol

Glycerol concn (% [wt])	Sp act (units/mg protein) ^a	Relative rate (%) ^b
6	10.8	5.26
10	18.0	8.77
16.7	29.7	14.5
20	35.1	17.1

^a The results of enzyme activity with potassium ferricyanide are shown.^b The enzymatic activity with ethanol is represented as 100%.

effect of glycerol concentration on mADH activity was examined, and higher dehydrogenase activity was observed within the range of 6 to 20% (wt/vol) glycerol. The results of enzymatic activities with PMS-DCIP also showed a similar tendency (data not shown). These results correspond to the fact that a higher initial glycerol concentration (e.g., 15 to 20% [wt/vol]) is an important factor in GA production. After the enzyme activity was measured, the reaction mixtures were filtered through a 0.45- μ m cellulose filter, and the samples were analyzed by HPLC. When 20% (wt/vol) glycerol was used as the substrate, 1 g/liter of glyceraldehyde was detected (retention time of 8.8 min). Together with the *adhA* disruptant observations, the obtained results strongly indicate that mADH is involved in GA production by *G. oxydans* IFO12528.

DISCUSSION

Until now, there have been few studies of microbial GA production, except from our group, and the maximal GA produced was 57 g/liter per batch (Daicel Chemical Industries, 1987, Japanese patent application 51069). In this study, we demonstrated that a strain of *G. frateurii* can produce 136.5 g/liter GA and that ED can efficiently concentrate glycerate to 236.4 g/liter from culture broth, leading to a straightforward crystallization of the GA calcium salt.

Acetic acid bacteria (*Acetobacteriaceae*) oxidize various sugar alcohols other than ethanol, including D-glucose, glycerol, and D-sorbitol; these oxidation reactions are termed oxidative fermentation (15), and the only known GA producers investigated so far belong to the acetic acid bacteria family. Therefore, we screened 162 strains of acetic acid bacteria for GA producers and investigated the GA productivity and enantiomeric composition of about 88 strains (see Table S1 in the supplemental material). Using a deep-well maximizer shaker without pH control, 52 and 9 strains among a total of 72 strains of *Gluconobacter* spp. produced 20 to 29.9 g/liter and 30 to 39.9 g/liter of GA, respectively, whereas among a total of 16 strains of *Acetobacter* spp. and *Gluconacetobacter* spp., none produced more than 20 g/liter of GA (Table 1). Because most of the *G. frateurii* strains produced more GA with 150 g/liter of initial glycerol than with 100 g/liter (see Table S1 in the supplemental material), we consider *G. frateurii* to be a suitable GA producer with a high glycerol concentration. However, three *Acetobacter* strains produced D-GA with an ee of more than 90%, whereas among *Gluconobacter* spp., only one strain produced D-GA with an ee of more than 90% (*G. frateurii* NBRC103471, 90% ee; Table 1). *Acetobacter* spp. appear to be suitable for pro-

TABLE 3. Comparison of features related to GA production in *Gluconobacter frateurii* NBRC103465 and *Acetobacter tropicalis* NBRC16470

Parameter	<i>G. frateurii</i> NBRC103465	<i>A. tropicalis</i> NBRC16470
GA production (6 days) (g/liter)	130.7	101.8
GA production (3 days) (g/liter)	105.9	45.0
Aeration rate in 5-liter jar (vvm)	0.5	2.5
D-GA ee (%)	73	99
Strain characteristics	Stable	Changeable phenotype

ducing a high D-GA enantiomeric composition, although the GA enantiomeric composition depends on the strain.

From these strains, we selected two different types of GA-producing strains, *G. frateurii* NBRC103465 and *A. tropicalis* NBRC16470. Investigation of the culture conditions for these strains with a jar fermentor revealed that the initial glycerol concentration, aeration rate, and pH control are important factors (see Fig. S1 in the supplemental material). We also developed a method to feed glycerol into the culture using a glycerol-containing alkaline solution as a pH control reagent. This method seemed to be effective for GA production, because *G. frateurii* NBRC103465 grown with this method produced 136.5 g/liter of GA (Fig. 3), whereas the same culture conditions, but with control of the pH to 6 using 10 M NaOH, resulted in just 51 g/liter of GA in the same period (data not shown). However, considering that *G. frateurii* showed only 33 g/liter of GA production with the same method but with 110 g/liter of initial glycerol (data not shown), an initial glycerol concentration of at least 15% (wt/vol) is necessary for efficient GA production. As shown in Fig. 3, the initial glycerol concentrations in the fermentor and glycerol fed from 5 M NaOH solution containing 50% (vol/vol) glycerol were 170 and 60 g/liter, respectively, and the remaining glycerol in the culture after a 7-day incubation was 56.3 g/liter. Considering that 136.5 g/liter of GA and 20.1 g/liter of DHA were produced within that period, GA and DHA yields of 0.68 and 0.12 mol/mol glycerol were obtained.

GA production by *A. tropicalis* NBRC16470 also requires a high initial glycerol concentration (approximately 200 g/liter) (9), but the aeration rate profile of this strain is completely different from that of *G. frateurii* NBRC103465. *A. tropicalis* NBRC16470 culture conditions consisting of 220 g/liter of initial glycerol, pH control (pH 6) with 10 M NaOH, a 2.5-vvm aeration rate, and a 500-rpm agitation speed produced 81.6 g/liter of GA (Fig. 4A); however, the same culture conditions, but with aeration rates of 0.5 and 5.0 vvm, resulted in just 1.5 and 10.8 g/liter of GA, respectively, within the same time period (data not shown). In the latter case, 95.4 g/liter of DHA was produced from 221.3 g/liter of initial glycerol during a 5-day incubation, which has not previously been shown for this strain (data not shown).

A comparison of the GA productivity levels of *G. frateurii* NBRC103465 and *A. tropicalis* NBRC16470 is shown in Table 3. The maximal amounts of GA production by *G. frateurii* and *A. tropicalis* were 130.7 and 101.8 g/liter, respectively, with 6-day incubations. Because the length of the oxidative fermentation is an important factor in the total process cost, we

compared the GA productivity during a 3-day incubation. As shown in Table 3, *G. frateurii* had an advantage during this period, and using a lower aeration rate was also favorable. In addition, *G. frateurii* is more stable during oxidative fermentation and is easier to handle than *A. tropicalis*, because *A. tropicalis* sometimes turns the culture brown during fermentation or after transfer, and in such cases does not produce GA. *Acetobacter* and *Gluconacetobacter* spp. are well known for frequent spontaneous mutations and the formation of two or more different colony types after serial transfer, which has also been observed with *A. tropicalis* SKU1100 (6). Considering the above findings, *G. frateurii* is more practical for GA production than *A. tropicalis*, but one problem is its rather low D-GA ee value (Table 3).

In order to know the mechanism of D-GA production, the identification of genes or enzymes involved in GA production is necessary. We examined the involvement of mADH in GA production using the *G. oxydans* $\Delta adhA$ mutant and found that the mutant almost lost GA productivity. In addition, we also clarified that the purified mADH can oxidize glycerol. To the best of our knowledge, this is the first report demonstrating that mADH catalyzes glycerol oxidation. Adachi et al. reported that purified mADH from *G. oxydans* IFO12528 (formerly *Gluconobacter suboxydans*) did not exhibit specific activities toward glycerol under the conditions used (1). Hence, for 30 years mADH has been thought to not catalyze the oxidation of glycerol. However, in the previous study, a relatively low concentration of substrate (100 μ mol of ethanol or glycerol in 1 ml of reaction mixture, approximately 1% [wt/vol]) was used to investigate the substrate specificity of mADH; in the present study we found that an increase in glycerol concentration (6 to 20% [wt/vol]) resulted in higher dehydrogenase activity. Therefore, we consider that the difference in substrate specificities toward glycerol was caused by substrate concentrations.

In conclusion, we demonstrated that more than 100 g/liter of D-GA can be produced by both *G. frateurii* and *A. tropicalis*, and the product can be easily concentrated by desalting ED and removing culture broth impurities. Considering that GA was previously known as a minor by-product of DHA production by *G. oxydans*, these results may provide a new application for surplus glycerol. It will be necessary to demonstrate the functionality of D-GA or its derivatives and increase its industrial output to add more value to D-GA.

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