Preliminary Evaluation of Glyceric Acid-producing Ability of *Acidomonas methanolica* NBRC104435 from Glycerol Containing Methanol

Shun Sato*, Dai Kitamoto and Hiroshi Habe†

Research Institute for Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN

† Present address: Environmental Management Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, JAPAN

Abstract: Some acetic acid bacteria produce large amounts of glyceric acid (GA) from glycerol in culture broth. However, methanol, which is a major contaminant of raw glycerol derived from the biodiesel fuel industry, sharply decreases cell growth and GA production [AMB Express, 3, 20, 2013]. Thus, we evaluated the methylotrophic acetic acid bacterium *Acidomonas methanolica* NBRC104435 for its ability to produce GA from glycerol containing methanol. This strain accumulated GA in its culture broth when 1–3 wt% glycerol was available as a carbon source. We observed improved cell growth and GA accumulation when 1 vol% methanol was added to the 3–5 wt% glycerol medium. The maximum concentration of GA was 12.8 g/L in medium containing 3 wt% glycerol plus 1 vol% methanol. In addition, the enantiomeric excess (ee) of the GA produced was revealed to be 44%, indicating that this strain converted glycerol to d-GA with a lower enantioselectivity than other acetic acid bacteria, which had 70–99% ee.

Key words: glyceric acid, methanol, *Acidomonas methanolica*, acetic acid bacteria, methylotroph

1 INTRODUCTION

Biomass resources are widely used to produce renewable energy fuels such as bioethanol and biodiesel fuel (BDF) to support the rapid expansion of renewable energy usage all over the world. Typical BDF is composed of fatty acid methyl esters, which are made from triacylglycerols (plant and animal oils) and methanol. The conventional method of BDF production uses an ester exchange reaction between triacylglycerol and methanol in the presence of alkali catalysts, and glycerol is produced at 10% of the initial triacylglycerol concentration. In general, excess methanol is used to increase the yield of fatty acid methyl esters, resulting in contamination of the glycerol byproduct with methanol. This byproduct is called raw glycerol, and can be refined into pure glycerol; however, raw glycerol is incinerated as industrial wastes in many cases. In terms of both sustainability and economics, raw glycerol is better employed as a feedstock for the production of valuable chemicals without refining. One promising process that uses raw glycerol as a feedstock for chemical production is microbial conversion. Many chemicals, such as ethanol, acetic acid, 2-propanol, and 1,3-propanediol have already been investigated for production by bacterial fermentation using raw glycerol as a carbon source. Our research group has focused on developing a biotechnological method to produce glyceric acid (GA; 2,3-dihydroxypropanoic acid) from raw glycerol using acetic acid bacteria. Results depended on the strain selected; *Gluconobacter frateurii* NBRC103465 accumulated 136 g/L of GA in its fermentation broth after 6 d of cultivation with pure glycerol, and *Acetobacter tropicalis* NBRC16470 accumulated optically pure d-GA at 101 g/L under the same conditions. However, GA production by *Gluconobacter* strains was severely affected by methanol in the culture medium. When the medium was supplemented with 1 vol% methanol, GA productivity of *Gluconobacter* strains decreased significantly, to half or less of the rate observed without methanol addition. To over-
come this problem, we developed a mutant strain of *G. frateurii* with the ability to produce GA in the presence of methanol. This mutant strain could produce 6.3 g/L of GA from 17 wt% glycerol in the presence of 5 vol% methanol, conditions under which the wild-type strain neither grew nor produced GA\(^9\). However, because GA production was insufficient and a high concentration of glycerol had to be maintained in the culture, another strategy needs to be developed for industrial use of raw glycerol containing methanol.

The genus *Acidomonas* was identified as acetic acid bacteria capable of growing on methanol as the sole carbon source. One species, *Acidomonas methanolica*, formerly known as *Acetobacter methanolicus*, is able to grow on methanol as a sole carbon and energy source because this strain has a methanol oxidase respiratory chain\(^{10,11}\). In comparison to other acetic acid bacteria, this unique feature of *A. methanolica* may improve its ability to produce GA from glycerol in the presence of methanol. However, GA production by this strain has not yet been investigated.

In this study, we evaluated the use of *A. methanolica* NBRC104435\(^7\) to produce GA from glycerol with or without methanol. *A. methanolica* NBRC104435 was found to produce GA well from the mixture of glycerol and methanol. In addition, evaluation of the enantiomeric purity of the GA produced resulted in characterization of this bacterium as a new type of GA producer.

### 2 EXPERIMENTAL PROCEDURES

#### 2.1 Bacterial strain and culture conditions

*A. methanolica* NBRC104435 was obtained from the National Institute of Technology and Evaluation (NITE) in Japan and was evaluated for GA production. Seed cultures of *A. methanolica* were prepared in 5 mL of glucose medium containing 0.5 wt% glucose, 0.5 wt% yeast extract (Difco Laboratories, Detroit, MI, USA), 0.5 wt% polypepton (Nihon Pharmaceutical, Tokyo, Japan), and 0.1% MgSO\(_4\)·7H\(_2\)O (pH 6.5) at 30°C and 200 rpm for 24 h. The seed cultures (1.5 mL) were then transferred to 300-mL Erlemmeyer flasks containing 30 mL of medium composed of 0.5 wt% yeast extract, 0.5 wt% polypepton, and 0.1 wt% MgSO\(_4\)·7H\(_2\)O (pH 6.5) plus appropriate concentrations of carbon sources (glycerol and/or methanol). The flasks were incubated at 30°C for 96 h and 200 rpm in a rotary shaker.

#### 2.2 Analytical procedures

Concentrations of glycerol, GA, and methanol in the culture broth were determined by high performance liquid chromatography (HPLC)\(^9\). Briefly, media samples were collected from the culture flasks and diluted with an appropriate amount of the mobile phase used in HPLC, followed by centrifugation to remove cell debris. After the supernatants were passed through 0.45-μm filters, 20 μL of each sample was injected into a Shimadzu LC-20 HPLC system (Shimadzu, Kyoto, Japan) equipped with refractive index detector RID-10 (Shimadzu) and Shodex SUGAR series column (Showa Denko K.K., Tokyo, Japan). A SUGAR SC1011 column with Milli-Q water as the mobile phase was used for glycerol analysis, while a SUGAR SH1011 with 5 mM H\(_2\)SO\(_4\) as the mobile phase was used for GA and methanol analyses. During analysis, columns were kept at 80°C for SC1011 and 65°C for SH1011. Flow rate of the mobile phase was 1 mL/min.

We evaluated the enantiomeric excess (ee) of the GA produced using HPLC with a chiral separation column. GA was recovered from culture broth by precipitation with ethanol into calcium salts\(^6\). After removal of cell debris from the culture by centrifugation, 1.1 equivalent of calcium chloride was added to the recovered supernatant, and then 2 volumes of ethanol were added to the mixture while stirring. After precipitation at room temperature, the mixture was kept at 4°C overnight. The precipitates were separated by filtration and collected, then the solids were dried under vacuum; this precipitation process was repeated twice prior to evaluation of enantioselectivity in the GA sample. The isolated glycerate calcium salts were diluted to approximately 1 g/L with a mobile phase of 0.45 mM CuSO\(_4\), and their 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 analysis, the column temperature was maintained at 21°C. L-GA and L-GA calcium salt dihydrate (Sigma-Aldrich) were used as standards.

Bacterial growth was evaluated by measuring optical density (OD) at 600 nm using a V-530 UV/VIS spectrophotometer (JASCO Corp., Tokyo, Japan).

#### 2.3 Chemicals

Unless otherwise noted above, all reagents and solvents were commercially available and of analytical grade from Wako Pure Chemicals or Tokyo Chemical Industries (Tokyo, Japan).

### 3 RESULTS AND DISCUSSION

#### 3.1 GA production from glycerol by *A. methanolica*

*A. methanolica* is able to grow on glycerol as a sole carbon source\(^9\); however, its ability to produce GA from glycerol has remained unclear. Thus, we first cultured *A. methanolica* in medium containing 0–10 wt% glycerol to evaluate its GA production. Figure 1 shows cell growth and
concentration of GA in the culture broth after 4 d of cultivation. A. methanolica grew well on 1 wt% glycerol, but cell growth decreased with increasing initial glycerol concentration. GA in the culture broth reached a maximum concentration of 6.9 g/L when 3 wt% glycerol was used, then accumulation of GA decreased with further increase in the initial glycerol concentration.

The effect of initial glycerol concentration on D-GA production by Acetobacter tropicalis NBRC16470 has previously been investigated using the same medium and cultivation time as the present study. Acetobacter tropicalis NBRC16470 accumulated only 0.7 g/L D-GA from 1 vol% glycerol, but produced 9.3 g/L D-GA when cultivated with 10 vol% glycerol, and the concentration of D-GA in the culture reached a maximum (10.5 g/L) with the addition of 15 vol% glycerol. However, such an increase in initial glycerol concentration decreased cell growth. Considering that other GA-producing acetic acid bacteria exhibit the same response to initial glycerol concentration, A. methanolica seemed to be very different from other acetic acid bacteria in its use of and tolerance for glycerol. It remains unknown why more than 3 wt% glycerol decreases growth and GA production by A. methanolica, but further research using its draft genome may help answer such questions by transcriptome analysis with the gene candidates capable of glycerol assimilation and GA production to compare gene expression profiles under different glycerol concentration.

3.2 Effect of methanol addition on GA production

After determining that A. methanolica could produce GA from glycerol, we next examined its ability to produce GA from 1–5 wt% glycerol containing 1–5 vol% methanol. Figure 2 shows the GA accumulation level after 4 d of cultivation in a medium containing glycerol and methanol. GA production was observed even when methanol was supplied to the culture, and a maximum concentration of GA obtained was 12.8 g/L when 3 wt% glycerol and 1 vol% methanol were used.

To elucidate changes in GA accumulation depending on methanol concentration, we investigated the time course of cell growth and accumulation of GA in broth containing 3 wt% glycerol plus 0–2 vol% methanol (Figs. 3A and 3B). Consumption of methanol and glycerol were also measured (Figs. 3C and 3D, respectively). As shown in Fig. 3C, cell growth increased with increasing methanol concentration. In the presence of 2 vol% methanol, the OD600 reached a maximum value of 19 after 3 d of cultivation, and good consumption of both glycerol and methanol was also observed. However, GA accumulation was only 1.5 g/L after 4 d of cultivation. On the other hand, when 1 vol% methanol was supplied to the culture, GA produced in the broth reached a maximum of 12.8 g/L after 4 d of cultivation. As shown in Fig. 3C, glycerol remained in the media containing 0–1 vol% methanol after 4 d of cultivation, whereas almost all glycerol was exhausted when 2 vol% methanol was supplied.

In a previous study, G. frateurii NBRC103465 produced...
28.2 g/L of GA from 170 g/L of glycerol in flask culture, but accumulated only 14.4 g/L GA when given the same concentration of glycerol plus 1 vol% methanol. Other *Gluconobacter* strains also decreased their accumulation of GA in the presence of methanol. In contrast with *Gluconobacter* strains, methanol increases GA production by *A. methanolica*. The OD600 value of *A. methanolica* culture containing 1 vol% methanol was greater than that of culture grown without methanol, suggesting that better growth of *A. methanolica* resulted in greater production of GA. Further increasing the methanol concentration in the culture resulted in more cell growth. *A. methanolica* utilize ribulose monophosphate pathway for methylotrophic growth, and methanol metabolism might be related with phosphoglycerate concentration via pentose phosphate pathway. This suggests that cell activity would be related with methanol and glycerol concentrations in the culture. Thus, *A. methanolica* has a unique ability to enhance GA production in the presence of methanol. This ability will be useful for the production of GA from raw glycerol containing methanol.

### 3.3 Enantioselectivity of GA

GA produced from 3 wt% glycerol plus 1 vol% methanol was recovered by precipitation as calcium salts. Chiral HPLC analysis revealed that the ee of D-GA obtained from *A. methanolica* culture was 44.2%. This value was much lower than those of *G. frateurii* NBRC103465 (72% ee) and *Acetobacter tropicalis* NBRC14760 (99% ee) (Fig. 4). To date, no acetic acid bacteria have been reported to produce D-GA with an ee below 50%. The value of 44% ee indicates that the GA produced in this study contains 28 mol% of L isomers. Previously, we developed a production method for L-GA from a racemic mixture of GA using optical bacterial resolution by newly isolated bacteria, in which the bacterial strains consume D-GA specifically and leave L-GA in the culture broth. A recent report indicated that L-GA will be useful in the stereospecific production of L-sugars for medical and pharmaceutical applications. The ability of *A. methanolica* to produce GA rich in L-isomers from raw glycerol could therefore provide a useful source of mixed D- and L-GAs for microbial resolution.
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4 CONCLUSION

The methylotrophic acetic acid bacterium A. methanolica produced GA from glycerol containing methanol, accumulating 12.8 g/L GA from 3 wt% glycerol plus 1 vol% methanol over 4 d of cultivation. In addition, the GA produced was revealed to have 44 % ee of the \( \text{D} \)-isomer, less than is produced by other GA-producing acetic acid bacteria. This is the first report of GA-producing acetic acid bacteria with an ee value less than 50 % indicating GA rich in the \( \text{L} \)-isomer. These results show that A. methanolica may be useful for production of GA from raw glycerol derived from the oil manufacturing processes, which generally contains methanol.

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