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L-Lactic acid production from glycerol coupled with acetic acid metabolism by *Enterococcus faecalis* without carbon loss

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Glycerol is a by-product in the biodiesel production process and considered as one of the prospective carbon sources for microbial fermentation including lactic acid fermentation, which has received considerable interest due to its potential application. *Enterococcus faecalis* isolated in our laboratory produced optically pure L-lactic acid from glycerol in the presence of acetic acid. Gas chromatography-mass spectrometry analysis using [1, 2-$^{13}C_2$] acetic acid proved that the *E. faecalis* strain QU 11 was capable of converting acetic acid to ethanol during lactic acid fermentation of glycerol. This indicated that strain QU 11 restored the redox balance by oxidizing excess NADH though acetic acid metabolism, during ethanol production, which resulted in lactic acid production from glycerol. The effects of pH control and substrate concentration on lactic acid fermentation were also investigated. Glycerol and acetic acid concentrations of 30 g/L and 10 g/L, respectively, were expected to be appropriate for lactic acid fermentation of glycerol by strain QU 11 at a pH of 6.5. Furthermore, fed-batch fermentation with 30 g/L glycerol and 10 g/L acetic acid wholly exhibited the best performance including lactic acid production (55.3 g/L), lactic acid yield (0.991 mol-lactic acid/mol-glycerol), total yield [1.08 mol-(lactic acid and ethanol)]/mol-(glycerol and acetic acid)], and total carbon yield [1.06 C-mol-(lactic acid and ethanol)/C-mol-(glycerol and acetic acid)] of lactic acid and ethanol. In summary, the strain QU 11 successfully produced lactic acid from glycerol with acetic acid metabolism, and an efficient fermentation system was established without carbon loss.

[**Key words:** Lactic acid fermentation; Glycerol; Acetic acid metabolism; Carbon loss; *Enterococcus faecalis*]

Bio-based chemical production from renewable resources has attracted considerable attention in recent years because of the serious energy issues and climate changes resulting from the mass consumption of fossil fuels. Recent studies have indicated that the production of platform chemicals, starting materials for chemical intermediates and polymers, via microbial fermentation is an important alternative to conventional production from petroleum (1). In recent years, lactic acid has received considerable interest because of its potential application in the development of poly-lactic acid, which is a class of biodegradable, biocompatible, and environmentally friendly plastics, and has been expected to substitute that derived from petrochemicals (2). Additionally, lactic acid has versatile applications in the food, chemical, cosmetic, and medical industries, leading to an increase in the demand for lactic acid (3,4). Chemical synthesis always produces a racemic mixture of lactic acid, while optically pure L(+), D(−), or racemic lactic acid is generally obtained by microbial fermentation using the appropriate bacterial strains, such as lactic acid bacteria (LAB), or genetically engineered strains (5). Therefore, a majority of the lactic acid production today can be attributed to microbial fermentation (4,5). The required raw materials generally account for the high cost of lactic acid production. Starch and refined sugar (derived from edible biomass) is currently being employed as a raw material in industrial lactic acid production, which might result in competition with the food-feed chain (2). Therefore, alternative inedible materials are required for large-scale lactic acid production (2). Many scientists have constructed efficient lactic acid fermentation systems using cheap materials, such as lignocellulosic biomass, because of their abundance and sustainability (4–6).

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On the other hand, glycerol, a by-product in a biodiesel production process that discharges 1 kg crude glycerol for every 10 kg of biodiesel produced, is available in abundant quantities, and is inedible (7,8). Furthermore, the price of crude glycerol has been steadily decreasing with the increase in biodiesel production (7). In addition, economically value-added products, such as lactic acid, 1,3-propandiol, propionic acid, and ethanol, can be produced from glycerol (7,9,10). Some researchers have reported that genetically engineered *Escherichia coli*, *Klebsiella pneumonia*, and *Rhizopus oryzae* produce lactic acid from glycerol under aerobic or microaerobic conditions (10–14). Oxidation power is required to produce lactic acid from glycerol, as glycerol has a higher reducing power compared to glucose (7). For example, genetically engineered *E. coli* is considered to oxidize NADH using oxygen (10), or by coupling with oxygen reduction (11), in order to produce lactic acid from glycerol.

Several reports have focused on the utilization of glycerol as the sole carbon source by LAB, which are gram-positive, non-spore-forming, catalase-negative, mesophilic (10–45°C) aerotolerant cocci or rods that cannot utilize oxygen as an electron acceptor, including *Lactobacillus panis*, *Lactobacillus rhamnosus*, and *Enterococcus faecalis* (15–17). A previous study has demonstrated that *L. panis* PM1 produced 1,3-propandiol from glycerol at a yield of 0.750 mol/mol (15); *L. rhamnosus* and *E. faecalis* were also observed to produce lactic acid from glycerol (16,17). However, *L. rhamnosus* produced lactic acid under aerobic conditions at a yield of 0.314 mol/mol, and with the formation of by-products including acetic acid, diacetyl, acetoin, and 2,3-butandiol (16). On the other hand, *E. faecalis* W11 anaerobically produced ethanol and formic acid as the primary products, and lactic acid as the by-product, using glycerol as the raw material, at a yield of 0.338 mol/mol; this indicated that the redox balance was mainly restored by *E. faecalis* W11 through the ethanol production pathway under anaerobic conditions (17). Consequently, lactic acid fermentation of glycerol by LAB, with a high yield (>0.4 mol/mol), has not been studied.

We considered a possibility of high lactic acid yield by LAB from glycerol with a coupled metabolism of another substrate with higher oxidizing power to restore the redox balance even under anaerobic condition. The aim of this study was the production of L-lactic acid from glycerol (with a high yield) by strain QU 11, which was isolated in our laboratory and identified as *E. faecalis*. Strain QU 11 enhanced L-lactic acid production (concentration and yield) from glycerol by reducing acetic acid to ethanol, restoring the redox balance. Furthermore, gas chromatography-mass spectrometry (GC–MS) analysis directly proved the ability of strain QU 11 to covert acetic acid to ethanol. Therefore, we successfully produced lactic acid from glycerol coupled with the acetic acid metabolic pathway, and constructed a fermentation system without carbon loss.

MATERIALS AND METHODS

Strains and medium Strain QU 11 used in this study was maintained as a stock culture at −80°C in 2 mL vials containing 15% (*v*/*v*) glycerol. Modified Man, Rogosa, and Sharpe (mMRS) medium, containing the following components per liter of distilled water: 20–80 g glycerol, 5–57 g sodium acetate trihydrate, 10 g peptone (Difco, Becton Dickinson and Co., Franklin Lakes, NJ, USA), 8 g beef extract (Nacalai Tesque, Kyoto, Japan), 4 g yeast extract (Difco, Becton Dickinson), 2 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.05 g $MnSO_4.4H_2O$, and 1 mL polyoxyethylene sorbitan monooleate (Tween 80), was used in this experiment. The refresh culture was grown in mMRS medium supplemented with 20 g/L glycerol, 10 g/L sodium acetate trihydrate, and citrate monohydrate (10 g/L); the pre-cultures were grown in mMRS medium supplemented with 20 g/L glycerol and 10 g/L sodium acetate trihydrate. The main culture medium consisted of mMRS medium supplemented with various concentrations of glycerol and acetic acid (as indicated for each experiment). The initial pH was adjusted to 6.5 using 1 M NaOH, and the media were sterilized at 115°C for 15 min.

Characterization and identification of strain QU 11 Strain QU 11 was isolated from fallen leaves collected at the Kyushu University campus. Strain QU 11 was identified based on the sugar fermentation pattern and 16S rDNA sequencing. The sugar fermentation pattern of strain QU 11 was determined using the API 50 CHL test kit (bioMerieux, Marcy l'Etoile, France), according to the manufacturer protocols. The obtained pattern was compared with those of reference strains described in previous studies (18). A partial 16S rRNA gene (16S rDNA) region of strain QU 11, corresponding to positions 8–1510 in the *E. coli* 16S rDNA, was analyzed using the primer set 8F and 1510R. The primer sequences were as follows: 8F, 5′-AGAGTTTGATCCTGGCTCAG-3′; and 1510R, 5′-CTGAAGCTTACGGTTACCTTGTTACGACTT-3′. The total genomic DNA was extracted from the cells using the Mag Extractor Kit (Toyobo, Osaka, Japan) according to the manufacturer protocols; this was used as the PCR template. PCR was performed using *Taq* DNA polymerase (Promega, Madison, WI, USA) under the following conditions: denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and polymerization at 72°C for 60 s. The amplified products were purified using the High Pure Product Purification Kit (Roche Diagnostics, Mannheim, Germany), as per the protocols detailed by the manufacturer. The purified PCR product was cloned into the pGEM-T vector (Promega), which was transformed into *E. coli* DH5α (Toyobo). DNA sequencing was carried out by FASMAC (Kanagawa, Japan). Similarities were identified in the GenBank database using the basic local alignment search tool (BLAST) algorithm.

Fermentation Batch cultures were performed without pH control at 30°C in 300 mL Erlenmeyer flasks, with a 100 mL working volume of mMRS medium supplemented with 20 g/L glycerol and 11 g/L sodium acetate trihydrate. A 1% (*v*/*v*) inoculum was transferred to the mMRS medium; subsequently, the batch culture was carried out statically under anaerobic conditions using AnaeroPack (Mitsubishi Gas Chemical Co., Tokyo, Japan). To enhance lactic acid production, batch cultures with pH control were carried out using mMRS medium (400 mL working volume including an inoculum volume of 10% (*v*/*v*)), supplemented with 20–80 g/L glycerol and 5–57 g/L sodium acetate trihydrate, at 30°C and 100 rpm, in a 1-L jar fermenter. NaOH (5 M) was added automatically to maintain a pH of 6.5 during the fermentation. Fed-batch cultures were carried out in mMRS medium supplemented with 30 g/L glycerol and 22 g/L sodium acetate trihydrate, under the same conditions described above. Glycerol and acetic acid solutions were fed to the jar fermenter when the glycerol and acetic acid concentrations reached 5 g/L and 2 g/L, respectively. Samples were periodically drawn for analysis.

Analytical method The optical density of all samples was monitored at 562 nm (OD_{562}) by spectrophotometry (UV–Visible Spectrophotometer BioSpec-1600; Shimadzu, Kyoto, Japan) in order to measure the cell density in the broth. Dry cell weight (DCW) was obtained as described in a previous study (19). OD_{562} was converted to DCW (g/L) using the appropriate calibration curve; 1.0 unit of OD_{562} corresponded to 0.345 g DCW/L. Samples of the cultures were centrifuged at 12,000 ×*g* for 10 min, and filtered using a membrane filter (Dismic-13HP, 0.45 μm; Advantec, Tokyo, Japan). The filtered supernatant was used for further analyses. The concentrations of glycerol, organic acids, and ethanol were determined by high-performance liquid chromatography (HPLC) using an US HPLC-1210 system (Jasco, Tokyo, Japan) and a RSpak SH-1011 column (Shodex, Tokyo, Japan) at 75°C, using 0.5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min, and an injection volume of 20 μL. The optical purity of lactic acid was measured using a BF-5 biosensor (Oji Scientific Instruments, Hyogo, Japan) according to the protocol provided by the manufacturer.

Elucidating the relationship between glycerol and acetic acid metabolism using [1, 2-$^{13}C_2$] acetic acid Test tube cultures were prepared in 15 mL screw-capped tubes, with a 5 mL working volume of mMRS medium supplemented with 20 g/L glycerol and 5 g/L [1, 2-$^{13}C_2$] acetic acid (Sigma–Aldrich, St Louis, MO, USA). One test tube culture was inoculated with a 1% (*v*/*v*) inoculum, and another was not inoculated (blank); these cultures were incubated statically at 30°C for 24 h. Each sample was defined as 24 h and a 0 h sample. Samples (5 mL) were centrifuged at 8000 ×*g* for 10 min, and filtered by membrane filters as described in the previous section. HCl (2 M) was added just before analysis to facilitate the volatilization of acetic acid. The samples were put into 12 mL headspace vials containing an internal standard (acetone). Solid phase microextraction (SPME) was performed according to the method described by Yang and Peppard (20). The headspace vial was conditioned to a pre-warming temperature of 40°C for 20 min; subsequently, the fiber containing an 85 μm polyacrylate coating (Spelco, Bellefonte, PA, USA) was injected to the headspace vial at 40°C for 20 min. ^{12}C-acetic acid, [1, 2-$^{13}C_2$] acetic acid (substrates), ^{12}C-ethanol, and ^{13}C-ethanol (products) content in the samples was quantified by GC–MS employing a GCMS-QP2010 Plus (Shimadzu) equipped with a DB-Wax column (30 m, 0.25 mm internal diameter, 0.5 μm film thickness; Agilent Technologies, Palo Alto, CA, USA). Helium was supplied as the carrier gas, at a flow rate of 40.8 cm/s. The initial oven temperature was maintained at 40°C for 3 min; the temperature was subsequently raised to 150°C at a rate of 10°C/min. The temperature of the injection port was maintained at 230°C. The ion source and interface temperatures were set at 200°C and 230°C, respectively. The SPME fiber was injected into the GC injector in the split injection mode, with a 1:20 split ratio. Metabolites were detected using the selected ion monitoring (SIM) mode, using the target fragment ion *m*/*z*: 45, 47, 58, 60, and 62.

Calculations The glycerol fermentation yields (mol/mol and C-mol/C-mol) were calculated as follows:

$$\text{Lactic acid yield [mol-lactic acid/mol-glycerol]} = (C_L/90.08)/(C_{Gly}/92.09) \quad (1)$$