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Utilization of High-Salinity Crude Glycerol Byproduct from Biodiesel Production for Biosynthesis of γ-Aminobutyric Acid in Engineered *Halomonas elongata*

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ABSTRACT

As the development of renewable energy has become imperative, biodiesel fuel (BDF) has been used as renewable biofuel with the advantage of having lower levels of greenhouse gas emissions. However, the transesterification reaction that produces BDF generates a high-salinity crude glycerol (CG) byproduct, which is difficult to recycle. *Halomonas elongata* is a moderately halophilic bacterium being used as a cell factory due to its ability to assimilate varieties of substrates for growth in high-salinity conditions. Previously, we engineered a recombinant *H. elongata* GOP-Gad to biosynthesize and accumulate γ-aminobutyric acid (GABA) as a high-value product. Here, we tested the ability of *H. elongata* GOP-Gad to use CG as a substrate for cell growth and GABA production. The CG byproduct was obtained from a BDF facility in Unzen City, Nagasaki, Japan, where geothermal energy catalyzed BDF production from waste cooking oil and biomethanol. Prior to use as the sole carbon (C) source in culture media, the CG byproduct was partially purified to remove soap substances and other impurities. Finally, we showed that *H. elongata* GOP-Gad could grow and accumulate GABA up to 28 µmol/g cell fresh weight in a minimal M63 medium containing 7% w/v NaCl with 4% w/v glycerol from the partially purified CG as a C source and 15 mM $(NH_4)_2SO_4$ as a nitrogen (N) source. This result demonstrates a new circulatory bioprocess of C and N, in which *H. elongata* GOP-Gad can use partially purified CG and $(NH_4)_2SO_4$ as the sole C and N sources for growth and GABA production.

1. INTRODUCTION

Global energy demand sharply increases as the human population grows. However, fossil fuel resources are limited. Adding to the problem are environmental and climate issues caused by the usage of fossil fuels. Therefore, the development of renewable energy has become imperative. Biodiesel fuel (BDF) is a renewable biofuel alternative to diesel fuel, offering the additional advantage of lower greenhouse gas emissions (Kosamia et al., 2020). BDF is formed as a methyl or ethyl ester of fatty acid and is usually produced by the transesterification reaction between vegetable oils or animal fats with short-chain alcohols under high-temperature conditions (Gerpen, 2005). During transesterification, 1 mole of triglyceride produces 3 moles of biodiesel and 1 mole of glycerol byproduct, which contains high concentrations of salt (Figure 1). Every batch of biodiesel production generates approximately 10% w/v glycerol and other impurities, which are harvested together as crude glycerol (CG) byproducts. The main impurities in CG are methanol, soap, free fatty acids, salt (inorganic salts residues from catalysts), unreacted mono-, di- and tri-glycerides, and water (Pagliaro and Rossi, 2010).

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Although the public and government of Unzen City, Nagasaki, Japan, aim to be more environmentally friendly by producing BDF with biomass-derived methanol, waste cooking oil, and hot spring geothermal energy (Nakagawa et al., 2007; Tominaga et al., 2014; Tominaga et al., 2015), the CG byproduct has not been used effectively. Due to its high salinity, CG byproducts generated in Nagasaki are generally only used as an additive to compost to promote fermentation (Tominaga et al., 2014; Tominaga et al., 2015).

Biotechnology for synthesizing high-value products, such as medicine, chemicals, and food additives, has been developed for decades, aiming to gradually replace the traditional petroleum-based industry. *H. elongata* strains are among the bacteria being used as microbial cell factories due to their ability to use different substrates to grow under high-salinity conditions (Schwibbert et al., 2011; Tanimura et al., 2013; Nakayama et al., 2020; Ye and Chen, 2021; Chen et al., 2022). For example, many *H. elongata* strains can utilize glucose, glycerol, or lignocellulosic biomass-derived sugars such as cellobiose, xylose, and arabinose as C sources and ammonium and amino acids as N sources (Vreeland et al., 1980; Ono et al., 1998; Tanimura et al., 2013). Unlike the *H. elongata* type-strain DSM 2581^T, the *H. elongata* OUT30018 (Ono et al., 1998; Ono et al., 1999) exhibits a unique ability to utilize putrefactive non-volatile amines, such as histamine and tyramine, derived from biomass waste as sources of carbon and nitrogen for cell growth. (Nakayama et al., 2020). *H. elongata* OUT30018 was also found to assimilate amino acids derived from biomass waste, such as CG byproducts of biodiesel industries (Bozbas, 2008; Ayoub and Abdullah, 2012). Therefore, *H. elongata* OUT30018 is one of the most promising bacterial strains for developing cell factories for producing fine chemicals from biomass waste.

Previously, we engineered a recombinant *H. elongata* GOP-Gad to biosynthesize and accumulate γ-aminobutyric acid (GABA) as a major osmolyte under high-salinity growth conditions (Zou et al., 2024). As *H. elongata* GOP-Gad has *H. elongata* OUT30018 genetic background, we explored the possibility of using KOH-neutralized high-salinity CG as a C source in the culture medium of *H. elongata* GOP-Gad for GABA production. As a result, we found that *H. elongata* GOP-Gad can use CG as the sole C source to produce GABA, which was accumulated in the cells up to 28 μmol/g cell fresh weight (CFW). In the future, we will continue to develop *H. elongata* strain, growth condition, and medium formula to increase GABA yield. The work presented here represents the initial phase in the development of *H. elongata* cell factory into single-cell feed additive. The cells will be used directly without further purification steps, creating a low-cost circulatory bioprocess of C and N to support a more recycling-oriented feed industry.

2. METHODOLOGY

2.1 Partial purification process of CG byproduct

KOH-neutralized CG byproduct of BDF production was obtained from a BDF production facility operated by the Social Welfare Corporation COSMOS association in Unzen City, Nagasaki, Japan, with the assistance of the Nagasaki Environmental Health Research Center, Omura City, Nagasaki, Japan. To remove soap substance and other impurities, 200 g of CG byproducts was mixed with 500 mL of 25% w/v NaCl solution in a salting-out process. The soap substance was separated from CG by centrifugation at 7,500×g for 10 min; then the CG solution was filtered through Qualitative filter paper (Advantec) to remove the remaining soap substance and other impurities. The resulting CG solution was filter-sterilized through a 0.22 μm pore size bottle top filter (Sartorius) and stored at room temperature until use. CG was diluted 100 times in pure water for High-performance liquid chromatography (HPLC) analysis or diluted 1,000 times in pure water and digested with nitric acid for analysis of Na and K ions content by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES; ICPS-7500, SHIMADZU) analysis. Because purified CG contains high Na concentration, the concentration of Na added to M63 minimal medium was adjusted accordingly.

2.2 Bacterial strain

In this study, *H. elongata* GOP-Gad (Zou et al., 2024) is used in all growth tests. The *H. elongata* GOP-Gad was generated from the glutamic acid overproducing mutant *H. elongata* GOP by introducing an artificial bicistronic *mCherry-HopGadmut* operon, which confers salt-inducible expression of 2 genes: The *mCherry* gene, which encodes a red fluorescent reporter mCherry protein that was used as a visual selection marker to facilitate transformant screening and as a marker to confirm transgene expression, and the *HopGadmut* gene, which encodes a mutant L-glutamic acid decarboxylase B (GadBmut) enzyme, which is active at a wide pH range to convert glutamate (Glu) to GABA (Figure 2).

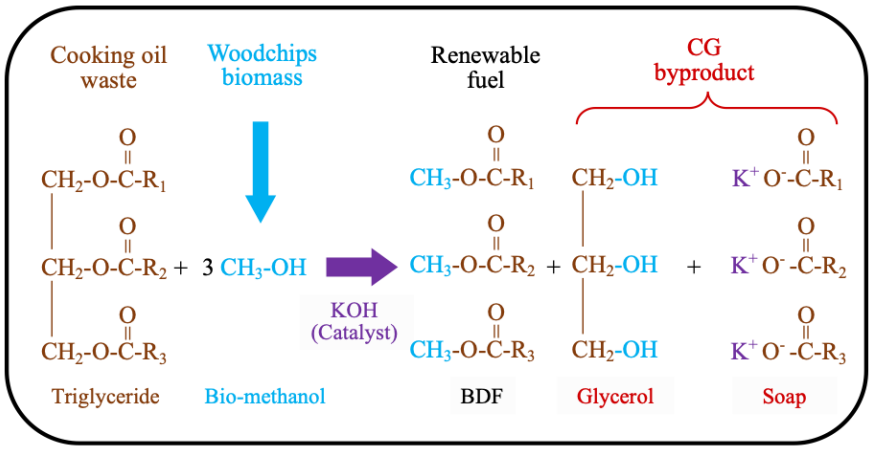

Figure 1. Biodiesel fuel (BDF) production process in a chamber heated by hot spring water at a BDF facility in Unzen City, Nagasaki, Japan.

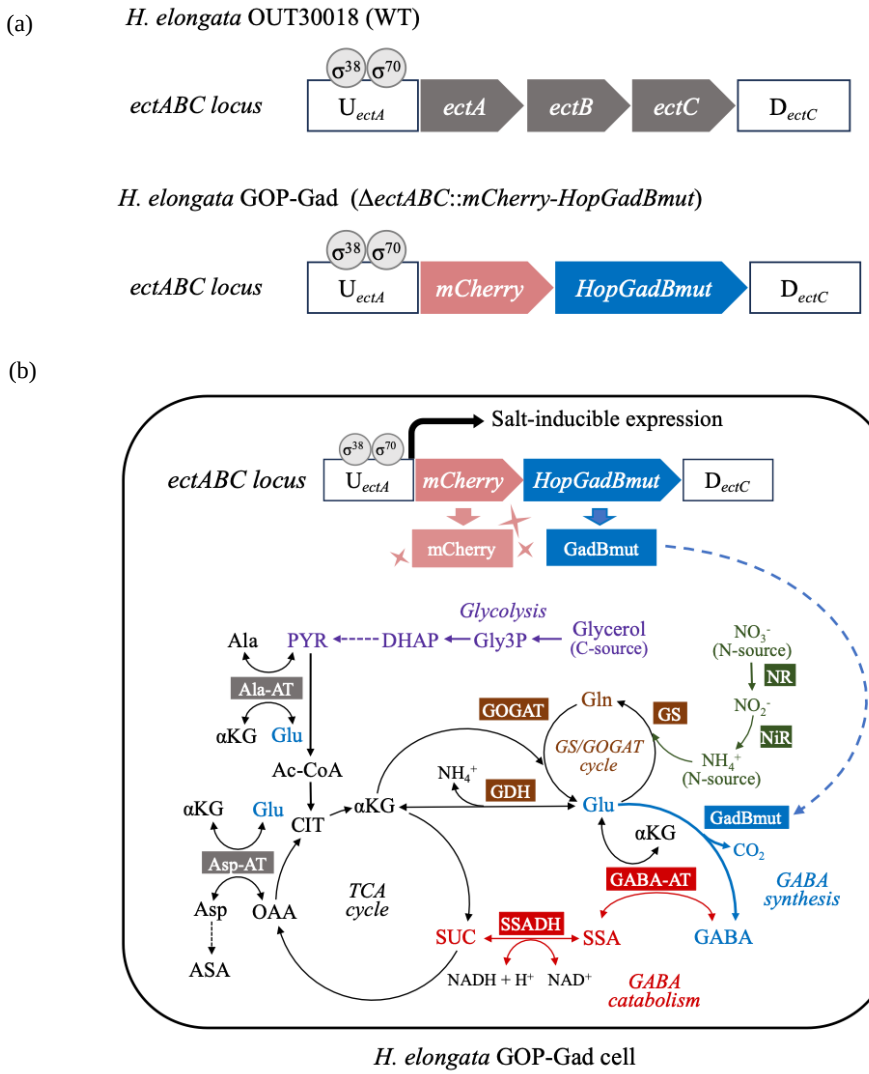

Figure 2. Engineered GABA metabolic pathway in *H. elongata* GOP-Gad. (a) Comparison of the genomic structures at the ectABC locus on the genome of the wild-type *H. elongata* OUT30018 and the engineered *H. elongata* GOP-Gad. UectA, A 1-kb upstream region of the ectA gene that contains an ectA promoter with putative binding sites for the osmotically induced sigma factor σ38 and the vegetative sigma factor σ70. This region was used as a target for homologous recombination at the ectABC locus; DectC, A 1-kb downstream region of the ectC gene that contains an ectC terminator. This region was used as a target for homologous recombination at the ectABC locus; ectA, a gene that encodes an L-2,4-diaminobutyric acid acetyltransferase; ectB, a gene that encodes a DABA transaminase; ectC, a gene that encodes an ectoine synthase; mCherry, a gene that encodes a red fluorescent reporter protein mCherry; HopGadBmut, a synthetic *H.*

elongata's codon-usage optimized (Hop) GadB mutant gene (HopGadBmut), which encodes a mutant L-glutamic acid decarboxylase B (GadBmut) with activity across broader pH ranges than the wild-type GAD. (b) GABA metabolic pathway in *H. elongata* GOP-Gad cell cultured in a medium containing glycerol as a C source and NH_4^+ or NO_3^- as an N source. Ac-CoA, acetyl coenzyme A; Ala, L-alanine; Ala-AT, L-alanine aminotransferase; α-KG, α-ketoglutaric acid; ASA, L-aspartate-β-semialdehyde; Asp, L-aspartic acid; Asp-AT, L-aspartic acid aminotransferase; CoA, coenzyme A; DHAP, dihydroxyacetone phosphate; GABA, γ- aminobutyric acid; GABA-AT, γ-aminobutyric acid aminotransferase; GadBmut, a mutant L-glutamic acid decarboxylase; Glu, L-glutamic acid/glutamate; GDH, L-glutamic acid dehydrogenase; GOGAT, L-glutamic acid synthetase; Gln, L-glutamine; GS: L-glutamine synthetase; Gly3P, glycerol 3-phosphate; NR, nitrate reductase; NiR; nitrite reductase OAA, oxaloacetate; PYR, pyruvate; SSA, succinate semialdehyde; SSADH, succinate semialdehyde dehydrogenase; and SUC, succinate.

2.3 Culture media

2.3.1 High-salinity LB media

As a complex medium for routine culture of *H. elongata* GOP-Gad, LB medium (Sambrook and Russel, 2001) was modified to contain high NaCl concentration (high-salinity LB medium), which contains 10 g/L Bacto-tryptone, 5 g/L Bacto-yeast extract, and 3% or 6% w/v NaCl. For solid medium, 15 g/L agarose was added before autoclave sterilization. A high-salinity LB medium containing 6% w/v NaCl was used for culturing the inoculum for the main culture in the M63 medium containing 7% w/v NaCl.

2.3.2 High-salinity M63 media containing pure glycerol (PG) or CG as the sole C source

The high-salinity M63 media was modified from the M63 minimal medium (Perroud and Le Rudulier, 1985) for culturing *H. elongata* GOP-Gad as described previously (Zou et al., 2024). High-salinity M63 media contain 100 mM KH_2PO_4, 1 mM $MgSO_4$, 3.9 µM $FeSO_4$, 3% or 7% w/v NaCl, 15 mM $(NH_4)_2SO_4$ or 30 mM $NaNO_3$ as the sole N source, and 4% w/v glycerol from PG, which contains 100% w/v glycerol, or CG, which contains 64% or 66% glycerol (Table 1), as the sole C source. A higher amount of CG was supplemented so that the final glycerol concentration of the medium was 4% w/v. The pH of the medium was adjusted to 7.2 with KOH solution before filter sterilization.

The C, N-free high-salinity M63 medium was prepared as described above without adding C and N sources.

2.4 Culture condition

H. elongata GOP-Gad was cultured in glass test tubes incubated in a 37°C water bath with 120 rpm agitation.

2.5 Extraction of major osmolytes accumulated in *H. elongata* GOP-Gad cells

Major osmolytes were extracted from *H. elongata* cells as previously described (Zou et al., 2024). In brief, *H. elongata* GOP-Gad was cultured in high-salinity LB medium until OD_{600} reached more than 1, when the cells were harvested by centrifugation at 10,000 × g for 3 min, and the weight of the cell pellets was recorded as cell fresh weight (CFW). Mimicking the bacterial milking process (Sauer and Galinski, 1998; Cánovas et al., 1997), the cell pellets were resuspended in pure water (20 µL/mg CFW) for hypo-osmotic extraction of major osmolytes from the cells. After centrifugation at 10,000 × g for 3 min, the supernatant containing the free amino acids and major osmolytes was collected as a major osmolytes sample.

2.6 Amino acids dabsylation

As previously described (Zou et al., 2024), 10 µL aliquot of free amino acids samples or standard amino acids was mixed with 2 µL of 2.5 mM internal standard norvaline and 8 µL of 1 M $NaHCO_3$ pH adjustment solution. Then, the sample was mixed with 40 µL of dabsylation reagents containing 2 mg/mL dabsyl chloride dissolved in acetonitrile and incubated at 70°C for 15 min. After the incubation, 440 µL of 250 mM $NaHCO_3$ solution was added, and the samples were centrifuged at 10,000 × g for 3 min. The supernatant of each sample was collected and filtered through a filter vial with 0.2-µm pore size polytetrafluoroethylene (PTFE) membrane (SEPARA Syringeless filter, GVS Japan K.K., Tokyo, Japan) prior to HPLC analysis.

2.7 HPLC analysis

Determination of dabsyl amino acids derived from *H. elongata* cells was carried out as previously described (Zou et al., 2024) using a high-performance liquid chromatography system (Shimazdu, Kyoto, Japan) equipped with an UV/VIS detector (SPD-10 A VP), an autosampler (SIL-10 AD VP), two pumps (LC-10 AD VP), degasser (DGU-14A), system controller (SCL-10A Vp), and column oven (CTO-10AC VP). LabSolutions LC software (Shimadzu, Kyoto, Japan) was used for system control and data acquisition. Chromatographic separation of dabsyl amino acids was achieved through an analytical C18 column (Poroshell 120 2.7 µm, EC-C18, 4.6×75 mm, Agilent Technologies Inc.) with C18 guard column

(Poroshell 120 2.7 µm Fast Guard, EC-C18, 4.6×5 mm, Agilent Technologies Inc.) using a mobile-phase gradient system consisting of 15% acetonitrile in 20 mM sodium acetate (pH 6.0) (mobile phase A) and 100% acetonitrile (mobile phase B). Dabsyl amino acids were determined by UV/VIS detector at 468 nm. The injection volume was 10 µL, the flow rate was 0.5 mL/min, and the column temperature was maintained at 27°C.

2.8 ICP-AES analysis

The concentrations of Na and K ions in the partially purified CG were determined by ICP-AES analysis. The PG and partially purified CG were diluted 1,000 times with distilled water, and 2.5 µL aliquot of each sample was transferred to a 50 mL polypropylene tubes. After mixing with 25 mL of Milli-Q water and 5 mL of 70% v/v HNO_3 (Nacalai Tesque Inc., Kyoto, Japan), the samples were incubated in a 65°C digestion block (DigiPREP jr., SCP Science, Quebec, Canada) for 15 min, followed by the incubation at 105°C for 120 min. After the samples cooled down to room temperature, 0.5 mL of 30% v/v H_2O_2 (Fujifilm Wako Pure Chemical, Ltd., Osaka, Japan) was added to each mixture, and the samples were further incubated at 105°C for 60 min. After the samples cooled down to room temperature, they were filtered through a 0.45 µm-pore-size Teflon membrane filter (DigiFILTER, SCP Science, Quebec, Canada). After rinsing the filter with Milli-Q water, the volume of each filtered sample was adjusted to 50 mL with Milli-Q water. Quantification of ions concentrations was performed on an ICP-AES (ICPS-7500, Shimadzu Corporation, Kyoto, Japan) following the manufacturer's instructions (Nakayama et al., 2019).

3. RESULTS AND DISCUSSION

3.1 Partial purification of CG by the salting-out process

As shown in Figure 1, at a BDF production facility in Unzen City, Nagasaki, Japan, heat generated by hot spring water facilitates a transesterification reaction between triglyceride in used cooking oil collected from households and restaurants in Nagasaki prefecture and biomethanol derived from wood chip biomass to produce BDF. KOH was used as an alkaline catalyst at this facility instead of NaOH because the resulting CG byproduct will be used as an additive to compost for agricultural fields that are sensitive to sodium salt. During the BDF production process, a saponification reaction partially occurs between triglyceride and KOH to form soap in the CG byproduct. As this study aims to use the CG in the BDF byproduct as the sole C source of *H. elongata* GOP-Gad's culture medium, soap must be removed due to its cytotoxic effect.

The first step in removing soap and other impurities was a salting-out step (Figure 3), in which the CG byproduct was mixed with 25% w/v NaCl solution. After mixing, the solution is centrifuged to separate the solution into two phases: the liquid phase, which contains the partially purified CG, and the solid phase, which contains soap and other impurities. After the solid phase, which floated on top of the liquid phase, was spooned out, the liquid phase was collected and further purified by filtration through a standard quantitative filter paper. The resulting partially purified CG contains 40% w/v CG. The concentrations of Na and K ions in the partially purified CG were analyzed by ICP-AES, and the glycerol concentration was analyzed by HPLC. The analysis results for different partially purified CG batches are shown in Table 1. Based on the results, NaCl concentrations of the partially purified 40% w/v CG batches were determined to be 17% w/v. With this information, the amount of NaCl added to the M63 minimal media used in the following growth tests was adjusted accordingly.

Table 1. Concentrations of Na^+, K^+, and glycerol in the partially purified crude glycerol (CG).

Chemical (unit)	Pure glycerol (PG)	CG-1	CG-2	CG-3
Na^+ (M)	ND	2.93	3.00	2.82
K^+ (M)	ND	0.41	0.42	0.39
Glycerol (% w/v)	100	66	64	66

The concentrations of Na and K ions in CG were analyzed by ICP-AES and the concentrations of glycerol in CG samples in different batches of partially purified CG (CG-1, CG-2, and CG-3) were analyzed by HPLC. CG samples were diluted 100 times in pure water for HPLC analysis or diluted 1,000 times in pure water and digested with nitric acid for ICP-AES analysis. ND, not detected.

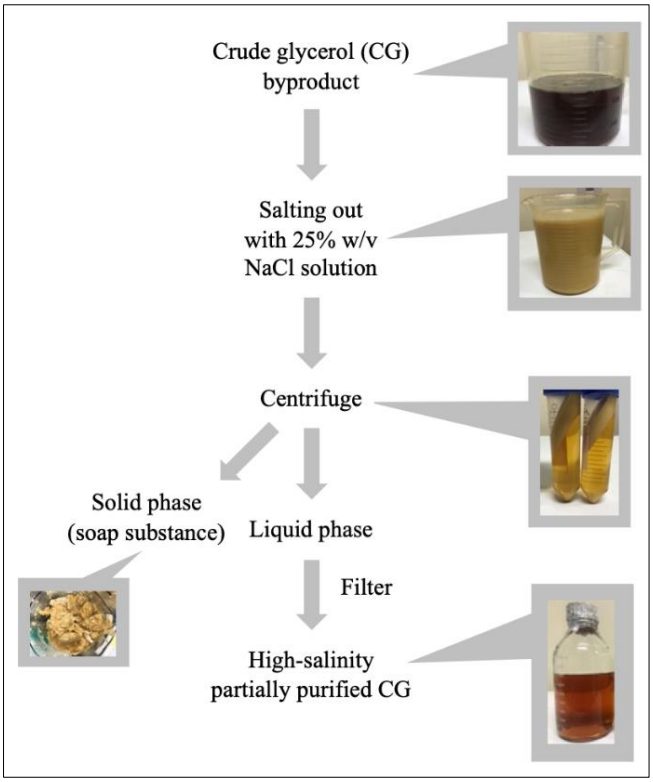

Figure 3. Partial purification of the crude glycerol (CG) byproduct. After salting-out treatment with a 25% w/v NaCl solution, the partially purified CG contains 40% w/v CG and high Na and K ions concentrations.

3.2 *H. elongata* GOP-Gad can utilize CG as the sole C source and ammonia or nitrate as the sole N source

To test whether the partially purified CG can work as the sole C source in a culture medium to sustain the growth and production of valuable compounds by bacterial cell factory, we tested the ability of the GABA-producing cell factory, *H. elongata* GOP-Gad (Zou et al., 2024), to produce GABA in a minimal M63 medium, which contains partially purified CG as its sole C source. As PG is known to be a good C source for bacterial growth, *H. elongata* GOP-Gad was precultured in the M63 minimal medium containing 3% w/v NaCl, 4% w/v PG as a C source, and 15 mM $(NH_4)_2SO_4$ as an N source. When the optical density at 600 nm (OD_{600}) of the culture reached the value of 1.00, the cells were collected and washed 3 times in the C, N-free M63 minimal medium containing 3% w/v NaCl before being used as a 5% v/v inoculum for the main cultures in the M63 minimal medium containing 3% w/v NaCl, 15 mM $(NH_4)_2SO_4$ or 30 mM $NaNO_3$, and 4% w/v glycerol derived from PG (100 w/v glycerol) or partially purified CG (64% or 66% w/v glycerol; Table 1). When the OD_{600} of the main cultures reached 0.90 to 1.20, major osmolytes in the *H. elongata* GOP-Gad cells were extracted and analyzed by HPLC. The expressions of transgenes were verified by the red fluorescent of the reporter mCherry protein (Figure 4(a)), and the profiles of major osmolytes accumulated in *H. elongata* GOP-Gad cells cultured in different media are shown in Figures 4(b) and 4(d). From these profiles, we found no significant difference in the amount of GABA accumulated in *H. elongata* GOP-Gad cells cultured in different media. However, when the total osmolytes inside the cells were compared (Figures 4(c) and 4(e)), it became more evident that *H. elongata* GOP-Gad cells cultured in the medium containing $(NH_4)_2SO_4$ as an N source accumulated more osmolytes than the cells cultured in the medium containing $NaNO_3$ as an N source. Interestingly, we also found that *H. elongata* GOP-Gad cannot survive in a medium containing 6% w/v NaCl when $NaNO_3$ was used as an N source (data not shown). Therefore, $(NH_4)_2SO_4$ was selected as an N source for our medium formula.

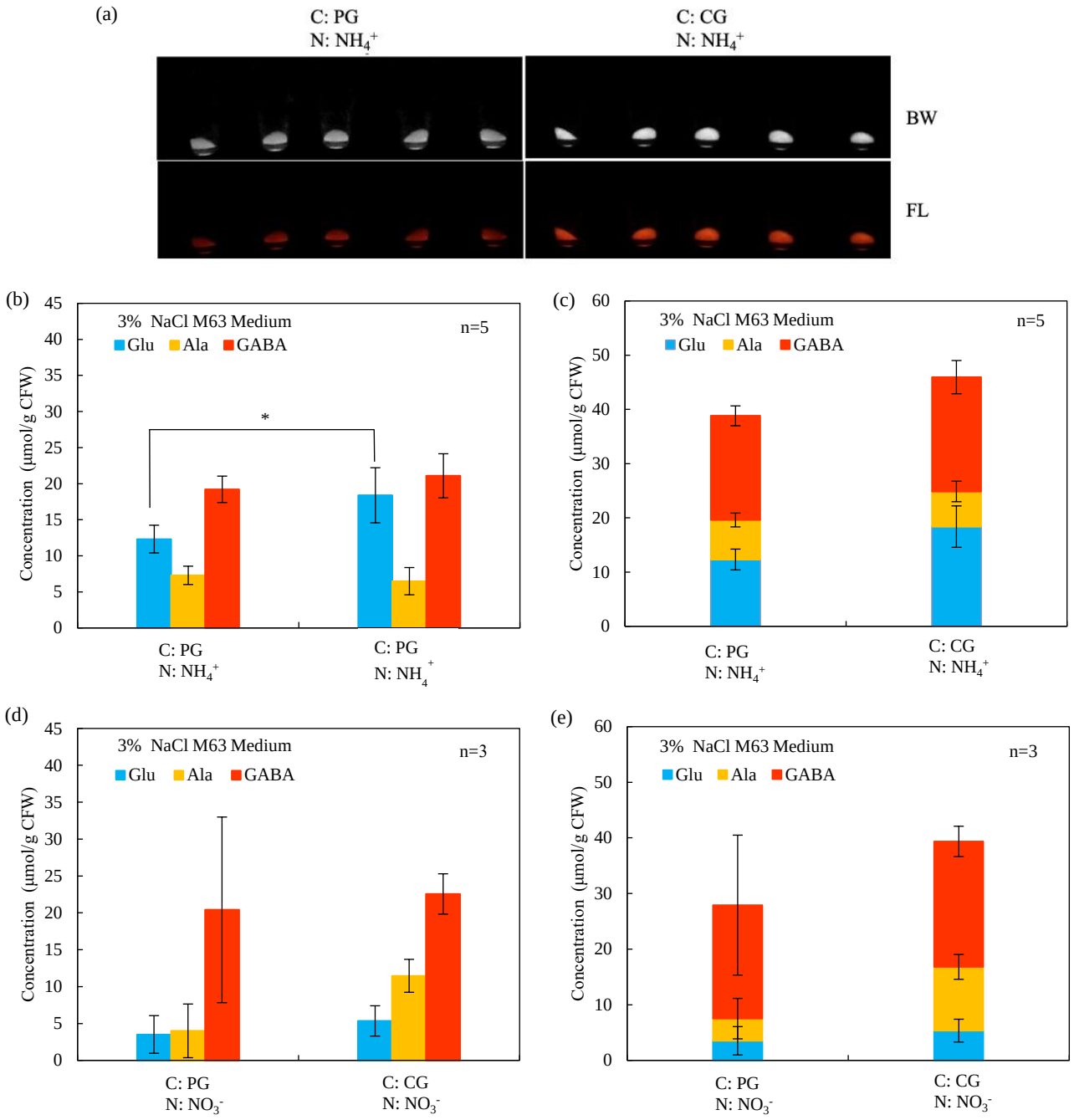

Figure 4. Profiles of major osmolytes in the cells of *H. elongata* GOP-Gad cultured in M63 media containing 3% w/v NaCl, 4% w/v glycerol derived from PG or CG as a C source, and 15 mM $(NH_4)_2SO_4$ or 30 mM $NaNO_3$ as an N source. Major osmolytes were extracted when OD_{600} of the main culture reached 0.9-1.2 and analyzed by HPLC. Data was normalized with internal standard norvaline. Values are mean±standard deviation (n=5). Glu, blue columns; Ala, yellow columns; GABA, red columns. *H. elongata* GOP-Gad was precultured in an M63 medium containing 3% w/v NaCl and 4% w/v PG. When the culture's OD_{600} reached 1.0, the cells were washed with a C, N-free M63 medium containing 3% w/v NaCl and used as 5% v/v inoculum for the main cultures. (a) Images of the pellets of *H. elongata* GOP-Gad cells. The cells were harvested when OD_{600} reached 1.0, and photos of the pellet were taken to visualize the red fluorescent of the reporter mCherry protein accumulated in *H. elongata* GOP-Gad as a marker to show correct expression from the salt-inducible *mCherry-HopGadBmut* operon. FL, fluorescent images of the cell pellets taken under blue light using an orange filter; BW, black and white images modified from FL images to show cell pellets. (b) Profiles of major osmolytes accumulated in *H. elongata* GOP-Gad cultured in M63 medium containing 4% w/v glycerol derived from PG or CG as a C source and 15 mM $(NH_4)_2SO_4$ as an N source. (c) Profiles of total osmolytes accumulated in *H. elongata* GOP-Gad cultured in M63 medium containing 4% w/v glycerol derived from PG or CG as a C source and 15 mM $(NH_4)_2SO_4$ as an N source. (d) Profiles of major osmolytes accumulated in *H. elongata* GOP-Gad cultured in M63 medium containing 4% w/v glycerol derived from PG or CG as a C source and 30 mM $NaNO_3$ as an N source. (e) Profiles of total osmolytes accumulated in *H. elongata* GOP-Gad cultured in M63 medium containing 4% w/v glycerol derived from PG or CG as a C source and 30 mM $NaNO_3$ as an N source.

3.3 Production of GABA by *H. elongata* GOP-Gad cultured in high-salinity medium containing CG and ammonia as the sole C and N sources

H. elongata GOP-Gad was precultured in LB medium containing 6% w/v NaCl to OD_{600} of more than 1.00, then the cells were washed 3 times in LB medium containing 6% w/v NaCl without C source and used as 10% v/v inoculum for the main culture in M63 minimal medium containing 7% w/v NaCl, 15 mM $(NH_4)_2SO_4$, and 4% w/v glycerol derived from PG or the partially purified CG. Major osmolytes in the cells were harvested when OD_{600} of the cultures reached 0.80 to 1.00. The expressions of transgenes were verified by the red fluorescent of the reporter mCherry protein (Figure 5a), and as shown in Figure 5b, the cells grown in high-salinity medium containing PG as C source accumulate GABA at the concentration of 26 µmol/g CFW, while those grown in high-salinity medium containing CG as C source accumulate GABA at the concentration of 28 µmol/g CFW. As expected, the concentration of GABA accumulated in *H. elongata* GOP-Gad cells cultured in higher salinity media (containing 7% w/v NaCl) is higher than those cultured in lower salinity (3% w/v NaCl) media (Figure 4). Based on data shown in Figures 4 and 5, *H. elongata* GOP-Gad can effectively use CG and ammonia as the sole C and N sources for GABA production.

Although an engineered *Escherichia coli* could produce 19.8 g of GABA per 1 L medium (Yu et al., 2019) and a recombinant *Corynebacterium glutamicum* expressing *E. coli*' GAD mutant could produce 38.6 g of GABA per 1 L medium (Choi et al., 2015), these strains cannot grow in a medium with high-salinity. Besides, these strains do not accumulate GABA in their cells. GABA produced by these strains is exported into the medium. Therefore, comparing our yield to those obtained by these strains is difficult. Further development of GABA-producing *H. elongata* strains, the medium formula, and the growth conditions are underway in our laboratory to improve GABA yield from *H. elongata* cell factory.

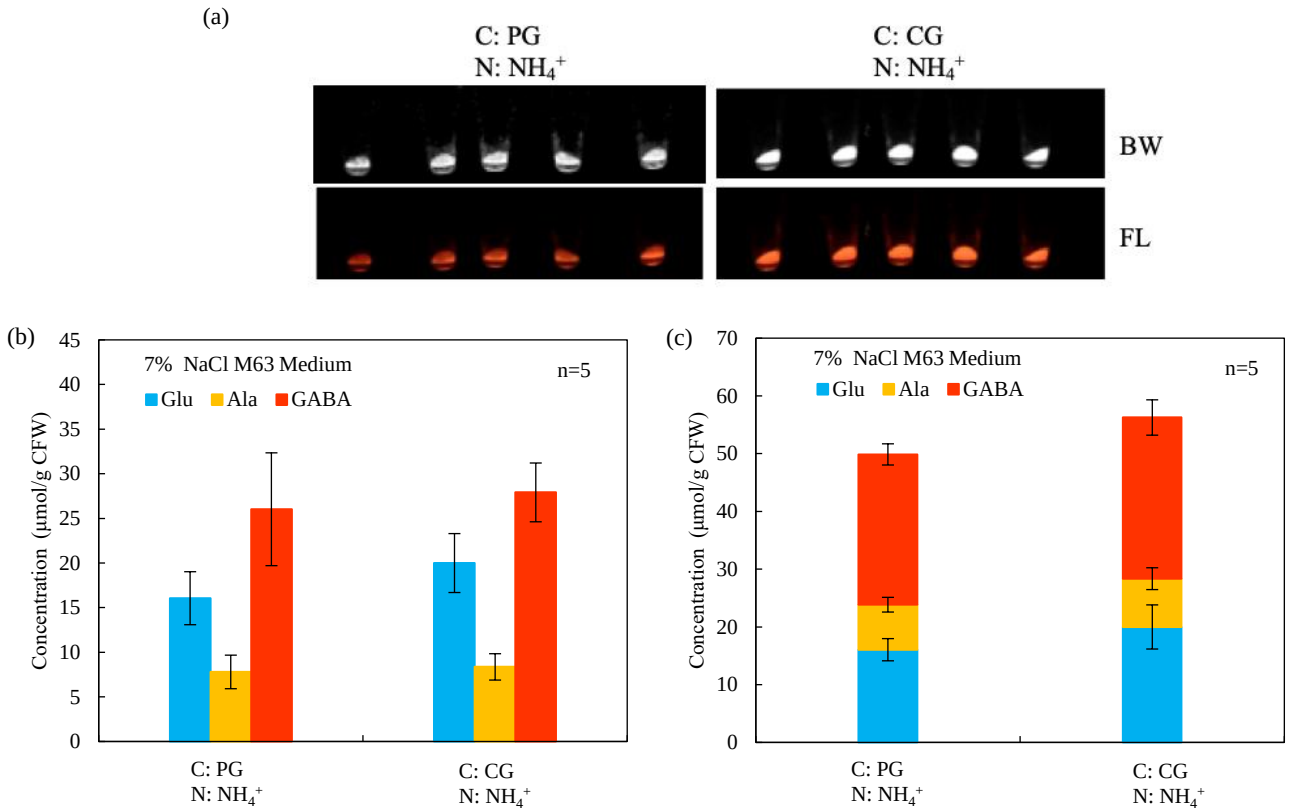

Figure 5. Profiles of major osmolytes in the cells of *H. elongata* GOP-Gad cultured in high-salinity M63 medium containing 6% w/v NaCl, 4% w/v glycerol from PG or CG as a C source, and 15 mM $(NH_4)_2SO_4$ as an N source. *H. elongata* GOP-Gad was precultured in high-salinity LB medium containing 6% w/v NaCl until the OD_{600} reached 1.0 when the cells were washed with C, N-free M63 medium containing 6% NaCl before being used as a 5% v/v inoculum for the main cultures in M63 medium containing 7% w/v NaCl, 4% w/v glycerol derived from PG or CG as a C source, and 15 mM $(NH_4)_2SO_4$ as an N source. Major osmolytes were extracted when OD_{600} of the main culture reached 0.8 to 1.0 and analyzed by HPLC. Data was normalized with internal standard norvaline. Values are mean±standard deviation (n=3 or 5 as indicated). Glu, blue columns; Ala, yellow columns; GABA, red columns. (a) Images of the pellets

of *H. elongata* GOP-Gad cells. The cells were harvested when OD_{600} reached 1.0, and photos were taken to visualize the red fluorescent of mCherry protein accumulated in *H. elongata* GOP-Gad as a marker to show correct expression from the salt-inducible *mCherry-HopGadBmut* operon. FL, fluorescent images of the cell pellets taken under blue light using an orange filter; BW, black and white images modified from FL images to show cell pellets. (b) Profiles of major osmolytes accumulated in *H. elongata* cells cultured in M63 medium containing 7% w/v NaCl, 4% w/v glycerol derived from PG or CG as a C source, and 15 mM $(NH_4)_2SO_4$ as an N source. (c) Profiles of total osmolytes accumulated in *H. elongata* cells cultured in M63 medium containing 7% w/v NaCl, 4% w/v glycerol derived from PG or CG as a C source, and 15 mM $(NH_4)_2SO_4$ as an N source.

4. CONCLUSION

BDF is currently in demand as a renewable energy source. For every gallon of BDF produced, approximately 0.3 kg of CG is generated (Ayoub and Abdullah, 2012; Bozbas, 2008). However, CG derived from the biodiesel production process contains high concentrations of salt and other impurities, which interfere with the downstream purification process, increasing the cost of CG application (Thompson and He, 2006). Here, we successfully used CG, partially purified by a simple and low-cost method, as a C source in the medium for culturing the GABA-producing *H. elongata* GOP-Gad. We confirmed that *H. elongata* GOP-Gad cultured in the high-salinity medium (7% w/v NaCl) containing CG as the sole C source could produce and accumulate GABA up to 28 µmol/g CFW. This concentration is comparable to that obtained from the *H. elongata* GOP-Gad cells cultured in the same medium supplemented with PG.

Industrial wastewater contains much higher N concentration than groundwater, and the most problematic N compounds in industrial wastewater are ammonia and nitrate (Islam and Suidan, 1998; Feleke and Sakakibara, 2002; Terada et al., 2003; Sumino et al., 2006). Our result shows that *H. elongata* GOP-Gad can use ammonia and nitrate as N sources for cell growth and GABA production. Therefore, N in industrial wastewater can be used directly in the production of GABA by *H. elongata* GOP-Gad without additional nitrification and denitrification processes, which would decrease the cost of wastewater treatment.

GABA is a natural feed supplement that benefits egg production (Zhang et al., 2012; Park and Kim, 2015). Therefore, GABA-producing *H. elongata* GOP-Gad cells have the potential to be further developed into an eco-friendly functional feed supplement for the poultry farming industry.

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