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# Continuous production of D-lactic acid from cellobiose in cell recycle fermentation using β-glucosidase-displaying *Escherichia coli*

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**The present study demonstrates continuous production of D-lactic acid from cellobiose in a cell recycle fermentation with a hollow fiber membrane using recombinant *Escherichia coli* constructed by deleting its pyruvate formate-lyase activating enzyme gene *pflA* and expressing a heterologous β-glucosidase on its cell surface. The β-glucosidase gene *bglC* from *Thermobifida fusca* YX was cloned into a cell surface display vector pGV3, resulting in pGV3-bglC. Recombinant *E. coli* JM109 harboring the pGV3-bglC showed β-glucosidase activity (18.9 ± 5.7 U/$OD_{600}$), indicating the cell surface functioning of mutant β-glucosidase. pH-stat cultivation using D-lactic acid producer *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC in minimum medium with 10 g/L cellobiose in a jar fermentor under anaerobic condition resulted in 5.2 ± 0.1 g/L of D-lactic acid was obtained after 84 h cultivation, indicating that the engineered *E. coli* produced D-lactic acid directly from cellobiose. For continuous D-lactic acid production, cell recycle fermentation was conducted under anaerobic condition and the culture was continuously ultrafiltrated with a hollow fiber cartridge. The permeate was drawn to the reservoir and a minimum medium containing 10 g/L cellobiose was fed to the fermentor at the same rate (dilution rate, 0.05 $h^{-1}$). Thus, this system maintained the D-lactic acid production (4.3–5.0 g/L), D-lactic acid production rate (0.22–0.25 g/L/h), and showed no residual cellobiose in the culture during 72 h operation. Interestingly, the D-lactic acid production rate in cell recycle fermentation was more than 3 times higher than that in the batch operation (0.06 ± 0.00 g/L/h).**



[**Key words:** Cellobiose; Cell recycle; *Escherichia coli*; Lactic acid; Surface display]

Lactic acid is one of the most versatile chemicals and shows two optically active isomers, D-lactic acid and L-lactic acid (1). Because the global market for L-lactic acid is currently greater than that for D-lactic acid, L-lactic acid has been used as a building block for manufacturing commodity products such as the bioplastic poly(L-lactic acid). Because poly(L-lactic acid) shows useful properties such as transparency, biocompatibility, and biodegradability, the polymer is believed to be a representative promising bioplastic for commercial use (2). Poly(L-lactic acid) is a crystallizable polymer and the melting temperature (Tm) of crystallized poly(L-lactic acid) is around 180°C. On the other hand, poly(L-lactic acid) and poly(D-lactic acid) can form a complex called stereocomplex poly(lactic acid) at a ratio of 1:1 (3,4). The crystallized stereocomplex has superior heat resistance (Tm = approximately 230°C) compared to poly(L-lactic acid). To promote the dissemination of poly(lactic acid) stereocomplex as a commodity bioplastic, enhancing the D-lactic acid production on a worldwide basis is a requirement.

To date, lactic acid bacteria from the genus *Lactobacillus* and molds from the genus *Rhizopus* have been generally employed in manufacturing lactic acid with high optical purity (5). Among these, homofermentative D-lactic acid bacteria such as *Lactobacillus delbrueckii* subsp. *bulgaricus* have been industrially used as D-lactic acid producers. Interestingly, deletion of pyruvate formate-lyase activating enzyme gene *pflA* in *Escherichia coli* is reported to result in D-lactic acid production from glucose (6). Most recently, it has been demonstrated to synthesize *in vivo* D-lactic-acid-containing polymers in *pflA*-deficient *E. coli* (7). The engineered *E. coli* produces no L-lactic acid because *E. coli* possesses D-lactate-specific dehydrogenase (8). Compared to lactic acid bacteria, *E. coli* seems to be a more suitable D-lactic acid producer because of its high growth rate; furthermore, gene manipulation in *E. coli* is easier than that in *L. delbrueckii* subsp. *bulgaricus* (9).

Since cellulose is the most abundant carbohydrate, use of cellulose as a substrate for fermentation has been demonstrated. Cellulose can be enzymatically degraded into glucose with a series of hydrolytic enzymes such as endoglucanase, cellobiohydrolase, and β-glucosidase (10). Cellobiose, a disaccharide consisting of two glucose units in β-1,4 linkage, is obtained during the hydrolysis of cellulose using endoglucanase and cellobiohydrolase. Cellobiose can be further degraded by β-glucosidase, to form glucose. During the hydrolytic process, cellobiose inhibits the enzymatic activities of endoglucanase and cellobiohydrolase (11). To reduce the usage of cellulose-degrading enzymes, it will be beneficial to use β-glucosidase-expressing *E. coli* for the hydrolytic process. Recently, cell surface display presents a unique expression system involving

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decoration of proteins on the cell surface (12). For instance, displaying α-amylase (EC 3.2.1.1) (13–17) and β-glucosidase (EC 3.2.1.21) on the cell surface (11,18–20) enables hydrolysis of starch and cellobiose and utilization of the resulting sugar to produce the desired end products. This technique can be applied for the direct production of D-lactic acid from cellobiose using β-glucosidase-expressing *E. coli*. As a similar research, the direct production of D-lactic acid from cellobiose using β-glucosidase-expressing *Schizosaccharomyces pombe* has been reported (21). D-Lactic acid was produced in metabolically engineered *S. pombe* by expressing a heterologous β-glucosidase from *Aspergillus aculaetus* on its cell surface, resulting in D-lactic acid production at 24.4 g/L from 30 g/L of cellobiose directly. We propose to develop a process for the direct production of D-lactic acid from cellobiose wherein recombinant *E. coli* with cell surface display of β-glucosidase facilitates cellobiose hydrolysis near the cell surface and utilize the resulting glucose to produce D-lactic acid.

Cell recycle fermentation has been demonstrated to improve lactic acid productivity (22–25). Cell recycle fermentation is one of the methods to achieve high cell density, resulting in higher productivity. Typically, an ultrafiltration module is used to separate cells from the culture. Since the permeate containing products is continuously obtained with no cells, separation functions as part of the downstream process. Additionally, continuous feeding of fresh medium to the fermentor at the same rate as the permeate allows continuous production. Although cell recycle fermentation is preferable to continuous D-lactic acid production, there is no report on continuous D-lactic acid production in cell recycle fermentation using recombinants.

Here we report the continuous production of D-lactic acid from cellobiose as the sole carbon source in cell recycle fermentation using *E. coli* expressing heterologous β-glucosidase. In this study, β-glucosidase gene *bglC* from a cellulose-degrading actinomycete *Thermobifida fusca* YX (26,27) was selected and expressed on the surface of *pflA*-deficient *E. coli* cells. Using the mutant, we demonstrated cell recycle fermentation with a hollow fiber membrane to produce D-lactic acid from cellobiose continuously.

## MATERIALS AND METHODS

**Bacterial strains and media** All *E. coli* strains were grown in LB medium or glucose-free M9 (12 g/L $Na_2HPO_4$, 6 g/L $KH_2PO_4$, 1 g/L NaCl, 2 g/L $NH_4Cl$, 0.24 g/L $MgSO_4$, 3.4 mg/L thiamine, 0.01 g/L $CaCl_2$) minimal medium containing 10 g/L glucose or cellobiose. When required, 100 mg/L spectinomycin was added to the medium for *E. coli* transformants. Recombinant *E. coli* cultivated in LB medium at 37°C for 18 h was inoculated in fresh LB medium, and cultured to an optical density at 600 nm ($OD_{600}$) of 0.1. The cultures were subsequently grown at 37°C until they reached $OD_{600}$ of 0.4–0.6; isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 1 mM for *bglC* expression.

**Plasmid construction** The expression vector pGV3 (17) was chosen for the cell surface display of β-glucosidase in this study. Based on the *bglC* sequence deposited as Tfu_0937 from *T. fusca* YX (GenBank database accession no. CP000088), the *bglC* gene codon-optimized for *E. coli* was synthesized by GENEWIZ, Inc. (Suzhou, China). The *bglC* gene was amplified by polymerase chain reaction (PCR) using KOD plus DNA polymerase (Toyobo, Osaka, Japan), the synthesized *bglC* as a template, and a primer set of bglC-F1 (5′-tgcgcatgcagatctctatgaccagccaaagtacc-3′) and bglC-R1 (5′-caagcttggctgcagttattcctggccaaaaatgc-3′) under the following conditions: denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 s, annealing at 50°C for 15 s, and polymerization at 68°C for 4 min. Under the same conditions, linearized pGV3 plasmid was also amplified by inverse PCR with pGV3 as a template and a primer set of pGV3-F1 (5′-ctgcagccaagcttggctgtttttggcgg-3′) and pGV3-R1 (5′-agatctgcatgcgcagggcacgctcgttag-3′). The amplified *bglC* gene was directionally cloned into the linearized pGV3 using GeneArt Seamless Cloning and Assembly kit (Invitrogen, Carlsbad, CA, USA), resulting in pGV3-bglC. DNA isolation and manipulation were performed according to the standard protocol (28). Transformation of *E. coli* was performed by electroporation with a 2.5 kV, 200 Ω, 25 μF electric pulse in a 0.2 cm cuvette using the ECM830 system (Harvard Apparatus, Holliston, MA, USA).

**Engineering of *E. coli*** The pyruvate formate-lyase gene (*pflA*) was deleted based on λ Red-mediated bacterial chromosome modification (29). Initially, *E. coli* JW0885, which was obtained from the National BioResource Project (National Institute of Genetics, Japan) and corresponds to *E. coli* BW25113 (*pflA*::$Km^r$), was transformed with pCP20, and the transformant was obtained by cultivation at 30°C. The transformant was incubated at 43°C for 18 h to express FLP recombinase for elimination of the kanamycin resistance marker ($Km^r$) from the genome. This procedure resulted in the BW25113 (*ΔpflA*) mutant. *E. coli* JM109 and *E. coli* BW25113 (*ΔpflA*) were transformed with pGV3 or pGV3-bglC, and the properties of the transformants were subsequently investigated.

**Fermentation experiments** After *E. coli* BW25113 (*ΔpflA*, pGV3-bglC) was first precultured in 2 mL LB medium in a test tube for 18 h at 37°C and 150 rpm, the cells were inoculated into 100 mL LB medium in a 500-mL Erlenmeyer flask and incubated at 37°C and 150 rpm. After the culture reached $OD_{600}$ of 0.4–0.6, IPTG was added at a final concentration of 1 mM for *bglC* expression and the culture was incubated for 12 h. The cells were then harvested and washed twice with saline solution. The cells were resuspended in M9 medium supplemented with 10 g/L glucose or cellobiose, 1 mM IPTG and 100 mg/L spectinomycin to an $OD_{600}$ of 0.1 before transferring into a jar fermentor. All batch cultivations were performed in a 2-L jar fermentor M–1000B (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) with a working volume of 1 L at an agitation speed of 100 rpm, at 37°C, $N_2$ flow rate at 0 and 0.2 L/min for microaerobic and anaerobic conditions, respectively, and pH maintained at 7.0 by automatic addition of 4M NaOH.

After D-lactic acid production in the batch cultivation ceased under anaerobic conditions with 10 g/L cellobiose, cell recycle fermentation was started under anaerobic conditions. Fig. 1 shows a schematic diagram of the cell recycle fermentation system used in this study. Cell recycle was continuously performed by ultrafiltration of the culture with a cross-flow type hollow fiber cartridge UFP-500-C-4A (GE healthcare Bio-Sciences, Uppsala, Sweden). The cartridge contains polysulfone hollow fibers (ID, 0.5 mm) with molecular weight cut-off of 50 kDa and a total membrane area of 0.065 $m^2$. The culture was drawn and then passed through the hollow fiber cartridge at 9 L/h with a peristaltic pump TP-205A (AS ONE, Osaka, Japan). The permeate was drawn from the hollow fiber cartridge to the reservoir and a minimum medium containing 2, 5, or 10 g/L cellobiose, 1 mM IPTG and 100 mg/L spectinomycin was fed to the fermentor at the same rate of 50 mL/h (dilution rate, 0.05 $h^{-1}$) with two peristaltic pumps (MR-2050, Tokyo Rikakikai Co., Tokyo, Japan).

**Analytical methods** D-Lactic acid produced in the culture supernatant was quantified using a Prominence HPLC system (Shimadzu, Kyoto, Japan) equipped with a chiral column, MCI GEL CRS10W (Mitsubishi Chemical, Tokyo, Japan). D-Lactic acid was eluted using 1 mM $CuSO_4$ solution with a flow rate of 0.5 mL/min. Absorbance of the eluate was monitored at 254 nm. Citric acid, pyruvic acid, and acetic acid in the culture supernatant were analyzed with an ion exclusion column SCR-102H (Shimazu) and 0.1% perchloric acid solution as the mobile phase with a flow rate of 0.9 mL/min. Absorbance of the eluate was monitored at 210 nm. Commercial D- and L-lactic acid purchased from Purac Japan (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively, were used as controls for D- and L-lactic acid quantification. Glucose concentration in the culture supernatant was measured using commercial kits: Glucose CII-Test Wako (Wako Pure Chemical Industries). Sugar concentration in the culture supernatant was determined by the phenol-sulfuric acid method (30). Glucose-free M9 medium and appropriate concentrations of cellobiose were used as controls for residual sugar concentrations.

After induction of *bglC* expression, *E. coli* cells were harvested followed by washing with 50 mM sodium phosphate buffer (pH 7.0). β-Glucosidase activity on the cell surface of *E. coli* strains was determined at 37°C for 30 min by using 10 μL of cell suspension and 500 μL of 10 mM *p*-nitrophenyl-β-D-glucopyranoside in 50 mM sodium phosphate buffer (pH 7.0). After the reaction was stopped by adding 1 mL of 2 M $Na_2CO_3$, the absorbance of the mixture was analyzed at 400 nm. One unit (U) of β-glucosidase activity was defined as the release of 1 μmol of *p*-nitrophenol

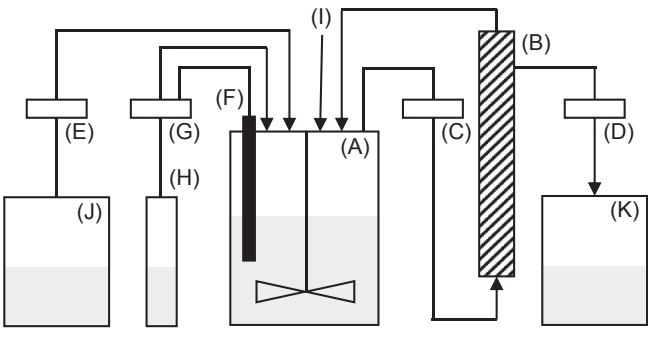


FIG. 1. Schematic diagram of the cell recycle fermentation system: A, fermentor; B, hollow fiber cartridge; C, recirculation pump; D, permeate control pump; E, medium feed control pump; F, pH electrode and controller; G, NaOH feed control pump; H, 4 M NaOH; I, $N_2$ flow; J, fresh medium reservoir; K, permeate reservoir.

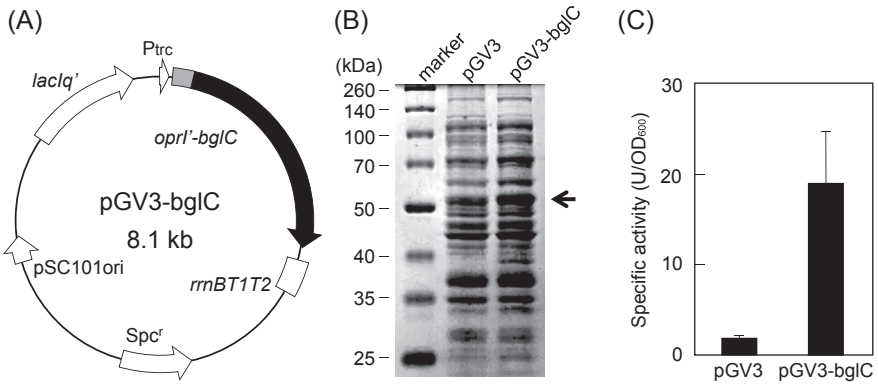


FIG. 2. Expression of β-glucosidase in *E. coli*. (A) Construction of the recombinant plasmid pGV3-bglC. The position and direction of transcription of recombinant genes are indicated by arrows. Spc$^r$, spectinomycin resistance gene. (B) SDS-PAGE analysis of BglC expression in *E. coli* JM109. Arrow indicates the band corresponding to the fusion proteins of OprI′-BglC (53.4 kDa). Expression of BglC in *E. coli* was performed after cultivation in 2 mL of LB medium at 37°C for 18 h following IPTG induction. *E. coli* JM109 harboring pGV3 was used as a control. (C) Specific BglC activities in *E. coli* JM109 harboring pGV3 or pGV3-bglC were measured after cultivation in 2 mL of LB medium at 37°C for 18 h as described in the materials and methods section. This assay was performed at 37°C in duplicate, and the mean value is presented with error bars.

($\varepsilon_{400} = 18.5\ \mathrm{mM}^{-1}$) within 1 min. The activity was normalized by dividing each value by the optical density ($OD_{600}$) of the cell suspension. β-glucosidase protein expression was confirmed by SDS-PAGE (12.5% polyacrylamide gel) using 10 μL of cells suspension. Before application to the gel, the cell concentration was adjusted to an $OD_{600}$ of 10 with Laemmli buffer (31) and the suspension was then denatured by heating at 100°C.

Dry cell weight (DCW) was calculated from optical density ($OD_{600}$) with a linear correlation factor (1 $OD_{600}$ = 0.36 g-DCW/L). Specific growth rate ($\mu$) was calculated as the slope of the regression line, from a plot between ln ($X/X_0$) and time ($t$) during the exponential growth period, where $X$ (g-DCW/L) and $X_0$ (g-DCW/L) are the cell concentrations at $t$ (h) and at the beginning of the exponential phase, respectively.

## RESULTS AND DISCUSSION

**β-Glucosidase expression using *E. coli*** In this study, we used the cell surface display vector pGV3 for *bglC* expression. This vector contains a truncated *Pseudomonas aeruginosa* lipoprotein gene *oprI′* as an anchor peptide gene for cell surface display. To express the fusion protein OprI′-BglC on the cell surface, we cloned *bglC* downstream of *oprI′* in-frame, resulting in pGV3-bglC (Fig. 2A). SDS-PAGE analysis showed the presence of OprI′-BglC fusion proteins expressed in *E. coli* JM109 harboring pGV3-bglC (Fig. 2B). To characterize the activity and localization of BglC, we performed an enzymatic assay using whole cells of *E. coli* JM109 harboring pGV3-bglC after induction using *p*-nitrophenyl-β-D-glucopyranoside as a substrate. As a result, *E. coli* JM109 harboring pGV3-bglC showed β-glucosidase activity (18.9 ± 5.7 U/$OD_{600}$), whereas the control strain, JM109 harboring pGV3, showed a negligible value (1.8 ± 0.3 U/$OD_{600}$) (Fig. 2C). This result suggests that OprI′ functioned as the anchor protein and that OprI′-BglC fusion protein was displayed on the outer cell membrane. *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC showed β-glucosidase activity (13.5 ± 0.2 U/$OD_{600}$) at a comparable level

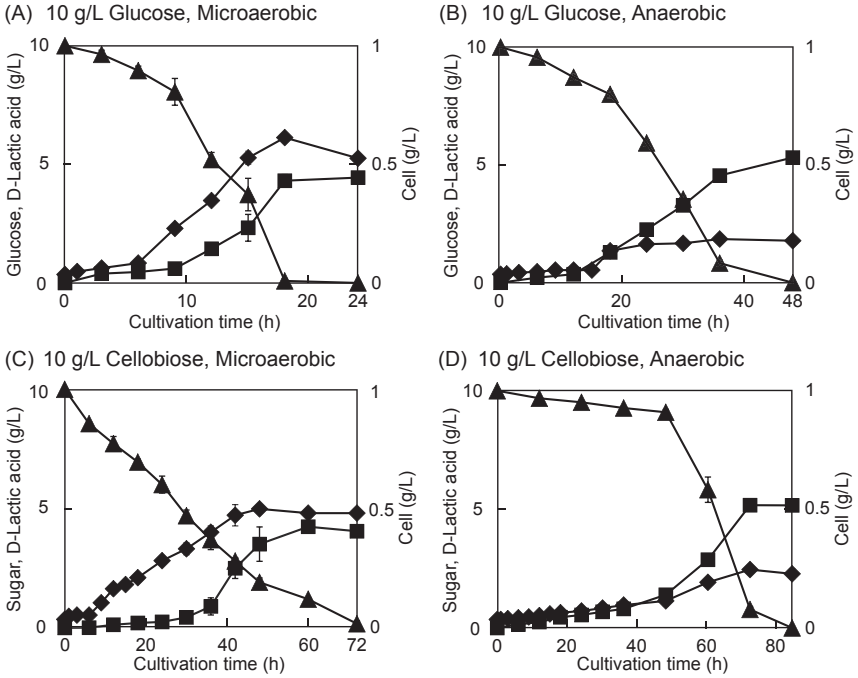


FIG. 3. Production of D-lactic acid in engineered *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC in batch fermentation. M9 minimum medium supplemented with 10 g/L glucose [(A) microaerobic; (B) anaerobic] or 10 g/L cellobiose [(C) microaerobic; (D) anaerobic] was used. Diamonds, cell growth; squares, D-lactic acid concentration; triangles, residual sugar. This cultivation was performed in duplicate, and the average is represented with error bars.

**TABLE 1.** Cell yield ($X$), specific growth rate ($\mu$), D-lactic acid (Dla) concentration ($C$), productivity (g-Dla/L/h) ($P$), yield of Dla to dry cell weight (DCW) ($Y_{P/X}$), and specific productivity ($SP_{P/X}$) from *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC in batch fermentation under different conditions.

| Conditions | | Cultivation time (h) | $X$ (g-DCW/L) | $\mu$ (1/h) | $C$ (g-Dla/L) | $P$ (g-Dla/L/h) | $Y_{P/X}$ (g-Dla/g-DCW) | $SP_{P/X}$ (g-Dla/g-DCW/h) |
|---|---|---|---|---|---|---|---|---|
| 10 g/L glucose | Microaerobic | 18 | 0.6 ± 0.0 | 0.17 ± 0.01 | 4.3 ± 0.3 | 0.24 ± 0.02 | 7.1 ± 0.4 | 0.24 ± 0.01 |
| | Anaerobic | 48 | 0.2 ± 0.0 | 0.05 ± 0.00 | 5.3 ± 0.1 | 0.11 ± 0.00 | 29.6 ± 0.5 | 0.11 ± 0.00 |
| 10 g/L cellobiose | Microaerobic | 72 | 0.5 ± 0.0 | 0.03 ± 0.00 | 4.1 ± 0.0 | 0.06 ± 0.00 | 8.4 ± 0.4 | 0.06 ± 0.00 |
| | Anaerobic | 84 | 0.2 ± 0.0 | 0.03 ± 0.00 | 5.2 ± 0.1 | 0.06 ± 0.00 | 22.5 ± 2.2 | 0.06 ± 0.00 |

Each experiment was repeated 2 times independently.

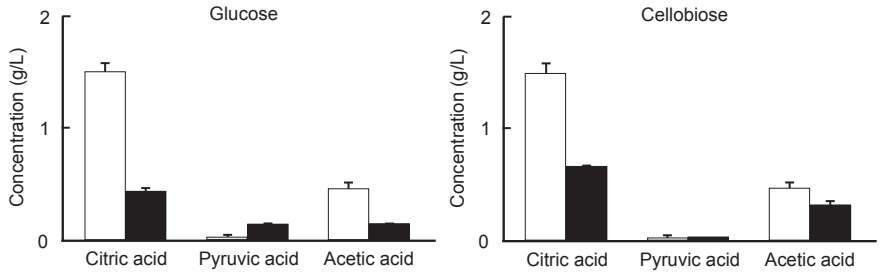


FIG. 4. Citric acid, pyruvic acid, and acetic acid production in engineered *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC in batch fermentation. M9 minimum medium supplemented with 10 g/L glucose or 10 g/L glucose was used (open bars, microaerobic; solid bars, anaerobic). This cultivation was performed in duplicate, and the average is represented with error bars.

whereas BW25113 (*ΔpflA*) harboring pGV3 showed a negligible value (0.0 ± 0.0 U/$OD_{600}$) (data not shown). Additionally, *E. coli* BW25113 (*ΔpflA*) harboring pGV3 showed no growth but *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC grew in a minimum medium supplemented with cellobiose as the sole carbon source (data not shown). This indicated that OprI′-BglC fusion protein worked as a cellobiose-degrading enzyme.

**D-Lactic acid production in a batch fermentation** PH-stat cultivation was conducted using the D-lactic acid producer *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC in a minimum medium with 10 g/L glucose in a jar fermentor under microaerobic and anaerobic conditions. As a result, 4.3 ± 0.3 g/L and 5.3 ± 0.1 g/L of D-lactic acid were obtained from 10 g/L glucose after 18 h and 48 h cultivation under microaerobic and anaerobic conditions, respectively (Fig. 3A and B). This result demonstrated that anaerobic conditions improved D-lactic acid production though the production rate and specific productivity were decreased more than two fold (0.11 ± 0.00 g/L/h) compared to that under microaerobic conditions (0.24 ± 0.00 g/L/h) (Table 1). In all fermentations, no L-lactic acid was detected in the cultures. Additionally, anaerobic conditions resulted in a dramatic decrease of by-products such as citric acid and acetic acid (Fig. 4). This indicated that anaerobic conditions improved the chemical purity of D-lactic acid. Furthermore, yield of D-lactic acid to DCW under anaerobic conditions was increased by more than four times (29.6 ± 0.5 g/g) compared to that under microaerobic conditions (7.1 ± 0.4 g/g). From these data, we concluded that anaerobic conditions are suitable for D-lactic acid production in *E. coli*. This finding was in agreement with that of a related study (6).

We demonstrated batch fermentation with 10 g/L cellobiose instead of glucose in the same manner. As a result, 4.1 ± 0.0 g/L and 5.2 ± 0.1 g/L of D-lactic acid were obtained from 10 g/L cellobiose after 72 h and 84 h cultivation under microaerobic and anaerobic conditions, respectively (Fig. 3C and D). Production of by-products including citric acid and acetic acid was similar to that when glucose was used (Fig. 4). The yield of D-lactic acid to DCW was 8.4 ± 0.4 g/g and 22.5 ± 2.2 g/g under microaerobic and anaerobic conditions, respectively (Table 1). These results indicate that the engineered *E. coli* directly produces D-lactic acid and that the yield of D-lactic acid to DCW was similar to that when glucose was used. D-Lactic acid productivity was 0.06 ± 0.00 g/L/h under both microaerobic and anaerobic conditions. The productivity was about twice decreased compared to that under anaerobic conditions with 10 g/L glucose. This suggested that cellobiose degradation is the rate-determining step in the engineered strain. It has been reported that BglC of *T. fusca* has a pH optimum of 7.0 and a temperature optimum of 50°C (26). To improve the cellobiose degradation in the engineered strain, the cultivation temperature would be increased to more than 37°C. In addition, instead of *trc* promoter on pGV3, other stronger promoters such as T7 would be preferable to enhance the expression level of BglC.

**Continuous D-lactic acid production in cell recycle fermentation** In the batch fermentation, D-lactic acid productivity was low due to a low cell concentration (0.2 g-DCW/L) under anaerobic condition. To improve the productivity, we demonstrated the continuous cell recycle fermentation at high cell density. To achieve continuous D-lactic acid production, we demonstrated cell recycle fermentation using the system shown in Fig. 1. To determine the optimum concentration of cellobiose supplied to this system during cell recycle fermentation, we preliminarily demonstrated

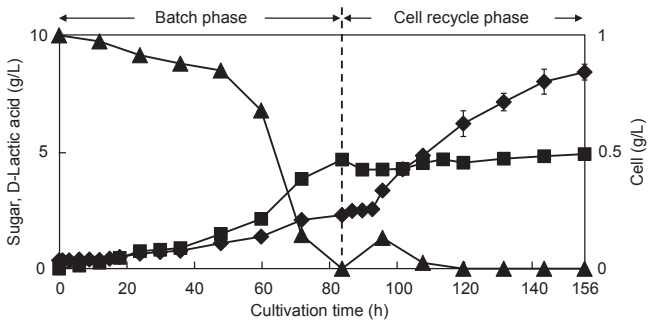


FIG. 5. Production of D-lactic acid in engineered *E. coli* BW25113 (*ΔpflA*) harboring pGV3-bglC in batch fermentation followed by cell recycle fermentation. The batch and cell recycle fermentations were performed under anaerobic conditions. The permeate was drawn to the reservoir and M9 minimum medium containing 10 g/L cellobiose was fed to the fermentor at the same rate (dilution rate, 0.05 $h^{-1}$). Cell recycle fermentation was demonstrated for 72 h after the batch fermentation was finished at 84 h. Diamonds, cell growth; squares, D-lactic acid concentration; triangles, residual sugar. This cultivation was performed in duplicate, and the average is represented with error bars.

**TABLE 2.** Comparison of D-lactic acid (Dla) production using recombinants.

| Organism | Fermentation mode | pH | Carbon source | Conditions | $C^a$ (g-Dla/L) | $P^a$ (g-Dla/L/h) | Reference |
|---|---|---|---|---|---|---|---|
| *Pediococcus acidilactici* | SSCF[b] | 5.5 | 25% corn stover | pH-stat, 42°C | 97.3 | 0.95 | 33 |
| *Lactobacillus plantarum* | SSCF | 5.5–6.0 | 20 g/L brown rice | 37°C | 117.1 | 0.81 | 34 |
| *Sporolactobacillus inulinus* | Batch | 5.0 | 100 g/L glucose | pH-stat | 93.4 | 1.37 | 35 |
| *Bacillus coagulans* | Batch | 5.0 | 110 g/L glucose | $Ca(OH)_2$ addition, 50°C | 99.1 | 1.38 | 36 |
| *Bacillus coagulans* | Fed-batch | 5.5–6.0 | 150 g/L glucose | $CaCO_3$ addition, 50°C | 145.0 | 1.50 | 37 |
| *Schizosaccharomyces pombe* | Batch | – | 30 g/L cellobiose | 30°C | 24.4 | 0.45 | 21 |
| *Saccharomyces cerevisiae* | Batch | – | 100 g/L glucose | 30°C | 60.3 | 2.80 | 38 |
| *Saccharomyces cerevisiae* | Fed-batch | – | 190 g/L glucose | $CaCO_3$ addition, 30°C | 112.0 | 2.20 | 39 |
| *Escherichia coli* | Batch | 7.0 | 100 g/L sucrose | pH-stat, 35°C | 96.5 | 0.80 | 40 |
| *Escherichia coli* | Batch | 7.0 | 10 g/L cellobiose | pH-stat, 37°C | 5.2 | 0.06 | This study |
| *Escherichia coli* | Continuous, cell-recycle via membrane | 7.0 | 10 g/L cellobiose | pH-stat, 37°C | 4.3–5.0 | 0.22–0.25 | This study |

[a] *C*, Dla concentration; *P*, Dla productivity (g-Dla/L/h).
[b] SSCF, simultaneous saccharification and co-fermentation.

cell recycle fermentation with a minimum medium containing 2, 5, and 10 g/L cellobiose at the same dilution rate (0.05 $h^{-1}$). As a result, D-lactic acid production rates were 0.04, 0.12, and 0.25 g/L/h when 2, 5, and 10 g/L cellobiose was used, respectively (data not shown). When 2 and 5 g/L cellobiose was used, the concentration of D-lactic acid in the fermentor gradually decreased due to the low amount of cellobiose supplied to the fermentor. On the other hand, use of 10 g/L cellobiose resulted in maintaining the concentration of D-lactic acid in the fermentor and no residual cellobiose during the operation. Therefore, we determined that the optimum cellobiose concentration for this system is 10 g/L. Cell recycle fermentation was demonstrated for 72 h at 0.05 $h^{-1}$ dilution rate after the batch fermentation was completed at 84 h (Fig. 5). During cell recycle fermentation, this system maintained the D-lactic acid concentration (4.3–5.0 g/L) and D-lactic acid productivity (0.22–0.25 g/L/h), without any residual cellobiose in the culture. Interestingly, cell recycle fermentation showed a more than 3-times higher D-lactic acid productivity compared to the batch operation (0.06 ± 0.00 g/L/h). Cell recycle fermentation significantly improved the D-lactic acid productivity because the system provided steady-state and continuous D-lactic acid production. After cell recycle fermentation for 72 h, 3.6 L of culture supernatant was obtained via the ultrafiltration module. The supernatant contained 4.6 ± 0.2 g/L of D-lactic acid, 0.5 ± 0.0 g/L of citric acid, 0.3 ± 0.0 g/L of acetic acid, and 0.2 ± 0.2 g/L of residual cellobiose without any cells (data not shown). This showed that the cell recycle system is also useful for the downstream process. The yield of D-lactic acid on cellobiose was calculated as 46.0 ± 2.0%. This value is comparable to that of the batch operation (52.0 ± 1.0%) as shown in Table 1, indicating that the cell recycle system does not affect the yield based on carbon source but enhances the productivity. The operational stability was maintained for 156 h at least (Fig. 5). This shows our system exhibited good operational stability in spite of use of recombinant harboring plasmid. It has been reported that continuous production of α-amylase in *Bacillus stearothermophilus* recombinant in non-selective medium was maintained for more than 300 generations without significant plasmid loss (32). In this study, the selective medium supplemented with spectinomycin was used for plasmid stability, but non-selective medium would be applicable.

D-Lactic acid production using recombinants have been reported so far. Some of which are summarized in Table 2. With several fermentation modes such as batch, fed-batch and simultaneous saccharification and co-fermentation, D-lactic acid production has been demonstrated using recombinants of lactic acid bacteria, yeasts, *Bacillus* and *E. coli* strains (21,33–40). The D-lactic acid concentration and productivity obtained in this study are low compared to those obtained in these related researches. This may be due to a low concentration and low assimilability of carbon source, cellobiose. Except for this study, there is no report on continuous fermentation for D-lactic acid production using recombinants. Therefore, this study provides new insight into performance, especially operational stability and cell recycle, in continuous fermentation for D-lactic acid production using recombinants.

In conclusion, it was clarified that cellobiose can be used as a carbon source for D-lactic acid production in β-glucosidase displaying *E. coli* and that D-lactic acid productivity is remarkably improved by cell recycle fermentation compared to that in batch fermentation.

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