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Enhanced production of 2′-fucosyllactose in engineered *Escherichia coli* BL21star(DE3) by modulation of lactose metabolism and fucosyltransferase

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ABSTRACT

2′-Fucosyllactose (2-FL) is one of most abundant functional oligosaccharides in human milk, which is involved in many biological functions for human health. To date, most microbial systems for 2-FL production have been limited to use *Escherichia coli* JM strains since they cannot metabolize lactose. In this study, *E. coli* BL21star(DE3) was engineered through deletion of the whole endogenous lactose operon and introduction of the modified lactose operon containing *lacZ*△*M15* from *E. coli* K-12. Expression of genes for guanosine 5′-diphosphate (GDP)-L-fucose biosynthetic enzymes and heterologous α-1,2-fucosyltransferase (FucT2) from *Helicobacter pylori* allowed the engineered *E. coli* BL21star(DE3) to produce 2-FL with 3-times enhanced yield than the non-engineered *E. coli* BL21star(DE3). In addition, the titer and yield of 2-FL were further improved by adding the three aspartate molecules at the N-terminal of FucT2. Overall, 6.4 g/L 2-FL with the yield of 0.225 g 2-FL/g lactose was obtained in fed-batch fermentation of the engineered *E. coli* BL21star(DE3) expressing GDP-L-fucose biosynthetic enzymes and three aspartate tagged FucT2.

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1. Introduction

Compared to other mammalian milks, human milk has very unique oligosaccharides which provide various biological activities on human health such as prebiotic effects, prevention of pathogenic infection and modulation of the immune system (Bode, 2012). In particular, 2′-fucosyllactose (2-FL) has been reported to be one of the main human milk oligosaccharides involved in biological functions as mentioned above, which led this functional sugar to receive great attention in terms of nutraceutical and pharmaceutical purpose (Castanys-Muñoz et al., 2013).

Biosynthesis of 2-FL in microbial systems has been proven to be superior to other systems such as chemical or enzymatic synthesis because it can allow large scale production of 2-FL with simple processes (Han et al., 2012). In the previous research, *E. coli* JM107(DE3) was engineered to produce 2-FL through the overexpression of RcsA [a positive regulator for the guanosine 5′-diphosphate (GDP)-L-fucose biosynthesis] and inactivation of WcaJ involved in colanic acid synthesis (Drouillard et al., 2006). In the other research, engineered *E. coli* JM109 or JM109(DE3) strains for whole cell production of 2-FL were developed by direct overexpression of the genes involved in *de novo* GDP-L-fucose biosynthesis and α-1,2-fucosyltransferase from *Helicobacter pylori* (Baumgärtner et al., 2013; Lee et al., 2012).

Microbial production of 2-FL is affected by a number of factors. One of them is to express heterologous α-1,2-fucosyltransferase which catalyzes the transfer of L-fucose molecule from GDP-L-fucose to an acceptor such as lactose. However, level of soluble α-1,2-fucosyltransferase (FucT2) from *H. pylori* expressed in recombinant *E. coli* is very low, which has disturbed easy and direct quantification of recombinant FucT2 (Lee et al., 2012; Wang et al., 1999). Fusion partners such as glutathione-*S*-transferase (GST), His_6-tagged propeptide sequence and thioredoxine peptide (Trx) were attached at the N-terminal of heterologous fucosyltransferase for enhancement of soluble expression in *E. coli* (Albermann et al., 2001; Dumon et al., 2004; Engels and Elling, 2014). Even though some fusion partners provide the solubility enhancement of FucT2 in *E. coli*, the fusion partners often have an inherency to disturb both the structure and function of the target proteins due to their big size (Kato et al., 2007). Moreover, some commonly used fusion

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Table 1
List of strains and plasmids used in this study.

Strains/Plasmids	Relevant description	Reference
E. coli TOP10	F⁻, *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) *ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)* 7697 *galU galK rpsL* (Str^R) *endA1 nupG*	Invitrogen (Carlsbad, CA, USA)
E. coli BL21star(DE3)	F⁻, *ompT*, *hsdSB*($r_B^-m_B^-$), *gal*, *dcm rne131* (DE3)	
△L	BL21star(DE3) △*lacZYA*	This study
△L M15	BL21star(DE3) △*lacZYA Tn7::lacZ△M15*	This study
pETDuet-1	Two T7 promoters, pBR322 replicon, Amp^R	Novagen
pCOLADuet-1	Two T7 promoters, ColA replicon, Kan^R	Novagen
pGRG36	Tn7 insertion vector, pSC101 replicon, Amp^R	McKenzie and Craig (2006)
pGlacZ△M15	pGRG36 + *lacZ△M15* (*Sma*I)	This study
BCGW	pETDuet-1 + *manC-manB* (*Nco*I/*Sac*I) + *gmd-wcaG* (*Nde*I/*Kpn*I)	Lee et al. (2009)
F	pCOLADuet-1 + *fucT2* (*Nco*I/*Sac*I)	Lee et al. (2012)
D3F	pCOLADuet-1 + three aspartate-fucT2 (*Nde*I/*Kpn*I)	This study
D4F	pCOLADuet-1 + four aspartate-fucT2 (*Nde*I/*Kpn*I)	This study
D5F	pCOLADuet-1 + five aspartate-fucT2 (*Nde*I/*Kpn*I)	This study
D6F	pCOLADuet-1 + six aspartate-fucT2 (*Nde*I/*Kpn*I)	This study
F-His	pCOLADuet-1 + *fucT2* (*Nco*I/*Sac*I) + His-tag	This study
D3F-His	pCOLADuet-1 + three aspartate-fucT2 (*Nde*I/*Kpn*I) + His-tag	This study

partners such as GST and maltose binding protein (MBP) cause a high metabolic burden. Contrary to fusion partner proteins, simple amino acid tags not only facilitate purification of their fusion partners but also enhance the solubility with less metabolic burden (Kweon et al., 2005; Waugh, 2005). The charges of amino acid tags are closely correlated with their solubilizing ability (Jung et al., 2011). Especially, N-terminal fusion of aspartate tag facilitated intracellular expression of heterologous protein in *E. coli* (Kim et al., 2015). This suggests that an appropriate amino acid tag have a beneficial influence on soluble expression of FucT2 and thus 2-FL production.

Intracellular availability of lactose is another essential factor for microbial production of 2-FL. In the previous research, the *lac* operon was disrupted and *lacY* was reintegrated in the chromosome of *E. coli* for efficient synthesis of lacto-*N*-tetraose, which is the third most abundant oligosaccharide in human milk (Baumgärtner et al., 2014). As mentioned above, most of previous studies employed the *E. coli* JM strains (*E. coli* K-12 derivatives) to produce 2-FL because it can take up lactose but cannot metabolize lactose as a carbon source. This unique phenotype seems to be due to complete deletion of the *lac* operon in the chromosome and introduction of the F' episome containing the modified *lac* operon (*lacI^q lacZΔM15*) (Baumgärtner et al., 2013; Drouillard et al., 2006; Lee et al., 2012).

However, considerable amounts of biofilm might be formed on the surface of JM109 due to the presence of conjugative plasmid F' that the strain contains (Ghigo, 2001; Ren et al., 2005). Biofilms are potential problem during the fermentation processes such as bioreactor operation, purification and filtration process. (Mattila-Sandholm and Wirtanen, 1992). In addition, inhibition of cell growth has been frequently observed when JM strains are grown for high cell density in order to increase productivity, which might be caused by either biofilms formations or the high level accumulation of acetate (Xia et al., 2008).

Compared to the JM strains, *E. coli* BL21 which does not contain F' has many advantages such as faster cell growth, lower acetate accumulation and better glucose utilization, which is ascribed to more active sugar metabolism such as glyoxylate shunt, gluconeogenesis, anaplerotic pathways and TCA cycle. In addition, BL21 exhibits less sensitivity to metabolic stress resulted from producing a large amount of heterologous proteins (Phue et al., 2008; Son et al., 2011).

Previously, production of 2-FL was attempted with engineered *E. coli* BL21star(DE3) able to synthesize GDP-L-fucose. However, only a small amount of 2-FL was produced because E. coli BL21star(DE3) assimilated lactose instead of being converted to 2-FL (Lee et al., 2012), which suggests that engineering of the lactose metabolism is required for efficient production of 2-FL in E. coli BL21star(DE3).

In this study, effects of modulation of *lac* operon on 2-FL production was investigated in *E. coli* BL21star(DE3). To alleviate the metabolism of lactose, the endogenous *lac* operon was replaced by the modified *lac* operon containing *lacZ△M15*. Afterwards, the effect of simple tagging of aspartate at the N-terminal of FucT2 on 2-FL production in engineered *E. coli* was also investigated in fed-batch fermentation.

2. Materials and methods

2.1. Strains and plasmids

All strains, plasmids, and oligonucleotides used in this study are listed in Table 1 and 2. *E. coli* TOP10 and *E. coli* BL21star(DE3) (Invitrogen, Carlsbad, CA, USA) were used for construction of plasmids and a host strain for production of 2-FL, respectively. To construct a *lac* operon knock-out strain (△L), the chromosomal region from 20 bp upstream of *lacI* to 40 bp downstream of *lacA* was deleted using the λ-red mediated recombination method with pKD46, pKD13 and pCP20 (Datsenko and Wanner, 2000). A PCR fragment containing a kanamycin resistance gene and sequences homologous to flanking regions of the *lac* operon was amplified from pKD13 using two primers, F_del_lac and R_del_lac. After transformation of plasmid pKD46 into BL21star(DE3), the 1.4 kb PCR product was introduced into BL21star(DE3) harboring pKD46 by electroporation. Upon recombination and selection for resistant colonies, the kanamycin-resistant gene was removed by transformation with pCP20 (containing the FLP recombinase gene). After incubation at 42 °C, the *lac* operon deleted strain without the kanamycin resistance gene and pCP20 was selected. The deletion of *lac* operon was verified by the colony PCR using two PCR primers of F_ch_lac and R_ch_lac.

The integration of the *lac* operon bearing *lacZ△M15* (*lacZ* deleted codon11–codon 42) into the *attTn7* locus was carried out using a Tn7-mediated site-specific transposition method (McKenzie and Craig, 2006). For construction of pGlacZ△M15, two DNA fragments were amplified from the *E. coli* K-12 genomic DNA (ATCC10798) with two pairs of primers P1_M15 lac/P2_M15 lac and P3_M15 lac/P4_M15 lac. The two PCR products were cloned simultaneously into pGRG36 (digested by *Sma*I) by *in vitro* homologous recom-

Table 2
Primers used in this study.

Name	Sequence
F_del_lac	CGAATGGCGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTGTAGGCTGGAGCTGCTTCG
R_del_lac	TCCTGCGCTTTGTTCATGCCGGATGCGGCTAATGTAGATCGCTGAACTTGATTCCGGGGATCCGTCGACC
F_ch_lac	CGAAGCGGCATGCATTTACG
R_ch_lac	CGCAGCTGTGGGTCAAAGAG
P1_M15 lac	AATTAATCAGATCCCGGGACCATCGAATGGCGCAAAACCTTTC
P2_M15 lac	GGTGCGGGCCACGACGGCCAGTGAATCCGTAATCA
P3_M15 lac	TGGCCGTCGTGGCCCGCACCGATCGCC
P4_M15 lac	GGCCGCTATTGACCCGGGGCTGTGGGTCAAAGAGGCATGATG
F_Tn7	GATGCTGGTGGCGAAGCTGT
R_Tn7	GATGACGGTTTGTCACATGGA
F_*Nde*I_fucT2	GGAATTC*CATATG*GCTTTTAAGGTGGTGC
F_*Nde*I_D3-fucT2	GGAATTC*CATATG*GATGATGATGCTTTTAA
F_*Nde*I_D4-fucT2	GGAATTC*CATATG***GATGATGATGAT**GCTTTTAA
F_*Nde*I_D5-fucT2	GGAATTC*CATATG***GATGATGATGATGAT**GCTTTTAA
F_*Nde*I_D6-fucT2	GGAATTC*CATATG***GATGATGATGATGAT**GATGCTTTTAA
R_*Kpn*I_fucT2	G*GGTAC*CATTAAGCGTTATACTTTTGGGATTTTACCT
R_*Kpn*I_fucT2-His	GG*GGTAC*CTTA**GTGGTGATGATGGTGATG**AGCGTTATACTTTTGGG

The underline nucleotides indicate the homologous recombination regions of *lac* operon in the *E. coli* chromosome. The italic- and bold sequences present the recognition sites of specific restriction enzymes and aspartate tags, respectively.

bination using In-Fusion HD Cloning Kit (TAKARA, Japan). After transformation of the pGlacZ△M15 into △L strain, transformants were selected at 30 °C. The transformants were grown in non-selective media to ensure the chromosomal integration and the pGlacZ△M15 were eliminated by incubation at 42 °C. The insertion of *lac* operon bearing *lacZ△M15* was confirmed by colony PCR using two primers, F_Tn7 and R_Tn7 (Table 2).

Plasmids BCGW and F were previously constructed for overexpression of the genes for GDP-L-fucose biosynthetic enzymes (ManB, ManC, Gmd and WcaG) and α-1,2 fucosyltransferase (FucT2) from *H. pylori*, respectively (Lee et al., 2009; Lee et al., 2012). For amplification of the aspartate tagged *fucT2* genes, DNA primers were formulated with a forward primer and R_*Kpn*I_fucT2. For example, D3-fucT2 (FucT2 with N-terminal three aspartate tag) was amplified using F_*Nde*I_D3-fucT2 and R_*Kpn*I_fucT2. The PCR products digested with *Nde*I and *Kpn*I were ligated with plasmid pCOLAduet-1 cut with the same restriction enzymes and therefore D3F, D4F, D5F and D6F were constructed. For the His-tag mediated purification of FucT2 and D3-FucT2, fucT2 genes were amplified with the forward primers, F_*Nde*I_fucT2 or F_*Nde*I_D3-fucT2 and the reverse primer, R_*Kpn*I_fucT2-His using plasmid F as the template. The PCR products were ligated with pCOLADuet-1 as described above and therefore F-His and D3F-His were constructed. All constructs were confirmed by the restriction enzyme digestion and DNA sequencing.

2.2. Culture conditions

Batch fermentations were performed in a 500 mL baffled flask (Nalgene) containing 100 mL of Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride) with appropriate antibiotics (ampicillin 50 μg/mL and kanamycin 50 μg/mL) at 25 °C. The agitation speed was maintained at 250 rpm. When optical density (OD_{600}) reached 0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) and lactose was added at a final concentration 0.1 mM and 20 g/L, respectively. Fed-batch fermentations were carried out in a 2.5 L bioreactor (Kobiotech, Incheon, Korea) containing 1.0 L of defined medium [13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid, 1.4 g/L $MgSO_4{\cdot}7H_2O$, 10 mL/L trace element solution (10 g/L Fe(III) citrate, 2.25 g/L $ZnSO_4{\cdot}7H_2O$, 1.0 g/L $CuSO_4{\cdot}5H_2O$, 0.35 g/L $MnSO_4{\cdot}H_2O$, 0.23 g/L $Na_2B_4O_7{\cdot}10H_2O$, 0.11 g/L $(NH_4)_6Mo_7O_{24}$, 2.0 g/L $CaCl_2{\cdot}2H_2O$), pH 6.8] containing 20 g/L glycerol and appropriate antibiotics at 25 °C. After complete depletion of glycerol added initially, feeding solution containing 800 g/L glycerol and 20 g/L $MgSO_4{\cdot}7H_2O$ was fed by a pH-stat mode. At the same time, IPTG and lactose were also added to a final concentration 0.1 mM and 20 g/L for induction of the T7 promoter-mediated gene expression and for production of 2-FL. For pH-stat feeding, the feeding solution was fed automatically into the bioreactor when the pH rose to a value higher than its set-point due to the depletion of glycerol. The pH of medium was controlled at 6.8 using a standard pH electrode (Mettler Toledo, USA) by addition of 28% NH_4OH. The pH-stat feeding strategy is based on direct coupling of carbon source consumption and concomitant export and import of proton and ammonium ion by the cell during growth. Whereas pH decreases by export of H^+ and import of NH_4^+ from the cells by consuming a carbon source, pH increases by import of H^+ and export of NH_4^+ from the cells when the carbon source is exhausted (Kim et al., 2004). Although glucose is mainly used for pH-stat feeding, glycerol could be also used for high density culture of *E. coli* by pH-stat (García-Arrazola et al., 2005; Wang et al., 2001). Agitation speed increased to 1200 rpm in order to prevent the deficiency of dissolved oxygen, and air flow rate was maintained at 2 vvm throughout the cultivation.

2.3. Determination of concentrations of cell and extracellular metabolites

Dry cell weight (DCW) was determined using optical density and a predetermined conversion factor (0.36). Optical density was measured at 600 nm absorbance using a spectrophotometer (Ultrospec 2000, Amersham Pharmacia Biotech, USA) after the samples were diluted to keep optical density between 0.1 and 0.5. Extracellular concentrations of 2-FL, lactose, glycerol, galactose and acetic acid were measured by a high performance liquid chromatography (HPLC) (Agilent 1100LC, USA) equipped with the Carbohydrate Analysis column (Rezex ROA-organic acid, Phenomenex, USA) and refractive index (RI) detector. The column heated at 60 °C was applied to analyze 20 μl of diluted culture broth. Five millimoles of H_2SO_4 solution was used as a mobile phase at a flow rate of 0.6 mL/min.

2.4. Analysis of expression patterns and purification of recombinant FucT2s

Total, soluble and insoluble protein fractions of FucT2-variants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide gel). After 12 h of 0.1 mM IPTG induction, cells were collected and concentrated to adjust its OD_{600} to be 10. The cells resuspended in 50 mM sodium

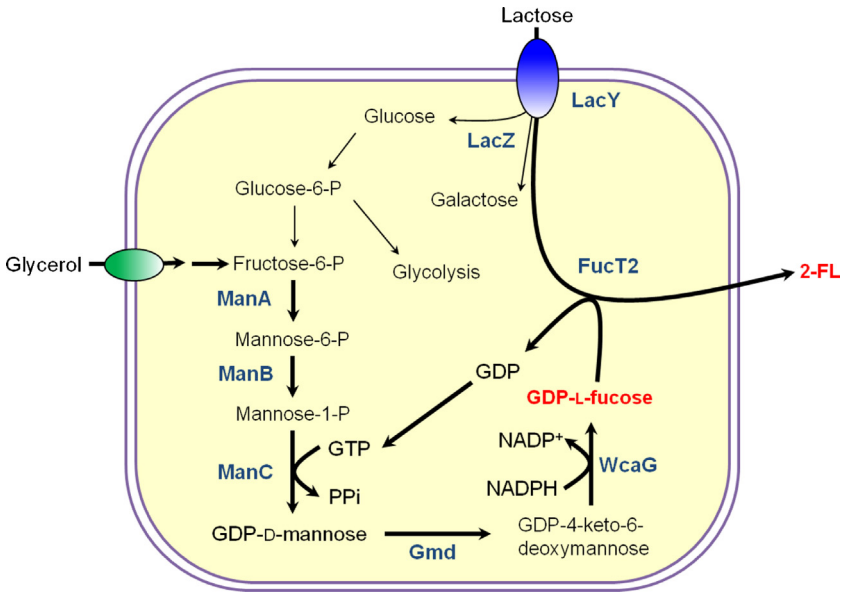

Fig. 1. The metabolic pathway for GDP-L-fucose and 2′-fucosyllactose (2-FL) biosynthesis in engineered *E. coli*. The names of proteins are abbreviated as follows; LacZ, β-galactosidase; LacY, lactose permease; ManA, mannose 6-phosphate isomerase; ManB, phosphomannomutase; ManC, mannose 1-phosphate guanylyltransferase; Gmd, GDP-D-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase; FucT2, α-1,2-fucosyltransferase. PPi and GTP denote diphosphate and guanosine 5′-triphosphate.

phosphate buffer (pH 7.0) were disrupted using an ultrasonic processor (Cole–Parmer, Vernon Hills, IL, USA). After collection of the supernatants (soluble fraction) and cell debris (insoluble fraction) by centrifugation (12,000 rpm, 4 °C, 10 min), each of 10 μl protein fractions were loaded onto the gel and visualized by staining the gels with Coomassie brilliant blue solution.

For purification of FucT2 and D3-FucT2, △L M15 strains harboring plasmid F-His or D3F-His were cultured in a flask containing 100 mL LB broth. After 12 h of 0.1 mM IPTG induction, the cells were harvested by centrifugation at 12,000 rpm and 4 °C for 10 min. The supernatant was removed and pellets were resuspended with the His-tag binding buffer containing 20 mM NaH_2PO_4 (pH 7.4), 0.5 M NaCl, and 30 mM imidazole. The prepared samples were loaded into a HisTrap FF column (1 mL) packed with the Ni Sepharose Fast Flow (GE Healthcare, Buckinghamshire, UK), followed by washing with 10 mL of the His-tag binding buffer. The bound proteins were eluted with elution buffer containing 20 mM NaH_2PO_4 (pH 7.4), 0.5 M NaCl, and 500 mM imidazole under constant level of immidazole concentration. The FucT2 and D3-FucT2 eluted from the column were collected and used for SDS-PAGE to investigate soluble expression levels and *in vitro* enzymatic reaction.

2.5. *β-galactosidase activity assay*

Cells were grown in 100 mL of the defined medium with 20 g/L glycerol at 25 °C and 250 rpm. After 6 h IPTG induction, an optical density of the culture broth was adjusted at 10 by appropriate dilution and concentration. Cells were resuspended in TE buffer [10 mM Tris–HCl (pH 8.0) and 1 mM EDTA (pH 8.0)] and disrupted by a ultrasonic processor (Cole-Parmer, IL, USA). Cells were centrifuged for 10 min at 12,000 rpm and 4 °C to separate soluble and insoluble fractions. Activity was determined by assaying soluble fractions using a β-Galactosidase Enzyme Assay System (Promega E2000). One unit of β-galactosidase was defined as the amount of enzyme able to reduce 1 μmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) to *o*-nitrophenol and galactose per minute at pH 7.5 and 25 °C. Protein concentration was determined by the protein assay kit (Bio-Rad, Richmond, CA, USA) using bovine serum albumin as a standard. Specific enzyme activity (U/mg protein) was obtained by dividing the enzyme activity by the total protein concentration of the crude enzyme solution. The assay was repeated independently in triplicate.

2.6. In vitro *enzymatic reaction*

The enzymatic reaction was carried out using HPLC based assay (Lee et al., 2013). The purified FucT2 and D3-FucT2 enzymes were assayed using 20 mM final concentration of GDP-L-fucose as a fucose donor, 20 mM of lactose as an acceptor, and 100 mM Tris–HCl (pH 7.5) buffer containing 20 mM $MgCl_2 \cdot 6H_2O$. The reaction was performed in a 200 μL final volume for 270 min at 25 °C and stopped by boiling the reaction mixture for a minute. Then, the reaction mixture was centrifuged at 12,000 rpm for 10 min at 4 °C to remove denatured proteins. The supernatant was filtered and analyzed by HPLC.

3. Results

3.1. *Effect of modulation of* lac *operon on 2-FL production*

Intracellular availability of lactose is one of the key factors for efficient biosynthesis of 2-FL in engineered *E. coli*. As illustrated in Fig. 1, β-galactosidase (encoded by the *lacZ*) catalyzes the first step of lactose metabolism, hydrolysis of lactose to glucose and galactose. In order to divert the lactose flux from the lactose utilizing pathway to the 2-FL biosynthetic pathway, partial deletion of the *lacZ* gene in *E. coli* BL21star(DE3) was attempted. However, no 2-FL production was observed in the *lacZ* deleted *E. coli* BL21star(DE3) strain, which suggested that transport of lactose into the cell was influenced by deletion of the *lacZ* gene (data not shown). Probably, deletion of the *lacZ* gene might be polar mutation, which affects the transcription or translation of the gene or operon downstream of the deletion site. It was reported that the polar mutations in *lacZ* lead to a decreased synthesis of lactose permease (LacY) and thiogalactoside transacetylase (LacA) (Malamy, 1966).

Since *E. coli* JM109 can take up lactose only (without metabolism), replacement of the endogenous *lac* operon in *E. coli* BL21star(DE3) with the *lac* operon bearing *lacZ△M15* was

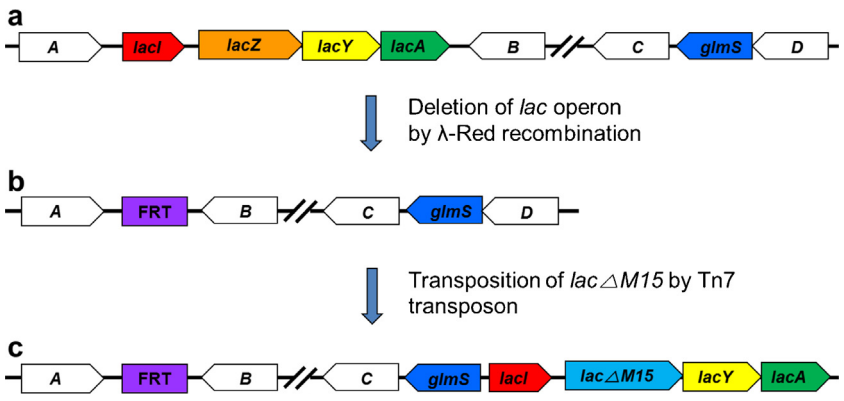

Fig. 2. Schematic of construction of the engineered *E. coli* strains (a) BL21star(DE3); (b) △L; (c) △L M15.

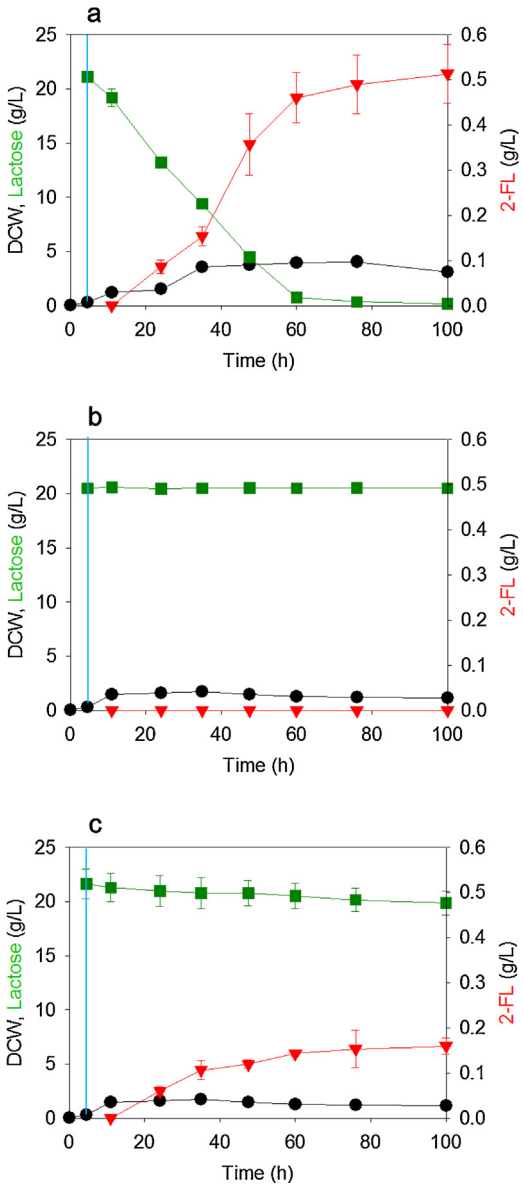

Fig. 3. Batch fermentations of engineered *E. coli* strains (a) BL21star(DE3) BCGW-F; (b) △L BCGW-F; (c) △L M15 BCGW-F. When optical density (OD_{600}) reached 0.8, IPTG and lactose was added to a final concentration 0.1 mM and 20 g/L, respectively (vertical line). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲. Error bars represent the standard deviation of three replicates.

Table 3
Specific activities of β-galactosidase (LacZ) of *lac* operon engineered *E. coli* strains.

Strains	LacZ activity (U/mg protein)
BL21star(DE3)	3.42 ± 0.04
△L	0.01 ± 0.00
△L M15	0.11 ± 0.01

The values in the table are averages determined from three independent experiments and standard deviations are shown.

attempted. Fig. 2 shows the scheme of deletion of the endogenous *lac* operon and introduction of the new *lac* operon in *E. coli* BL21star(DE3). First, the region of the endogenous *lac* operon was deleted by λ-red recombination, which resulted in the *E. coli* △L strain (*lac* operon deficient strain). Second, the new *lac* operon fragment containing *lacZ△M15* was inserted the downstream site of the *glmS* gene called *attTn7* by Tn7 based transposition, which resulted in the △L M15 strain (*lacZ△M15* knock-in strain). As a result of replacement of the *lac* operon, the β-galactosidase activity of △L M15 has decreased by 97% compared to wild type *E. coli* BL21star(DE3) (Table 3).

Subsequently, the effect of *lac* operon replacement on 2-FL production was investigated with three strains, wild type *E. coli* BL21star(DE3), △L and △L M15 in batch fermentations. The control strain, wild type BL21star(DE3), consumed most of lactose initially added within 60 h to produce 0.51 g/L 2-FL with a yield of 0.025 g 2-FL/g lactose (Fig. 3a). Most of the consumed lactose might be used for cell growth since the control strain showed the highest value of final dry cell mass compared to other strains. In contrast to the control strain, the *lac* operon deficient strain (△L) could not consume lactose at all and therefore, could not produce 2-FL (Fig. 3b). It might be due to the absence of lactose permease. The *lacZ△M15* knock-in strain consumed approximately 1.8 g/L lactose to produce 0.16 g/L 2-FL. However, the yield of 0.091 g 2-FL/g of lactose in the *lacZ△M15* knock-in strain corresponded to a 3.6 fold increase compared with the control strain (Fig. 3c), which was almost the same as the 2-FL yield of JM109(DE3) (Table 4). The *E. coli* BL21star(DE3) derived strain able to metabolize lactose slowly was constructed, indicating that replacement of the endogenous *lac* operon with the modified one is effective in 2-FL production. The results of batch fermentations are summarized in Table 4.

3.2. *Effect of aspartate tagged FucT2 on 2-FL production*

Simple amino acids such as aspartate (Asp, D) were known to influence the expression of a heterologous gene in *E. coli* (Kim et al., 2015). The effect of FucT2s fused with three to six aspartate tags (D3-D6 tags) on 2-FL production was investigated via

Table 4
Summary of batch fermentations of engineered *E. coli* strains.

Strains	Maximum dry cell weight (g/L)	Maximum 2-FL concentration[b] (g/L)	Lactose consumed[b] (g/L)	Yield (g 2-FL/g lactose)
JM109(DE3)[a] BCGW-F	1.70 ± 0.28	1.23 ± 0.011	13.7 ± 0.067	0.090 ± 0.004
BL21star(DE3) BCGW-F	4.06 ± 0.09	0.51 ± 0.065	20.9 ± 0.447	0.025 ± 0.003
△L BCGW-F	1.74 ± 0.32	N.D.	0.0	–
△L M15 BCGW-F	1.23 ± 0.13	0.16 ± 0.017	1.76 ± 0.263	0.091 ± 0.004
△L M15 BCGW-D3F	1.54 ± 0.11	0.34 ± 0.010	1.35 ± 0.026	0.252 ± 0.012

The values in the table are averages determined from three independent experiments and standard deviations are shown.
[a] The result of batch fermentation of JM109(DE3) BCGW-F strain was cited in Lee et al. (2012).
[b] Extracellular concentrations of lactose and 2-FL were determined and used for the calculation of lactose consumption and 2-FL production.

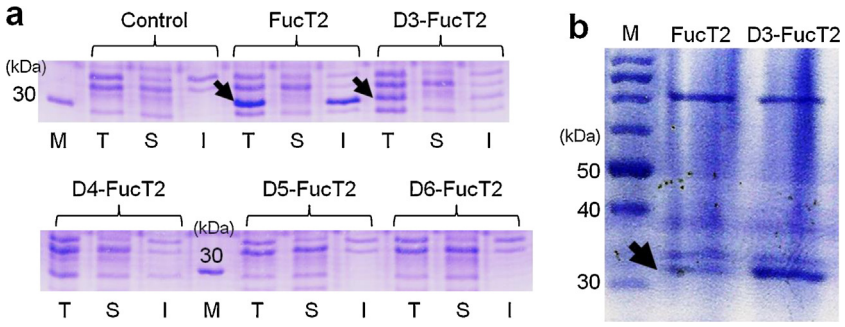

Fig. 4. SDS-PAGE analysis of crude extracts (a) and purified enzymes (b) to investigate the effects of aspartate tags on the expression patterns of FucT2 variants in engineered *E. coli* strains. The abbreviations were defined as follows: arrows; FucT2, T; total protein fraction, S; soluble protein fraction, I; insoluble protein fraction, M; molecular weight marker.

batch fermentations of four engineered *E. coli* △L M15 BCGW strains expressing D3-FucT2, D4-FucT2, D5-FucT2 or D6-FucT2, respectively. Even though the cell growth pattern and lactose uptake of the four strains were similar to each other, different 2-FL production patterns of engineered *E. coli* expressing aspartate tagged FucT2 were observed. Production of 2-FL was not detected in the cultures of the engineered strains except for the culture of △L M15 BCGW-D3F (data not shown). In batch fermentation of △L M15 BCGW-D3F strain, 0.34 g/L of 2-FL was produced with a yield of 0.252 g/g lactose corresponding to 2.1- and 2.8 times higher than those of the control strain, △L M15 BCGW-F (Table 4). To elucidate if these 2-FL production patterns are related with soluble expression of aspartate tagged FucT2, expression of D3-FucT2, D4-FucT2, D5-FucT2 and D6-FucT2 was analyzed by SDS-PAGE. The *E. coli* harboring pCOLADuet-1 and plasmid F were used as a negative and a positive control, respectively. As shown in Fig. 4, expression of D4-FucT2, D5-FucT2 and D6-FucT2 could not be confirmed. Most FucT2 was expressed as insoluble form in the *E. coli* expressing FucT2, which is consistent with the previous study (Lee et al., 2012). The total- and insoluble levels of FucT2 expression significantly decreased in the D3-FucT2 strain, however, the levels of soluble FucT2 and D3-FucT2 were invisible in the SDS-PAGE analysis with crude extracts. To confirm the soluble level of expression, purification of FucT2 and D3-FucT2 with His-tag was performed. As a result, soluble expression of FucT2 and D3-FucT2 was confirmed and the level of D3-FucT2 was much higher than those of FucT2 (Fig. 4b). Subsequently, *in vitro* enzymatic synthesis of 2-FL was carried out to verify that the enzyme activity of D3-FucT2 is higher than that of FucT2. As expected, 10.4 μmol 2-FL was synthesized in the reaction mixture of D3-FucT2, which is corresponding to about 3.4 fold higher than that of wild type FucT2 (Fig. 5).

Therefore, improved soluble expression of FucT2 by fusion of the three aspartate tag might lead to higher 2-FL production with enhanced yield than the control strain.

3.3. Production of 2-FL by fed-batch fermentation

As glycerol is known to be a by-product generated from biodiesel production processes, development of microbial systems converting glycerol into value-added products can be advantageous and promising (Yazdani and Gonzalez, 2007). In addition, glycerol can be utilized simultaneously with lactose since both carbon sources are transported into the cell through the non-PTS system (Postma et al., 1993).

In order to supply a carbon source for biosynthesis of GDP-L-fucose as well as cell growth, glycerol fed-batch fermentation of engineered *E. coli* △L M15 BCGW-F was carried out with intermittent lactose addition (Fig. 6a). After complete depletion of glycerol added initially, glycerol was fed by a pH-stat mode. Production of 2-FL was launched by IPTG induction for expression of 2-FL biosynthetic enzymes as well as addition of lactose as an acceptor for fucose. After depletion of initial lactose in 47 h of culture, the same amount of lactose was dumped to the fermentation broth in order to maintain 2-FL biosynthesis. Acetate accumulation was not observed throughout the fed-batch fermentations. Through the assimilation of glycerol, cell growth was maintained to reach 73.1 g/L of final cell concentration. As a result, 2.6 g/L of 2-FL with a yield of 0.063 g 2-FL/g lactose was obtained at the end of fed-batch fermentation. The intracellular concentration of 2-FL was approximately 10% (<0.4 g/L) of a total amount of 2-FL (data not shown).

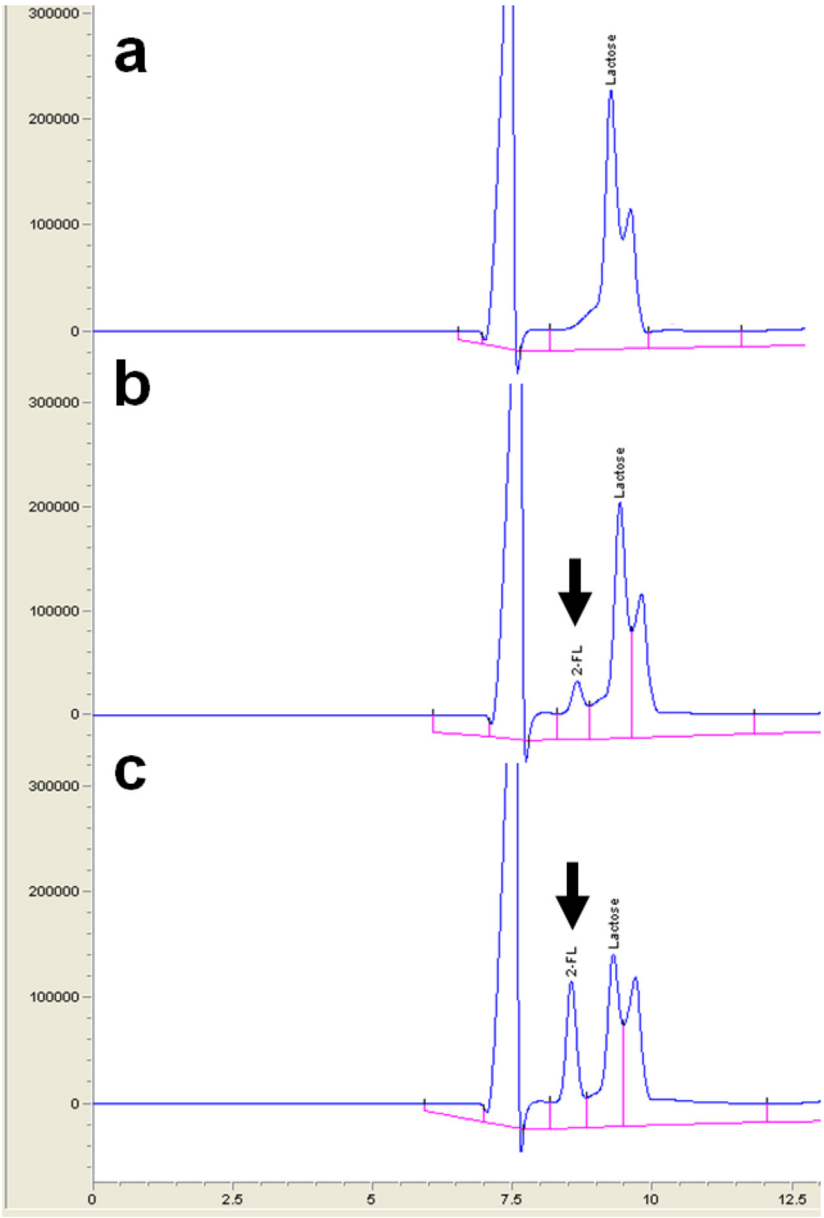

Fig. 5. HPLC analysis of *in vitro* enzymatic reaction mixtures with control (a), FucT2 (b) and D3-FucT2 (c). The arrow points the peaks of 2-FL.

Table 5
Comparison of results of fed-batch fermentations of engineered *E. coli* BL21star(DE3) strains.

Strains	Maximum dry cell weight (g/L)	Maximum 2-FL concentration[b] (g/L)	Yield (g 2-FL/g lactose)	Productivity[a] (g/L·h)
△L M15 BCGW-F	73.1	2.6	0.063	0.043
△L M15 BCGW-D3F	71.1	6.4	0.225	0.118

[a] 2-FL productivity was estimated during the 2-FL production period after IPTG induction and lactose dumping.
[b] Extracellular concentrations of lactose and 2-FL were determined and used for the calculation of lactose consumption and 2-FL production.

To verify the beneficial effects of the aspartate tag attached at the N-terminal of FucT2 on production of 2-FL, fed-batch fermentation was also performed by controlling the glycerol concentration while dumping lactose. After IPTG induction, the cell growth pattern of the engineered *E. coli* △L M15 BCGW-D3F was similar to the control strain, and its final dry cell weight reached 71.1 g/L. After lactose addition, 2-FL concentration increased linearly up to 6.4 g/L in 78 h culture with a yield of 0.225 g/g lactose, which was 2.5 and 3.6 fold higher than those of the control strain. The results of fed-batch fermentations are summarized in Table 5.

4. Discussion

It was previously observed that engineered *E. coli* JM109(DE3) produced a considerable amount of 2-FL through slow metabolism of lactose while engineered *E. coli* BL21star(DE3) produced low con-

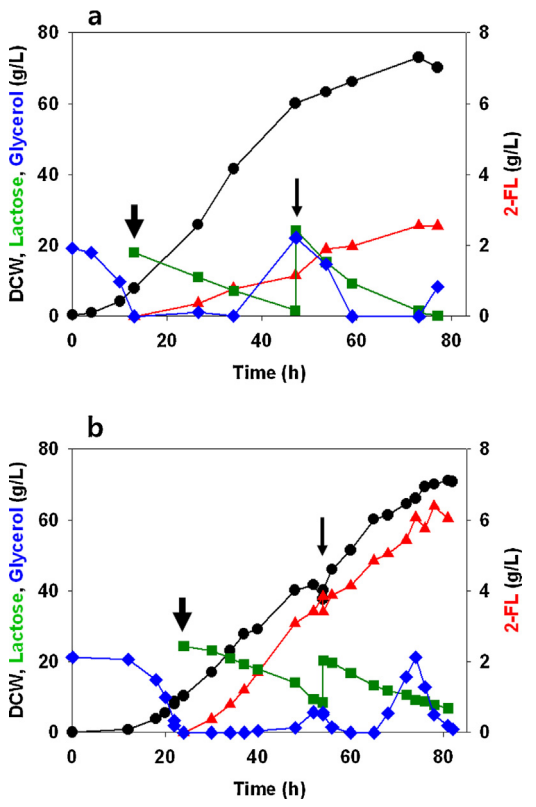

Fig. 6. Fed-batch fermentations of △L M15 (a) BCGW-F; (b) BCGW-D3F. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (thick arrow). 200 g/L lactose solution was further dumped after depletion of lactose (thin arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ♦.

centration of 2-FL because of fast lactose metabolism (Lee et al., 2012). In the case of the JM109(DE3) strain, the α-peptide of β-galactosidase (synthesized from beginning of the *lacZ* gene of DE3 cassette in the genome) is combined with the ω-peptide (synthesized from *lacZ△M15* in F' episome) to result in complementation of β-galactosidase activity, which is called α-complementation. It was reported that the complemented enzyme has at least 50% of the specific activity of native β-galactosidase (Langley et al., 1975). Consequently, a decrease in lactose hydrolysis activity in the *E. coli* JM109(DE3) strain might allow higher 2-FL production than *E. coli* BL21star(DE3). However, JM109 formed biofilms that cause serious problems in fermentation. This is owing to the conjugative plasmid F' in JM109 (Teodósio et al., 2012). Moreover, it is difficult to grow *E. coli* JM109(DE3) to high cell density because several problems such as inhibition of cell growth and significant accumulation of acetate were observed in fed-batch type cultivation (Shiloach et al., 1996). Meanwhile, *E. coli* BL21star(DE3) does not have conjugative plasmids and it is relatively easy to grow *E. coli* BL21star(DE3) to high cell density because the BL21star(DE3) strain exhibits active sugar metabolism and tolerance against metabolic stress, which is a reason why *E. coli* BL21star(DE3) was developed for 2-FL production instead of JM109(DE3).

In order to increase intracellular lactose availability and to enhance the 2-FL yield from lactose, the *lac* operon of BL21star(DE3) in the chromosome was engineered by mimicking the *lac* operon of JM109(DE3) in F' episome. As expected, substitution of the *lac* operon with *lacZ△M15* led to an improvement of 2-FL yield from lactose substantially when compared with the control strain and the *lac* operon deficient strain (Fig. 3, Table 4). It seemed that intracellular lactose availability was improved by reducing β-galactosidase activity significantly while maintaining *lacY* and *lacA* activities.

Even though the control strain produced 2-FL more than the △L M15 strain in batch fermentation, the △L M15 strain was thought to be more efficient for 2-FL production because 2-FL yield was improved by a 3-fold. In addition, the control strain produced a very small amount of 2-FL in the glycerol fed-batch fermentation (data not shown) while the △L M15 strain produced 2.6 g/L of 2-FL in the glycerol fed-batch fermentation.

It was reported that fusion of simple amino acid tags enhanced soluble expression of heterologous lipase in recombinant *E. coli*. Among the various tags used, negative-charged amino acid tags, especially three to six aspartate tags were most effective in improved expression of *Candida antarctica* lipase (Kim et al., 2015). Therefore, attachment of three to six aspartate tags was applied to FucT2 expression in this study. The engineered *E. coli* expressing D3 fused FucT2 increased 2-FL production by a 2.1 fold and yield by a 2.8 fold compared to the engineered *E. coli* expressing FucT2. The soluble expression of FucT2 by fusion of three aspartates improved 2-FL production compared to the control strain. Interestingly, more than four aspartates inhibited FucT2 expression, indicating that three molecules of aspartate are the optimum amino acid tag for FucT2 expression.

To develop an efficient microbial process for 2-FL production without biofilm formation, the *E. coli* BL21star(DE3) was employed in this study. The endogenous lactose metabolism of *E. coli* was modulated by replacement of the endogenous *lac* operon with the modified one containing *lacZΔM15* in order to increase intracellular lactose availability. In addition, aspartate fused FucT2 enhanced 2-FL production more. To improve the 2-FL titer and yield based on lactose consumed, further research is in progress to introduce novel α-1,2-fucosyltransferase.

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References

Albermann, C., Piepersberg, W., Wehmeier, U., 2001. Synthesis of the milk oligosaccharide 2′-fucosyllactose using recombinant bacterial enzymes. Carbohyd. Res. 334, 97–103.
Baumgärtner, F., Conrad, J., Sprenger, G.A., Albermann, C., 2014. Synthesis of the human milk oligosaccharide lacto-*N*-tetraose in metabolically engineered plasmid-free *E. coli*. Chembiochem 15, 1896–1900.
Baumgärtner, F., Seitz, L., Sprenger, G.A., Albermann, C., 2013. Construction of *E. coli* strains with chromosomally integrated expression cassettes for the synthesis of 2′-fucosyllactose. Microb. Cell Fac. 12, 40.
Bode, L., 2012. Human milk oligosaccharides: every baby needs a sugar mama. Glycobiology 22, 1147–1162.
Castanys-Muñoz, E., Martin, M.J., Prieto, P.A., 2013. 2′-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk. Nutr. Rev. 71 (12), 773–789.
Datsenko, K.A., Wanner, B.L., 2000. One-step inactivation of chromosomal genes in *E. coli K-12 using PCR products*. Proc. Natl. Acad. Sci. U. S. A 97, 6640–6645.
Drouillard, S., Driguez, H., Samain, E., 2006. Large-scale synthesis of H-antigen oligosaccharides by expressing *Helicobacter pylori* α-1,2-fucosyltransferase in metabolically engineered *E. coli* cells. Angew. Chem. 118, 1810–1812.
Dumon, C., Samain, E., Priem, B., 2004. Assessment of the two Helicobacter pylori alpha-1, 3-fucosyltransferase ortholog genes for the large-scale synthesis of LewisX human milk oligosaccharides by metabolically engineered *E. coli*. Biotechnol. Prog. 20 (2), 412–419.
Engels, L., Elling, L., 2014. WbgL: a novel bacterial α1, 2-fucosyltransferase for the synthesis of 2′-fucosyllactose. Glycobiology 24, 170–178.
Ghigo, J.-M., 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature 412, 442–445.
Han, N.S., Kim, T.J., Park, Y.C., Kim, J., Seo, J.H., 2012. Biotechnological production of human milk oligosaccharides. Biotechnol. Adv. 30, 1268–1278.

Jung, H.-J., Kim, S.-K., Min, W.-K., Lee, S.-S., Park, K., Park, Y.-C., Seo, J.-H., 2011. Polycationic amino acid tags enhance soluble expression of Candida antarctica lipase B in recombinant *E. coli*. Bioproc. Biosys. Eng. 34, 833–839.

Kato, A., Maki, K., Ebina, T., Kuwajima, K., Soda, K., Kuroda, Y., 2007. Mutational analysis of protein solubility enhancement using short peptide tags. Biopolymers 85, 12–18.

Kim, S.K., Park, Y.C., Lee, H.H., Jeon, S.T., Min, W.K., Seo, J.H., 2015. Simple amino acid tags improve both expression and secretion of Candida antarctica lipase B in recombinant *E. coli*. Biotechnol. Bioeng. 112 (2), 346–355.

Kweon, D.H., Kim, S.G., Han, N.S., Lee, J.H., Chung, K.M., Seo, J.H., 2005. Immobilization of Bacillus macerans cyclodextrin glycosyltransferase fused with poly-lysine using cation exchanger. Enzyme Microb. Tech. 36, 571–578.

Langley, K.E., Fowler, A.V., Zabin, I., 1975. Amino acid sequence of beta-galactosidase. IV. Sequence of an alpha-complementing cyanogen bromide peptide, residues 3–92. J. Biol. Chem. 250, 2587–2592.

Lee, J., Kim, D., Sohng, J., 2013. Cloning and functional characterization of an α-1,3-fucosyltransferase from *Bacteroides fragilis*. Biotechnol. Bioprocess Eng. 18 (5), 843–849.

Lee, W.H., Han, N.S., Park, Y.C., Seo, J.H., 2009. Modulation of guanosine 5′-diphosphate-D-mannose metabolism in recombinant *E. coli* for production of guanosine 5′-diphosphate-L-fucose. Bioresource Technol. 100, 6143–6148.

Lee, W.H., Pathanibul, P., Quarterman, J., Jo, J.H., Han, N.S., Miller, M.J., Jin, Y.S., Seo, J.H., 2012. Whole cell biosynthesis of a functional oligosaccharide, 2′-fucosyllactose, using engineered *E. coli*. Microb. Cell Fac. 11, 48.

Malamy, M.H., 1966. Frameshift mutations in the lactose operon of *E. coli*. Cold Spring Harbor symposia on quantitative biology. Cold Spring Harbor Laboratory Press, 189–201.

Mattila-Sandholm, T., Wirtanen, G., 1992. Biofilm formation in the industry: a review. Food Rev. Int. 8, 573–603.

McKenzie, G.J., Craig, N.L., 2006. Fast, easy and efficient: site-specific insertion of transgenes into enterobacterial chromosomes using Tn7 without need for selection of the insertion event. BMC Microbial. 6, 39.

Phue, J.N., Lee, S.J., Trinh, L., Shiloach, J., 2008. Modified *E. coli* B (BL21), a superior producer of plasmid DNA compared with *E. coli* K (DH5α). Biotechnol. Bioeng. 101, 831–836.

Postma, P., Lengeler, J., Jacobson, G., 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. 57, 543.

Ren, D., Zuo, R., Barrios, A.F.G., Bedzyk, L.A., Eldridge, G.R., Pasmore, M.E., Wood, T.K., 2005. Differential gene expression for investigation of *E. coli* biofilm inhibition by plant extract ursolic acid. Appl. Environ. Microbiol. 71, 4022–4034.

Shiloach, J., Kaufman, J., Guillard, A., Fass, R., 1996. Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by *E. coli* BL21 ((DE3) and *E. coli* JM109. Biotechnol. Bioeng. 49, 421–428.

Son, Y.-J., Phue, J.-N., Trinh, L.B., Lee, S.J., Shiloach, J., 2011. The role of Cra in regulating acetate excretion and osmotic tolerance in E. coli K-12 and *E. coli* B at high density growth. Microb. Cell Fac. 10, 52.

Teodósio, J., Simões, M., Mergulhão, F., 2012. The influence of nonconjugative *E. coli plasmids on biofilm formation and resistance*. J. Appl. Microbiol. 113, 373–382.

Wang, G., Boulton, P.G., Chan, N.W.C., Palcic, M.M., Taylor, D.E., 1999. Novel Helicobacter pylori α1, 2-fucosyltransferase, a key enzyme in the synthesis of Lewis antigens. Microbiology 145, 3245–3253.

Waugh, D.S., 2005. Making the most of affinity tags. Trends Biotechnol. 23, 316–320.

Xia, X.X., Han, M.J., Lee, S.Y., Yoo, J.S., 2008. Comparison of the extracellular proteomes of *E. coli B and K-12 strains during high cell density cultivation*. Proteomics 8, 2089–2103.

Yazdani, S.S., Gonzalez, R., 2007. Anaerobic fermentation of glycerol: a path to economic viability for the biofuels industry. Curr. Opin. Biotech. 18, 213–219.