농학박사학위논문

# 대장균의 대사과정 재설계에 의한 2'-fucosyllactose 의 생산

# Production of 2'-Fucosyllactose, a Human Milk Oligosaccharide by Metabolically Engineered *Escherichia coli*

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A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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**February 2016**


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# Production of 2'-Fucosyllactose, a Human Milk Oligosaccharide by Metabolically Engineered *Escherichia coli*

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**the requirements for the degree of**

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**by**

**Young-Wook Chin**

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이 논문을 농학박사학위논문으로 제출함

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**ABSTRACT**

Human milk has many biological functions including prebiotic effects, prevention of pathogenic infection, modulation of immune systems and anti-inflammatory effects since it contains a high dose of unique oligosaccharides which are relatively rare in other mammalian milks. Especially, 2'-fucosyllactose (2-FL) which is one of the most abundant ones among 200 different oligosaccharides has received great attention in terms of nutraceutical and pharmaceutical purpose. As biosynthesis of 2-FL with microbial systems has been proven to be more efficient than chemical or enzymatic synthesis, several studies on microbial production of 2-FL have been reported recently.

Biosynthesis of 2-FL is influenced by several factors including intracellular lactose availability, activity of α-1,2-fucosyltransferase and supply of guanosine 5'-diphosphate (GDP)-L-fucose as a donor of fucose. In this study, *Escherichia coli* BL21star(DE3) was engineered by focusing on the key factors for efficient production of 2-FL. Specifically, the activity of β-galactosidase was attenuated through deletion of the whole endogenous lactose operon and introduction of the modified lactose operon containing *lacZΔM15*. Expression of α-1,2-fucosyltransferase (FucT2) from *Helicobacter pylori* along with the genes for the *de novo* pathway of GDP-L-fucose (*manB*, *manC*, *gmd*

and *wcaG*) allowed engineered *E. coli* BL21star(DE3) to produce 2-FL with 3-times enhanced yield of 0.091 g 2-FL/g lactose than the non-engineered *E. coli* BL21star(DE3) strain.

The titer and yield of 2-FL were improved by adding the three aspartate molecules at the N-terminal of FucT2. 6.4 g/L of a maximum concentration of 2-FL was obtained in fed-batch fermentation of the β-galactosidase attenuated *E. coli* strain (ΔL M15) expressing three aspartate tagged FucT2 with the genes involved in the *de novo* pathway of GDP-L-fucose. Moreover, expression of putative α-1,2-fucosyltransferase (WcfB) from *Bacteroides fragilis* in the ΔL M15 strain led to a 4-fold enhancement in 2-FL concentration compared to that of ΔL M15 exspressing FucT2. However, an unknown by-product also accumulated in the media together with 2-FL. Since the unknown by-product was composed of galactose, complete elimination of the residual β-galactosidase activity was attempted. Finally, 2-FL titer was further increased up to 15.4 g/L with 2-FL yield of 0.858 g/g lactose and productivity of 0.530 g/L/h by fed-batch fermentation of the β-galactosidase deleted *E. coli* strain (ΔL YA) expressing WcfB and the enzymes involved in the *de novo* pathway for GDP-L-fucose biosynthesis.

An engineered *E. coli* strain able to produce 2-FL from fucose, lactose

and glycerol was also constructed by introducing the *fkp* gene coding for fucokinase/GDP-L-fucose pyrophosphorylase from *B. fragilis* involved in the *salvage* GDP-L-fucose biosynthetic pathway and expressing FucT2 from *H. pylori*. Employment of the LacZ-attenuation mutant (ΔL M15) and LacZ-deletion mutant (ΔL YA) allowed a significant improvement of 2-FL titer and yield based on lactose. In addition, 2-FL titer and yield based on fucose were further increased by deletion of the *fucI-fucK* gene cluster encoding fucose isomerase and fuculose kinase in the chromosomes of the LacZ mutants. Fed-batch fermentation of the ΔL YA strain deleted *fucI-fucK* and expressing Fkp and FucT2 resulted in 23.1 g/L of 2-FL concentration with yields of 0.37 mole 2-FL/mole lactose, 0.36 mole 2-FL/mole fucose and productivity of 0.39 g/L/h. These results suggest that microbial production of 2-FL might be a promising method for mass production of 2-FL.

**Keywords**: 2'-Fucosyllactose (2-FL); engineered *Escherichia coli*; GDP-L-fucose; *lac* operon; fucosyltransferase; aspartate tag; fucose

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# Chapter 1

# Literature review:

## 1.1. Human milk

In the late 1800s, when first-year mortality rates of the total infant were as high as 30%, it was found that breast-fed infants had a much higher chance of survival and had lower pathogenesis of infectious diarrhea and many other diseases than formula-fed infants. Some pediatricians and microbiologists had begun to recognize the "mysterious effects of human milk" and discovered differences in the bacterial population in the feces of breast-fed compared with formula-fed infants. However, which components of human milk affect the bacterial population in the infant's intestine had remained as mystery until more than half a century later. During this period, breast feeding in Europe and America decreased rapidly (Riordan & Countryman, 1980). By the 1950s, many mothers had considered breastfeeding was something practiced by the uneducated and those of lower classes. On the other hands, infant formula feeding was considered superior to breast feeding (Nathoo & Ostry, 2009). Since 1960s, the functionality has been unveiled and breast feeding was revived in Canada and the US, especially among more educated and affluent women. Nowadays, the World Health Organization (WHO) recommends exclusive breastfeeding for the first six months of an infant`s life.

Human milk is a very complex fluid with a number of components and multiple functions. The composition of human milk varies with the

stage of lactation period, as well as the mother`s age, food consumption, parity, and environment (Kulski & Hartmann, 1981). However, mature human milk typically contains 30-40 g/L fat, 15-17 g/L protein, 60-70 g/L carbohydrate (Armand et al., 1996), with these roughly categorized components subdivided into many valuable constituents.

Human milk supplies the primary source of nutrition for newborns and furthermore, breastfeeding continues to provide health benefits into and after early childhood. These benefits include prebiotic effects, inhibition of pathogen infection, reduction of inflammatory disease, beneficial effects of neurological development, and enhancement of vaccine responses (Boehm & Stahl, 2007; Hahn-Zoric et al., 2008; Lanting et al., 1994; Severin & Wenshui, 2005).

As shown in Table 1.1, contents of the most of human milk constituents are similar to those of bovine milk, however, oligosaccharides content of human milk is a 100 - 300 fold much higher than that of bovine milk.

## 1.2. Human milk oligosaccharides (HMOs)

### 1.2.1. Structure of HMOs

Compared with milk from other species, human milk is unique in terms of its content of complex oligosaccharides (Kunz & Rudloff, 1993). Quantitatively, the oligosaccharides are the third largest component in human milk after lactose and fat. Based on numerous research, the general agreement is that this key component represents 5-15 g/L of mature milk and approximately 22 g/L of colostrum (Kunz et al., 2000; Newburg, 1997; Rivero-Urgell & Santamaria-Orleans, 2001).

Up to date, approximately 200 different kinds of HMOs have been reported and more than 90 HMO structures have been published (Ninonuevo et al., 2006). Biological functions of oligosaccharides are closely related to their conformation. Because HMOs are not digested in the infant small intestine, they maintain the functions that depend on their structural configuration, which explains why such a large number of different HMOs are present (Miller & McVeagh, 2007). Basically, HMOs are composed of the five monosaccharides; D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose, and sialic acid [*N*-acetylneuraminic acid (NeuAc)] with lactose usually found at the reducing end. Synthesis of HMOs in the breasts begins with a lactose molecule. The lactose is elongated by an enzymatic attachment

of GlcNAc residues linked in $\beta$ 1-3 or $\beta$ 1-6 linkage to a Gal residue followed by further addition of Gal in a $\beta$ 1-3 (lacto-*N*-biose) or $\beta$ 1-4 bond (*N*-acetyllactosamine) (Fig 1.1A). In this way, the core structures of HMO are synthesized. Additional modification results from attachments of lactosamine, fucose, and/or NeuAc residues at different positions of the core region and the core elongation chain (Kunz et al., 2000; McVeagh & Miller, 2008). Extension with lacto-*N*-biose appears to terminate the chain, while *N*-acetyllactosamine can be further elongated by adding one of the two disaccharides. A β1-6 linkage between two disaccharide units introduces chain branching. Branched structures are designated as iso-HMO; linear structures without branches as para-HMO (Fig 1.1B). Lactose or the elongated oligosaccharide chain can be fucosylated in α1-2, α1-3 or α1-4 linkage and/or sialylated in α2-3 or α2-6 linkage (Fig 1.1C–E). Some HMOs occur in several isomeric forms, e.g. lacto-*N*-fucopentaose (LNFP, Figure 1.1D) or sialyllacto-*N*-tetraose (LST, Fig 1.1E) (Bode, 2012).

### 1.2.2. Functionalities of HMOs

To date, numerous conclusive studies have been reported that HMOs have many beneficial effects on human's health (Bode, 2012; Castanys-Muñoz et al., 2013a; Kunz & Rudloff, 2006; Newburg & Grave, 2014). The functionality revealed at first was a prebiotic effect.

Prebiotics are defined as “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health” (Roberfroid, 2007). To be prebiotics there are some prerequisites; prebiotics are resistant to gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption. HMOs meet all criteria given that an absorption rate of ~1% can be neglected in this specific context and that the great majority of HMOs reach the distal small intestine and colon intact at high concentrations (Bode, 2012). HMOs are a bifidus (bifidogenic) factor which stimulates growth of *Bifidobacterium* and the effects of HMOs on growth of *Bifidobacterium* is depending on the strain and type of HMO. For example, *B. longum* subsp. *infantis* grows well when HMOs are offered as a sole carbon source and *B. infantis* consumes HMOs completely along with mono- and disaccharide degraded from HMOs. Compared with *B. infantis*, *B. bifidum* grows slightly slower on HMOs and leaves behind at least some of the monosaccharide degradation products. In contrast, *B. longum* subsp. *longum* and *B. breve* hardly grow on HMOs at all and metabolize only lacto-*N*-tetraose (Asakuma et al., 2011; LoCascio et al., 2007; Marcobal et al., 2010).

The other major function of HMOs is an inhibitory effect against

infection by pathogens such as bacteria, viruses, toxins and parasites. Most pathogens need to adhere to mucosal surfaces for colonization or invasion to the host and cause disease. In the initial stage of the infective process, HMOs act as inhibitors for adhesion of pathogenic bacteria and viruses to epithelial surfaces as HMOs are soluble receptor analogues of epithelial cell-surface carbohydrates (Bode, 2006; Boehm & Stahl, 2007; Kunz & Rudloff, 2006). For example, an antiadhesive effect against *Campylobacter jejuni* infection which is one of the most common pathogens causing bacterial diarrhea and infant mortality is the most representative antibacterial example (Morrow et al., 2004; Ruiz-Palacios et al., 2003). The antiadhesive effects can be also applied to certain protozoan parasites like *Entamoeba histolytica*, which causes amoebic dysentery or amoebic liver abscess (Jantscher-Krenn et al., 2012; Pritt & Clark, 2008). The fucosylated HMOs also interact with the guanylyl cyclase receptor for the stable toxin of *E. coli*, thereby inhibiting toxin binding (Crane et al., 1994). Moreover, HMOs possess binding epitopes of selectin ligands such as sialyl Lewis X and sialyl Lewis a. Therefore, they reduce leukocyte binding to endothelial cells and serve as anti-inflammatory components influencing inflammatory processes (Kunz & Rudloff, 2006; Kunz et al., 2000; Schumacher et al., 2006).

Lastly, HMOs potentially act as immune modulators and nutrients for brain development. HMOs modulate lymphocyte cytokine production, potentially leading to a more balanced Th1/Th2 response. HMOs also provide sialic acid as potentially essential nutrients for brain development and cognition (Bode, 2012).

## 1.3. 2'-Fucosyllactose (2-FL)

Among the 200 different kinds of HMOs, 50-80% percent of the HMOs are fucosylated and 10-20% are sialylated (Kunz et al., 2000; Ninonuevo et al., 2006). Especially, 2-FL is the most abundant oligosaccharide in human milk and thereby it is a major sugar involving a number of various physiological properties of HMOs (Castanys-Muñoz et al., 2013b; Chaturvedi et al., 2001). The growing recognition of the roles of 2-FL in fundamental biological processes and their potential applications as functional foods and therapeutics has generated a need for larger amounts of 2-FL (Han et al., 2012).

Methods for the chemical synthesis of 2-FL have been developed (Gokhale et al., 1990; Kameyama et al., 1991; Kretzschmar & Stahl, 1998; Zhao et al., 2010). However, the structural complexity makes the chemical synthesis of 2-FL a challenging task with low yields and use of highly toxic organic solvents. Moreover, the synthesis is expensive, time-consuming and requires multiple protection and de-protection steps (Bulter & Elling, 1999; Kretzschmar & Stahl, 1998). Enzymatic synthesis using fucosyltransferase is more efficient because fucosyltransferase can carry out highly stereoselective and regioselective bond formation. However, this method also has the significant weakness that GDP-L-fucose is one of the most expensive

nucleotide sugars used as a fucose donor (Dumon et al., 2004). Moreover a purification process of fucosyltransferase is needed. On the other hand, microbial production is more feasible for industrial production of 2-FL because it only needs a simple fermentation process with cheap substrate.

## 1.4. Biosynthesis of GDP-L-fucose and 2-FL

2-FL can be synthesized by enzymatic fucosylation of lactose with GDP-L-fucose used as fucose donor by α-1,2-fucosyltransferase (Fig. 1.3). Since GDP-L-fucose is a very expensive nucleotide sugar ($85/mg), supplementation of GDP-L-fucose is a crucial factor for 2-FL production. GDP-L-fucose can be synthesized through two kinds of metabolic pathways, which take place in the cytoplasm (Fig. 1.4) (Becker & Lowe, 2003). These two pathways are referred to the *de novo* pathway and the *salvage* pathway (Tonetti et al., 1998). The *salvage* pathway, accounting for approximately 10% of GDP-L-fucose biosynthesis, utilizes the free cytosolic fucose as a substrate, which is derived from lysosomal degradation or from an extracellular source. In contrast, the *de novo* pathway, accounting for 90% of the total GDP-L-fucose production (Becker & Lowe, 2003), utilizes GDP-D-mannose as a substrate, which is synthesized via the fructose and mannose metabolism. As depicted in Fig. 1.4, the *de novo* pathway is catalyzed by three enzymes [mannose-6-phosphate isomerase (ManA, E.C. 5.3.1.8), phosphomannomutase (ManB, E.C. 5.4.2.8) and mannose-1-phosphate guanylyltransferase (ManC, E.C. 2.7.7.13)] and converts fructose-6-phosphate to GDP-D-mannose. The *de novo* pathway has been identified in many organisms including bacteria, plants and mammals (Ginsburg, 1960; Liao & Barber, 1971; Overton & Serif,

1981). The *de novo* pathway includes conversion of GDP-D-mannose to GDP-L-fucose through three reactions catalyzed by two enzymes, GDP-D-mannose-4,6-dehydratase (GMD, E.C. 4.2.1.47) and GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase (GMER, also known as GDP-L-fucose synthetase and WcaG, FX in *E. coli*, human, respectively, E.C. 1.1.1.271) (Andrianopoulos et al., 1998; Sullivan et al., 1998; Tonetti et al., 1996; Tonetti et al., 1998). In the first step, GMD converts GDP-D-mannose to an unstable intermediate, GDP-4-keto-6-deoxy-D-mannose, by a dehydration reaction. This enzymatic reaction involves oxidation of a hydroxyl group at C-4 of mannose to a keto group and reduction of a hydroxyl group at C-6 of mannose to a methyl residue. This concerted oxidation and reduction involves an intramolecular hydride ($H^+$) transfer from the C-4 to the C-6 on the mannose ring by a tightly bound cofactor nicotinamide adenine dinucleotide phosphate ($NADP^+$) (Oths et al., 1990). The product of GMD, GDP-4-keto-6-deoxymannose, is generated by catalysis with the dual functional epimerase-reductase enzyme WcaG (Andrianopoulos et al., 1998). In the first step, GDP-4-keto-6-deoxy-D-mannose is epimerized at C-3 and C-5, which leads to a change from D- to L-configuration and produces GDP-4-keto-6-deoxy-L-galactose. The 4-reductase activity of the WcaG protein catalyzes a hydride transfer from the NADPH cofactor to a keto group at C-4, yielding GDP-L-

fucose and $NADP^+$ (Menon et al., 1999).

An alternative pathway of the GDP-L-fucose biosynthesis is the *salvage* pathway that reutilizes L-fucose generating from the degradation of glycoproteins and glycolipids (Coffey et al., 1964). This pathway involves the phosphorylation of L-fucose by L-fucokinase (Fuk, EC 2.7.1.52) at the anomeric position to form L-fucose-1-phosphate with ATP as a cofactor (Butler & Serif, 1985; Park et al., 1998). And then, L-fucose-1-phosphate with GTP is condensed by L-fucose-1-phosphate guanylyltransferase (Fpgt, EC 2.7.7.30) to form GDP-L-fucose (Ishihara & Heath, 1968). The *salvage* pathway had been considered to exist only in eukaryotes until the discovery of L-fucokinase/GDP-L-fucose pyrophosphorylase (Fkp) in a human symbiont bacteria, *B. fragilis* (Coyne et al., 2005). The Fkp enzyme is a bifunctional enzyme catalizing both reactions in the *salvage* pathway of GDP-L-fucose. Introduction of Fkp and α-1,3-fucosyltransferase from *Helicobacter pylori* has been led to synthesis of a library of fucosylated trisaccharide bearing a wide variety of functional groups at the fucose C-5 position using fucose analogues as precursors (Wang et al., 2009).

## 1.5. Previous studies on microbial production of GDP-L-fucose and 2-FL

Microbial production would be more feasible for commercial scale production of 2-FL than chemical and enzymatic methods. Thus, a number of studies for microbial production have been reported to produce 2-FL and GDP-L-fucose, its key precursor. *Saccharomyces cerevisiae* was employed as a GDP-L-fucose producer since *S. cerevisiae* is known to have a rich pool of GDP-D-mannose. The expression of the *E. coli* genes coding for Gmd and WcaG was controlled by the galactose-inducible promoters of GAL1 and GAL10, respectively. As a result of flask culture with galactose as a carbon source, 0.2 mg/L GDP-L-fucose was produced by recombinant *S. cerevisiae* (Mattila et al., 2000). In the other research, batch fermentation of engineered *E.coli* BL21star(DE3) overexpressing the *gmd* and *wcaG* genes from *E. coli* K12 resulted in 39 mg/L GDP-L-fucose with glucose as a sole carbon source (Byun et al., 2007). Coexpression of ManB and ManC involved in the GDP-D-mannose metabolism with Gmd and WcaG in recombinant *E. coli* gave the production of 170 mg/L GDP-L-fucose titer in fed-batch fermentation from glucose (Lee et al., 2009b). The final step catalyzed by WcaG requires NADPH as a reducing power, sufficient supply of NADPH

which was assessed by overexpression of NADPH-dependent metabolic enzymes such as isocitrate dehydrogenase (Icd), malate dehydrogenase (MaeB) and G6PDH. The optimized strain of *E. coli* BL21star (DE3) overexpressing ManB, ManC, Gmd, WcaG and G6PDH produced 235 mg/L GDP-L-fucose (Lee et al., 2011). In addition, it was examined that which guanosine nucleotide biosynthetic enzyme is most effective in GDP-L-fucose biosynthesis since guanosine 5'-triphosphate is used in the step catalyzed by ManC as a source of guanosine nucleotide. As a result, GDP-L-fucose titer increased up to 305 mg/L in fed-batch fermentation by coexpression of guanosine-inosine kinase (Gsk) (Lee et al., 2012b). Using GMP and mannose as starting materials, 18.4 g/L of GDP-L-fucose accumulated in a bioconversion process by a bacterial coupling system composed of three engineered *E. coli* strains overexpressing the GDP-L-fucose biosynthetic enzymes and *Corynebacterium ammoniagenes* producing GTP (Koizumi et al., 2000).

In previous research concerning production of 2-FL, it was reported that *E. coli* JM107(DE3) overexpressing RcsA (a positive regulator for the *de novo* GDP-L-fucose biosynthesis) and deleting WcaJ produced up to 11 g/L of 2-FL extracellularly (Drouillard et al., 2006). It was also reported that engineered *E. coli* JM109 or JM109(DE3) strains for

whole cell biosynthesis of 2-FL were constructed by direct overexpression of the genes involved in the *de novo* GDP-L-fucose biosynthesis and α-1,2-fucosyltransferase from *H. pylori* and therefore, 10 g/L of 2-FL accumulated in the medium (Baumgärtner et al., 2013a).

Several attempts have been made to produce GDP-L-fucose or fucosyllactose from fucose via the *salvage* pathway. Recombinant *Saccharomyces cerevisiae* expressing fucokinase/fucose-1-phosphate guanylyltransferase (Fkp) produced 0.14 mg of GDP-L-fucose from exogenous 2.46 g/L of L-fucose in the culture medium (Liu et al., 2011). Engineered *E. coli* BW25113(DE3) overexpressing fucose permease (FucP), Fkp and deleting the fuculose-1-phosphate aldolase (FucA) gene was able to convert L-fucose to 0.22 g/L GDP-fucose. The additional expression of α-1,2- or α-1,3-fucosyltransferase led to biosynthesis of 2′- or 3′-fucosyllactose (Hüfner et al., 2009). Chromosomal integration of the *fkp* gene along with six genes involved in the *de novo* pathway (*manB*, *manC*, *gmd* and *wcaG*) and fucosyltransferase (*futC*) was attempted in *E. coli* JM109 and therefore, 388 mg 2-FL per g cell was produced in flask cultivation (Baumgärtner et al., 2013a). In another report, 122 mg/L of GDP-L-fucose was produced by a fed-batch fermentation of the engineered *E. coli* expressing Fkp with the genes involved in the guanosine nucleotides

biosynthesis (*gpt*, *gmk* and *ndk*) (Zhai et al., 2015).

## 1.6. Objectives of the dissertation

This dissertation was focused on development of microbial systems for efficient production of 2-FL by metabolically engineered *E. coli*. The specific objectives of this research are listed:

1) To attenuate the β-galactosidase activity of *E. coli* BL21star(DE3) expressing the genes involved in the *de novo* GDP-L-fucose biosynthetic pathway (*gmd*, *wcaG*, *manB* and *manC*) and the gene (*fucT2*) coding for α-1,2-fucosyltransferase from *H. pylori* for production of 2-FL from glycerol and lactose,
2) To investigate the effects of attachment of the aspartate tags to α-1,2-fucosyltransferase (FucT2) from *H. pylori* on 2-FL production,
3) To improve 2-FL production through replacement of α-1,2-fucosyltransferase from *B. fragilis* and complete deletion of *lacZ*,
4) To produce 2-FL from fucose, glycerol and lactose via the *salvage* pathway of GDP-L-fucose biosynthesis

Table 1.1. Composition of human and bovine milk

| Contents | Human milk | Bovine milk |
|---|---|---|
| **Fat (g/L)** | | |
| **Total (g/L)** | **42** | **38** |
| Fatty acids-length ≤8C (%) | trace | 6 |
| Polyunsaturated fatty acids (%) | 14 | 3 |
| **Protein (g/L)** | | |
| **Total** | **11** | **33** |
| Casein 0.4 | 3 | 25 |
| a-lactalbumin | 3 | 1 |
| Lactoferrin | 2 | Trace |
| IgA | 1 | 0.03 |
| IgG | 0.01 | 0.6 |
| Lysozyme | 0.5 | Trace |
| Serum albumin | 0.5 | 0.3 |
| β-lactoglobulin | - | 3 |
| **Carbohydrate (g/L)** | | |
| Lactose | 70 | 48 |
| **Oligosaccharides** | **5 - 15** | **0.05** |
| **Minerals (g/L)** | | |
| Calcium | 0.3 | 1.25 |
| Phosphorus | 0.14 | 0.93 |
| Sodium | 0.15 | 0.47 |
| Potassium | 0.55 | 1.55 |
| Chlorine | 0.43 | 1.03 |

Table 1.2. Contents of major carbohydrates in human milk (Smilowitz et al., 2013)

| Metabolite | Contents (μmole/L) |
|---|---|
| 2'-Fucosyllactose (2-FL) | $2.50 \times 10^3 \pm 1.70 \times 10^3$ |
| 3'-Fucosyllactose (3-FL) | $2.10 \times 10^3 \pm 1.20 \times 10^3$ |
| 3'-Sialyllactose (3-SL) | 144 ± 43.7 |
| 6'-Sialyllactose (6-SL) | 119 ± 54.9 |
| Fucose | 182 ± 135 |
| Galactose | 92.3 ± 49.1 |
| Glucose | $1.50 \times 10^3 \pm 530$ |
| Lactodifucotetraose (LDFT) | 266 ± 199 |
| Lacto-N-neotetraose (LNnT) | 121 ± 67.5 |
| Lacto-N-fucopentaose (LNFP Ⅰ) | 189 ± 159 |
| Lacto-N-fucopentaose (LNFP Ⅱ) | 210 ± 168 |
| Lacto-N-fucopentaose (LNFP Ⅲ) | 233 ± 74.0 |
| Lacto-N-tetraose (LNT) | 506 ± 284 |
| Lactose | $170 \times 10^3 \pm 7.30 \times 10^3$ |

Table 1.3. Previous reports on microbial production of GDP-L-fucose

| Host strain | Substrate | Culture type | GDP-L-fucose | | Characteristics | Reference |
|---|---|---|---|---|---|---|
| | | | Concentration (g/L) | Productivity (g/L-h) | | |
| *E. coli* NM522 | GMP Mannose | Bioconversion by two-step reaction | 18.4 | 0.84 | Bacterial coupling of *Corynebacterium ammoniagenes* and three recombinant *E. coli* strains | (Koizumi et al., 2000) |
| *S. cerevisiae* | Dextrose Galactose | Batch | $0.02 \times 10^{-2}$ | - | Expression of the *gmd* and *wcaG* genes from *E. coli* | (Mattila et al., 2000) |
| *E. coli* BL21s(DE3) | Glucose | Fed-batch | 0.35 | 0.02 | Coexpression of the *gsk* gene coding guanosine-inosine kinase | (Lee et al., 2012b) |
| *C. glutamicum* | Glucose Mannose | Batch | 0.09 | - | Expression of the *gmd* and *wcaG* genes from *E. coli* | (Chin et al., 2013) |
| *E. coli* JM109(DE3) | Fucose | Batch | 0.22 | - | Deletion of *fucA,* overexpression of *fkp* and *fucP* | (Hüfner et al., 2009) |
| *S. cerevisiae* | Fucose | Batch | $0.14 \times 10^{-3}$ | - | Expression of *fkp* | (Liu et al., 2011) |
| *E. coli* JM109(DE3) | Fucose | Batch | 0.39 | - | Expression of *fkp* | (Baumgärtner et al., 2013b) |
| *E. coli* BL21s(DE3) | Glucose Fucose | Fed-batch | 0.12 | - | Overexpression of *fkp* with genes involved in guanosine nucleotide biosynthesis (*gpt*, *gmk* and *ndk*) | (Zhai et al., 2015) |

Table 1.4. Previous reports on microbial production of 2-FL

| Strain | Substrate | Culture type | Extracellular 2-FL | | Characteristics | Reference |
|---|---|---|---|---|---|---|
| | | | Concentration (g/L) | Productivity (g/L-h) | | |
| *E. coli* JM107 | Glucose Lactose | Fed-batch | 11.0 (3.00)[a] | 0.24 | Overexpression of *rcsA* and mutant *fucT2* and disruption of *wcaJ* | (Drouillard et al., 2006) |
| *E. coli* JM109(DE3) | LB Lactose | Batch | 1.23 | - | Overexpression of *manB*, *manC*, *gmd* and *wcaG* with *fucT2* | (Lee et al., 2012a) |
| *E. coli* JM109 | Glycerol Lactose | Fed-batch | 10.2 (10.2)[a] | 0.43 | Chromosomal expression of *manB*, *manC*, *gmd* and *wcaG* with *fucT2* | (Baumgärtner et al., 2013a) |

[a]Intracellular concentration of 2-FL

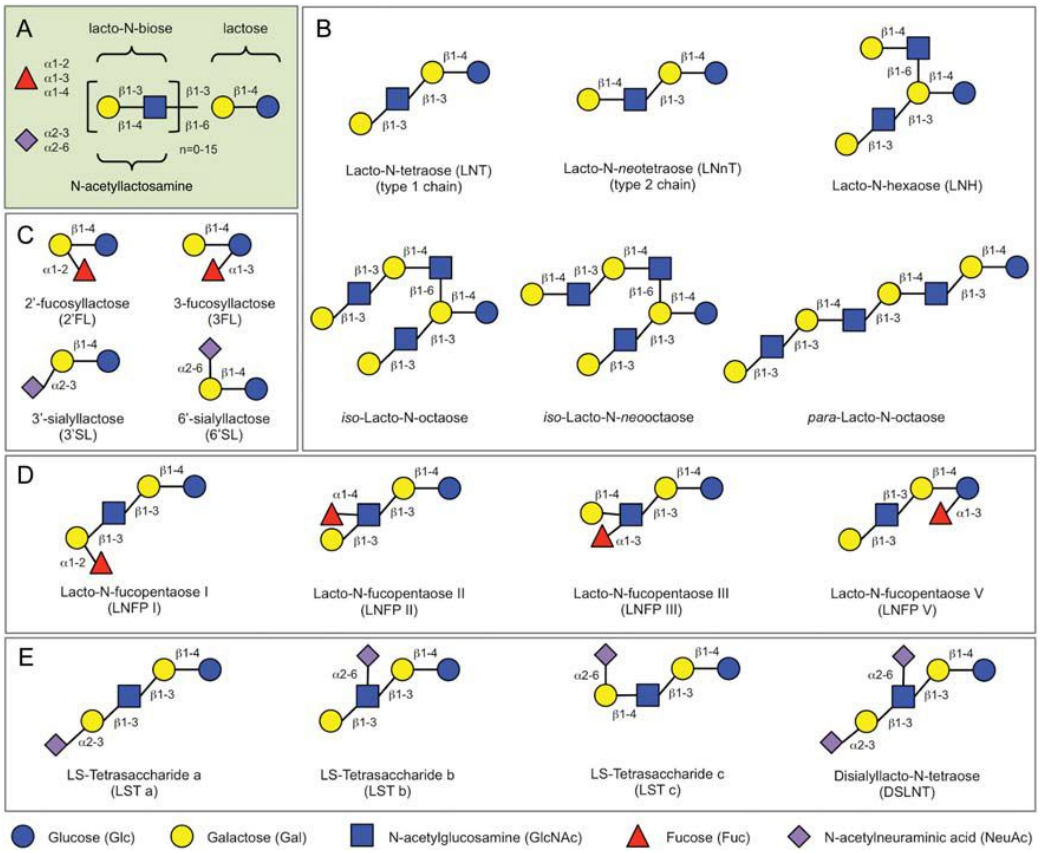

A
lacto-N-biose
lactose
α1-2
α1-3
α1-4
α2-3
α2-6
β1-3
β1-4
β1-6
n=0-15
N-acetyllactosamine
B
Lacto-N-tetraose (LNT)
(type 1 chain)
Lacto-N-neotetraose (LNnT)
(type 2 chain)
Lacto-N-hexaose (LNH)
iso-Lacto-N-octaose
iso-Lacto-N-neooctaose
para-Lacto-N-octaose
C
2'-fucosyllactose
(2'FL)
3-fucosyllactose
(3FL)
3'-sialyllactose
(3'SL)
6'-sialyllactose
(6'SL)
D
Lacto-N-fucopentaose I
(LNFP I)
Lacto-N-fucopentaose II
(LNFP II)
Lacto-N-fucopentaose III
(LNFP III)
Lacto-N-fucopentaose V
(LNFP V)
E
LS-Tetrasaccharide a
(LST a)
LS-Tetrasaccharide b
(LST b)
LS-Tetrasaccharide c
(LST c)
Disialyllacto-N-tetraose
(DSLNT)
Glucose (Glc)
Galactose (Gal)
N-acetylglucosamine (GlcNAc)
Fucose (Fuc)
N-acetylneuraminic acid (NeuAc)

Fig. 1.1. Representative HMO structures. (A) HMOs follow a basic structural blueprint. (Monosaccharide key is shown at the bottom of the Figure.) (B) Lactose can be fucosylated or sialylated in different linkages to generate trisaccharides. (C) Lactose can be elongated by addition of either lacto-*N*-biose (type I) or *N*-acetyllactosamine (type II) disaccharides. Addition of disaccharides to each other in the β1-3 linkage leads to linear chain elongation (para-HMO); a β1-6 linkage between two disaccharides introduces chain branching (iso-HMO). (D) Elongated type I or II chains can be fucosylated in different linkages to form a variety of structural isomers, some of which have Le blood group specificity (Fig 3). (E) The elongated chains can also be sialylated in different linkages to form structural isomers. Disialylated lacto-*N*-tetraose (bottom right) prevents NEC in neonatal rats (Bode, 2012).

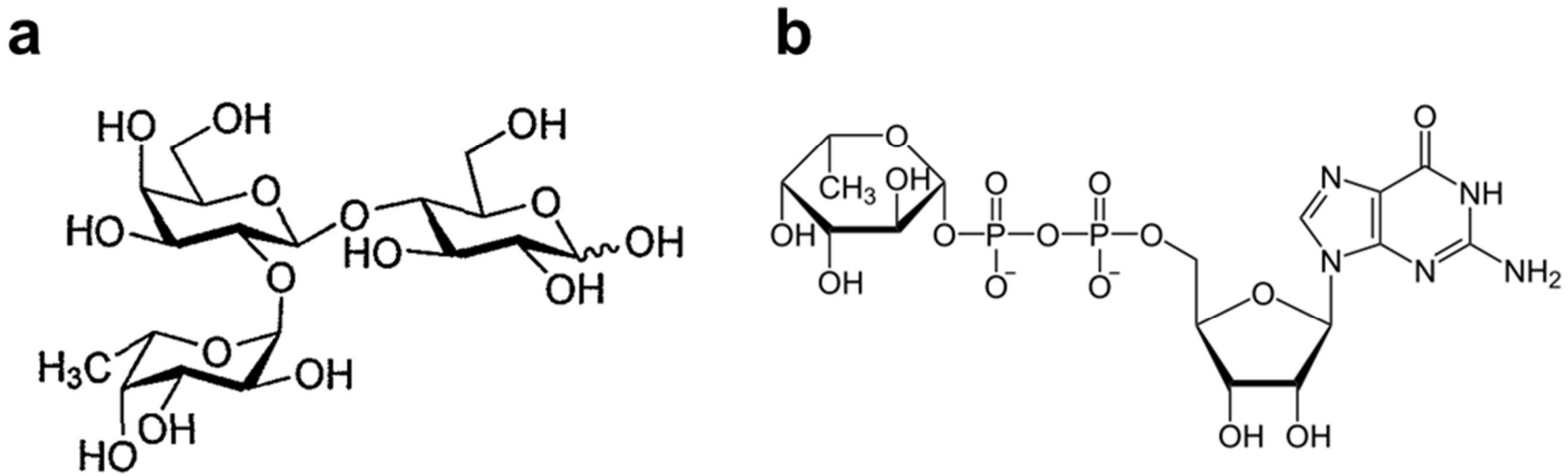



Fig. 1.2. Structure of 2-FL (a) and GDP-L-fucsoe (b)

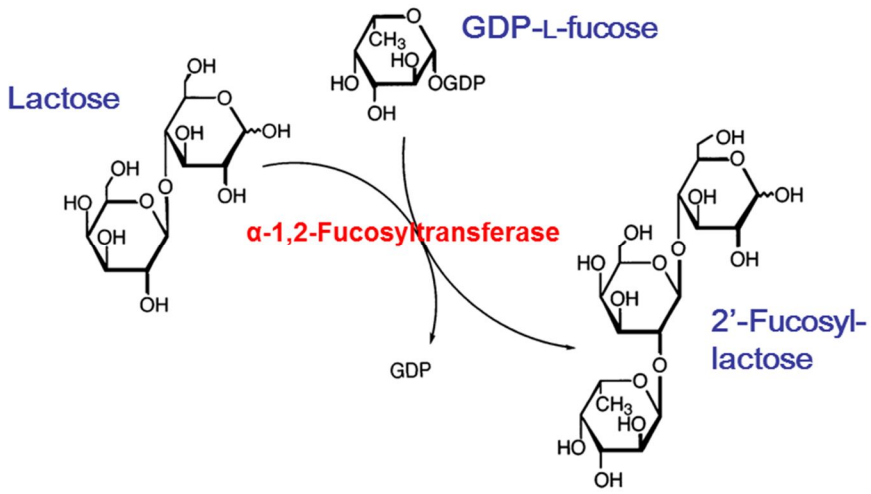



Fig. 1.3. Enzymatic fucosylation of lactose by α-1,2-fucosyltransferase

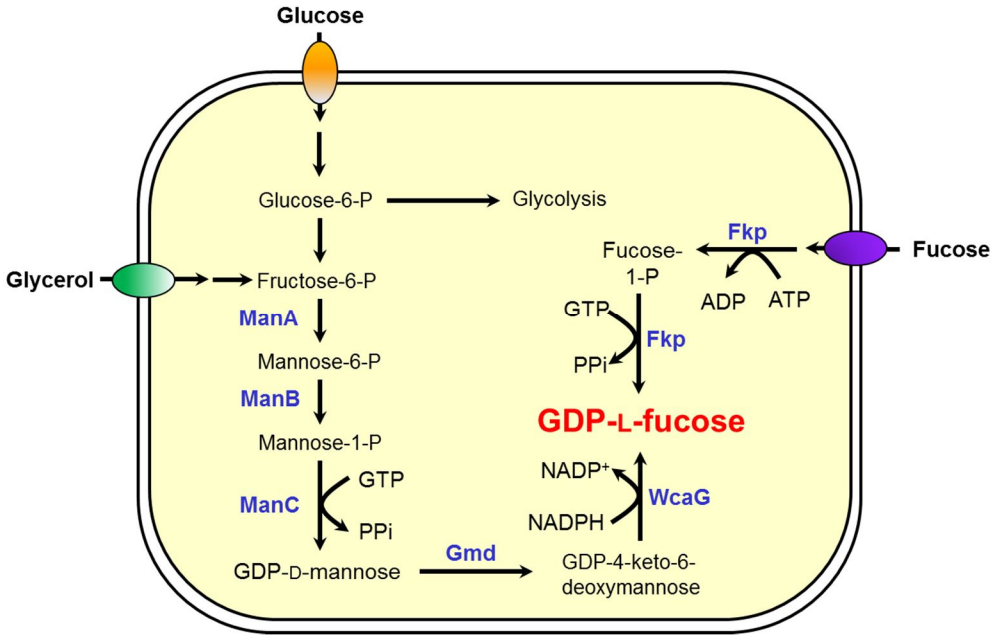



Fig. 1.4. Biosynthesis of GDP-L-fucose via *de novo* and *salvage* pathway. The names of enzymes are abbreviated as follows; ManA, mannose 6-phosphate isomerase; ManB, phosphomannomutase; ManC, mannose 1-phosphate guanylyltransferase; Gmd, GDP-D-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase; Fkp, fucokinase/fucose-1-phosphate guanylyltransferase. PPi and GTP denote diphosphate and guanosine 5'-triphosphate.

# Chapter 2

# Production of 2-FL through modulation of lactose metabolism of engineered *Escherichia coli* BL21star(DE3)

## 2.1. Summary

2-FL is the most abundant oligosaccharide in human milk. To date, most of microbial systems for 2-FL production have been limited to *E. coli* JM strains since they cannot metabolize lactose. In this chapter, *E. coli* BL21star(DE3) was employed as a host for efficient production of 2-FL. To minimize the β-galactosidase activity, the *lac* operon was engineered through deletion of the whole endogenous *lac* operon in *E. coli* BL21star(DE3) and introduction of the modified *lac* 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 *H. 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). As a result, a final 2-FL concentration of 2.6 g/L was obtained in the fed-batch fermentation of the engineered *E. coli* with the modified *lac* operon containing *lacZ⊿M15* expressing ManB, ManC, Gme, WcaG and FucT2.

## 2.2. 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-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., 2013a).

Biosynthesis of 2-FL using 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 relatively 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 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 the *de novo* GDP-L-fucose biosynthesis and α-1,2-fucosyltransferase from *H. pylori* (Baumgärtner et al., 2013a; Lee et al., 2012a).

Intracellular availability of lactose is an essential factor for microbial production of 2-FL. As mentioned above, most of previous studies employed the *E. coli* JM strains (*E. coli* K-12 derivatives) as a host for 2-FL production 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., 2013a; Drouillard et al., 2006; Lee et al., 2012a).

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 a potential problem during the fermentation processes such as bioreactor operation, purification and filtration process. (Mattila-Sandholm & 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., 2012a), suggesting that engineering of the lactose metabolism is required for efficient production of 2-FL in *E. coli* BL21star(DE3).

In this chapter, effects of modulation of the *lac* operon on 2-FL production were 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, fermentation of the engineered *E. coli* expressing Gmd, WcaG, ManB, ManC and FucT2 was carried out in a bioreactor.

## 2.3. Materials and methods

### 2.3.1. Strains and plasmids

All strains, plasmids, and oligonucleotides used in this study are listed in Table 2.1 and 2.2. *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 (ΔL), 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 & Wanner, 2000). A PCR fragment containing the 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 the *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 to codon 42) into the *attTn7* locus was carried out using the Tn7-mediated site-specific transposition method (McKenzie & 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 recombination using the In-Fusion HD Cloning Kit (TAKARA, Japan). After transformation of pGlacZ⊿M15 into the ⊿L strain, transformants were selected at 30°C. The transformants were grown in non-selective media to ensure the chromosomal integration and plasmid pGlacZ⊿M15 was eliminated by incubation at 42°C. The insertion of the *lac* operon bearing *lacZ⊿M15* was confirmed by colony PCR using two primers, F_Tn7 and R_Tn7.

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., 2009a; Lee et al., 2012a). All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

### 2.3.2. 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 were 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 determined using a standard pH electrode (Mettler Toledo, USA) and controlled at 6.8 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 1,200 rpm in order to prevent the deficiency of dissolved oxygen, and air flow rate was maintained at 2 vvm throughout the cultivation.

### 2.3.3. 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 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.

### 2.3.4. 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, 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 an ultrasonic processor (Cole-Parmer, IL, USA). Cells were centrifuged for 10 minutes at 12,000 rpm and 4°C to separate soluble and insoluble fractions. Activity was determined by assaying soluble fractions using the β-Galactosidase Enzyme Assay System (Promega E2000). One unit

of β-galactosidase was defined as the amount of an enzyme able to hydrolyze 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.

## 2.4. Results

### 2.4.1. Construction of LacZ attenuated *E. coli* strain

Intracellular availability of lactose is one of the key factors for efficient biosynthesis of 2-FL in engineered *E. coli*. As illustrated in Fig. 2.1, β-galactosidase (encoded by *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, suggesting 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 mutation in *lacZ* 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 attempted. Fig. 2.2 shows the scheme of deletion of the endogenous *lac* operon and introduction

of the new *lac* operon into *E. coli* BL21star(DE3). First, the region of the endogenous *lac* operon in wild type *E. coli* BL21star(DE3) was deleted by λ-red recombination, which resulted in the *E. coli* ΔL strain (*lac* operon deficient strain). Second, the new *lac* operon fragment containing *lacZΔM15* from *E. coli* K-12 was inserted the downstream site of the *glmS* gene called *attTn7* by Tn7 based transposition, which resulted in the ΔL M15 strain (*lacZΔM15* knock-in strain). As a result of replacement of the *lac* operon, the β-galactosidase activity of ΔL M15 has decreased by 97% compared to that of wild type *E. coli* BL21star(DE3) (Table 2.3).

### 2.4.2. Effects of modulation of *lac* operon on 2-FL production

Subsequently, the effect of *lac* operon replacement on 2-FL production was investigated with three strains, wild type *E. coli* BL21star(DE3), ΔL and ΔL 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. 2.3a). Most of the consumed lactose might be used for cell growth since the control strain showed the highest dry cell mass compared to other strains. In contrast to the control strain, the *lac* operon deficient strain (ΔL) could not consume lactose at all and therefore, could not

produce 2-FL (Fig. 2.3b). 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. 2.3c), which was almost the same as the 2-FL yield of JM109(DE3) (Table 2.4). 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 2.4.

### 2.4.3. Fed-batch fermentation of engineered *E. coli* ⊿L M15 BCGW-F using glycerol pH-stat

Since 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 & Gonzalez, 2007). In addition, glycerol can be utilized simultaneously with lactose because 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* ΔL M15 BCGW-F was carried out with intermittent lactose addition (Fig. 2.4). After complete utilization of glycerol added initially, glycerol was fed by a pH-stat mode. Production of 2-FL was launched by IPTG induction for expression of the 2-FL biosynthetic enzymes as well as addition of lactose as an acceptor for fucosylation. 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 the total amount of 2-FL (data not shown).

## 2.5. 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 concentration of 2-FL because of fast lactose metabolism (Lee et al., 2012a). In the case of the JM109(DE3) strain, the α-peptide of β-galactosidase (synthesized from the 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 with high cell density 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 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 is relatively easy to grow 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) is 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. 2.3, Table 2.4). It seemed that intracellular lactose availability was improved by reducing β-galactosidase activity significantly while maintaining lactose permease (LacY) activity.

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. The FucT2 was expressed as inclusion bodies, suggesting that engineering or replacement of FucT2 might be necessary for more efficient production of 2-FL.

Table 2.1. List of strains and plasmids used in Chapter 2

| 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) | |
| ΔL | BL21star(DE3) *ΔlacZYA* | This study |
| ΔL M15 | BL21star(DE3) *ΔlacZYA Tn7::lacZΔM15* | 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 & Craig, 2006) |
| pGlacZΔM15 | pGRG36 + *lacZΔM15* (*Sma*I) | This study |
| BCGW (pmBCGW) | pETDuet-1 + *manC-manB* (*Nco*I/*Sac*I) + *gmd-wcaG* (*Nde*I/*Kpn*I) | (Lee et al., 2009a) |
| F (pHfucT2) | pCOLADuet-1 + *fucT2* (*Nco*I/*Sac*I) | (Lee et al., 2012a) |

Table 2.2. List of primers used in Chapter 2

| Name | Sequence |
|---|---|
| F_del_lac | CGAATGGCGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGA GTGTAGGCTGGAGCTGCTTCG |
| R_del_lac | TCCTGCGCTTTGTTCATGCCGGATGCGGCTAATGTAGATCGCTGAACTTG ATTCCGGGGATCCGTCGACC |
| 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 |
| R_*Kpn*I_fucT2 | G*GGTACC*ATTAAGCGTTATACTTTTGGGATTTTACCT |

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.

Table 2.3. Specific activities of β-galactosidase of the *lac* operon engineered *E. coli* strains

| Strains | β-Galactosidase activity (U/mg protein) |
|---|---|
| BL21star(DE3) | 3.42 ± 0.04 |
| ΔL | 0.01 ± 0.00 |
| ΔL M15 | 0.11±0.01 |

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

Table 2.4. Summary of batch fermentations of engineered *E. coli* strains bearing modified *lac* operons

| 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 |
| ΔL BCGW-F | 1.74 ± 0.32 | N.D. | 0.0 | - |
| ΔL M15 BCGW-F | 1.23 ± 0.13 | 0.16 ± 0.017 | 1.76 ± 0.263 | 0.091 ± 0.004 |

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.

Table 2.5. Result of fed-batch fermentation of engineered *E. coli* BL21star(DE3) harboring BCGW and F

| Strain | Maximum dry cell weight (g/L) | Maximum 2-FL concentration[b] (g/L) | Yield (g 2-FL/g lactose) | Productivity[a] (g/L·h) |
|---|---|---|---|---|
| ΔL M15 BCGW-F | 73.1 | 2.6 | 0.063 | 0.043 |

[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.

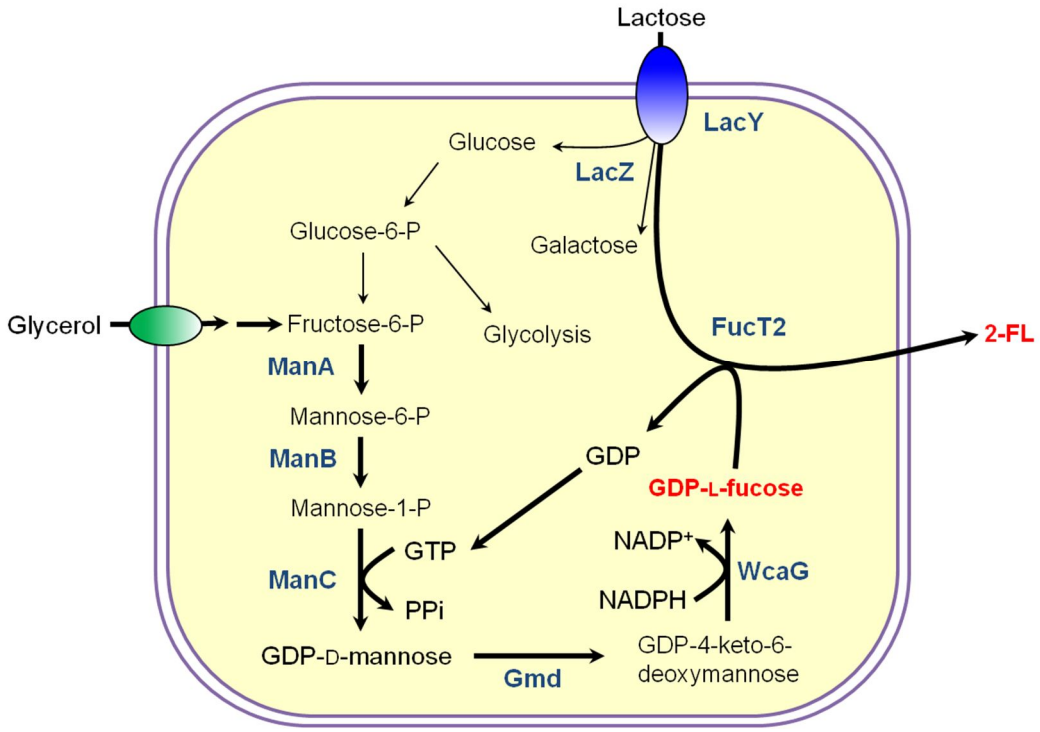



Fig. 2.1. The strategy for production of GDP-L-fucose and 2-FL in engineered *E. coli* ΔL M15 harboring BCGW and F. The names of proteins are abbreviated as follows; LacZ, β-galactosidase; LacY, lactose permease; ManA, mannose 6-phosphate isomerase; ManB, phosphomannomutase; ManC, mannose 1-phosphate guanylyl-transferase; 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.

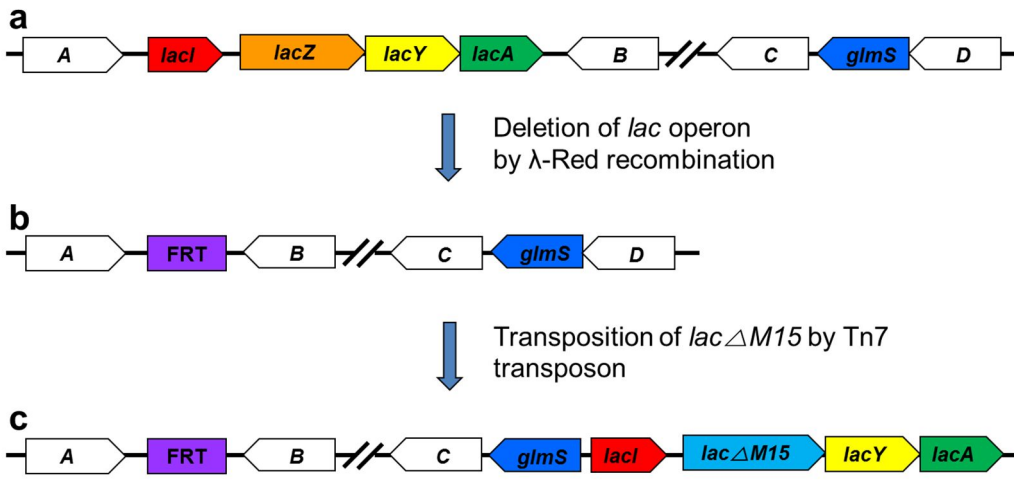
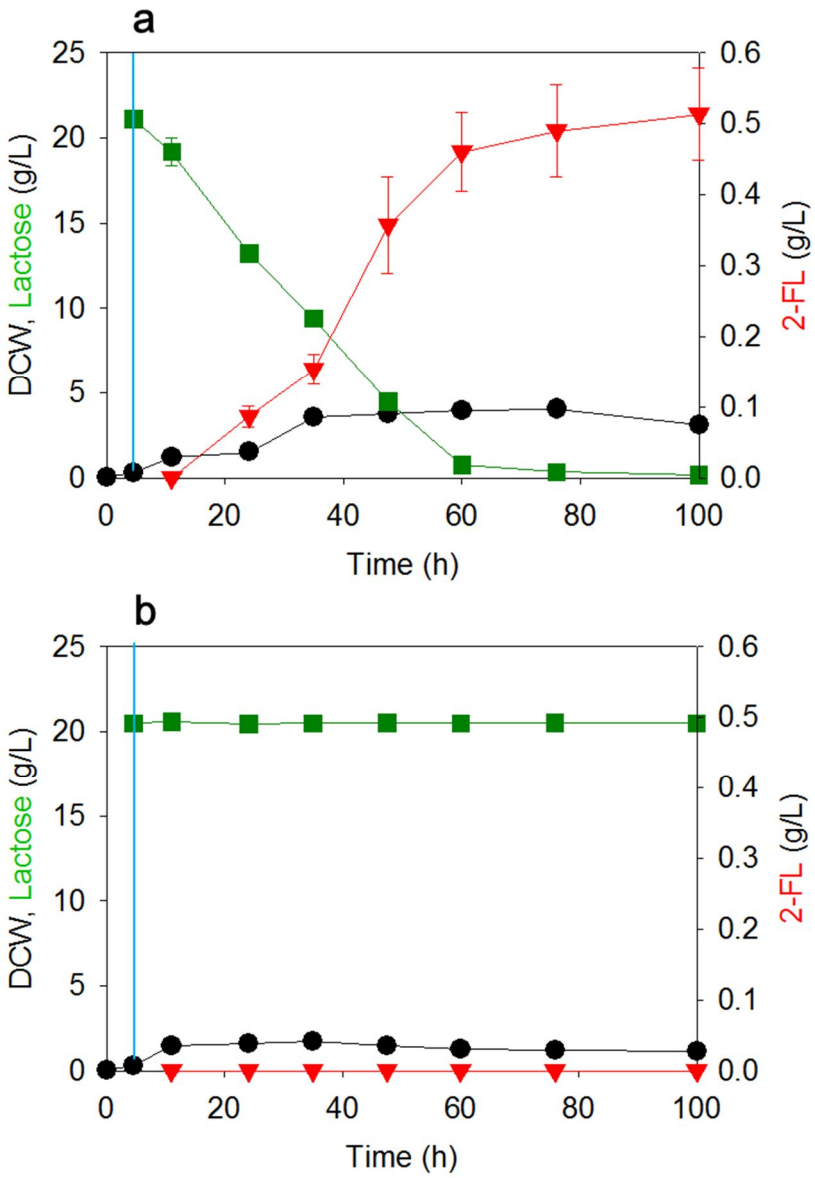



Fig. 2.2. Schematic of construction of the engineered *E. coli* strains (a) BL21star(DE3); (b) ΔL; (c) ΔL M15


a
DCW, Lactose (g/L)
2-FL (g/L)
Time (h)
b
DCW, Lactose (g/L)
2-FL (g/L)
Time (h)

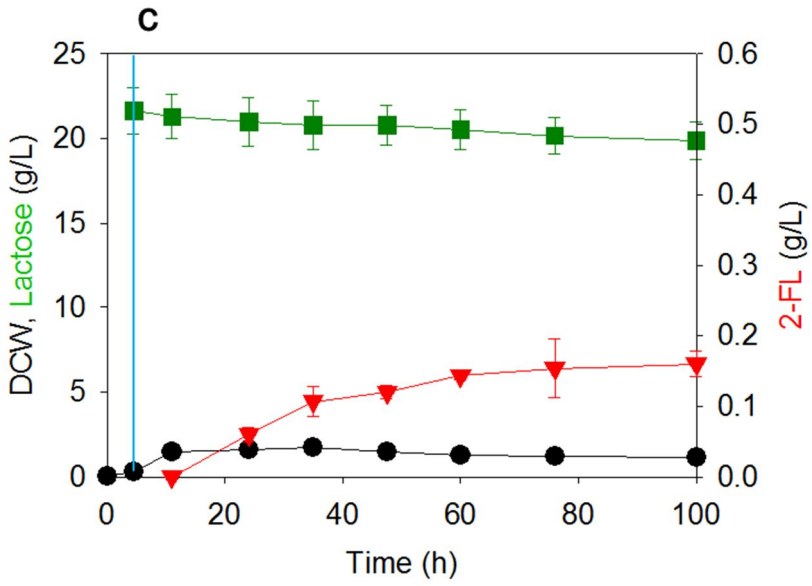



Fig. 2.3. Batch fermentations of engineered *E. coli* strains (a) BL21star(DE3) BCGW-F; (b) ΔL BCGW-F; (c) ΔL M15 BCGW-F. When optical density ($OD_{600}$) reached 0.8, IPTG and lactose was added to a final concentration 0.1 mM and 20 g/L, respectively (vertical line). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▼. Error bars represent the standard deviation of three replicates.

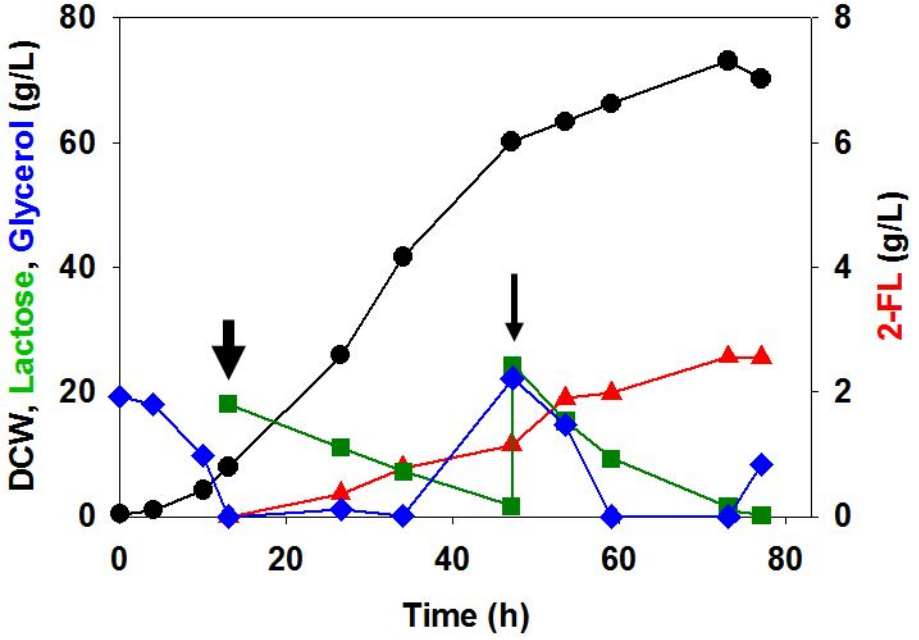



Fig. 2.4. Fed-batch fermentation of ΔL M15 BCGW-F. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (thick arrow). 200 g/L lactose solution was further dumped after depletion of lactose (thin arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆.

# Chapter 3

# Investigation of the effects of the attachment of aspartate tags to α-1,2-fucosyltransferase on 2-FL production in engineered *E. coli*

## 3.1. Summary

2-FL is one of the most important oligosaccharides in human milk, which is involved in many biological functions for infant`s health. To date, most enzymatic and microbial systems for 2-FL production have been limited to use α-1,2-fucosyltransferase (FucT2) from *H. pylori* since this enzyme has been functionally well-characterized. However, the level of soluble α-1,2-FucT2 from *H. pylori* expressed in *E. coli* is very low, which has limited 2-FL production. In this chapter, fusion of aspartate molecules to FucT2 was attempted to improve soluble expression of FucT2 and hence, to enhance 2-FL production. Fusion of aspartate molecules with FucT2 led to an enhancement in both of the level of soluble expression and fucosylation activity. Overall, final concentration of 6.4 g/L 2-FL with the yield of 0.225 g 2-FL/g lactose was gained in fed-batch fermentation of the engineered *E. coli* BL21star(DE3) expressing GDP-L-fucose biosynthetic enzymes and three aspartate tagged FucT2.

## 3.2. Introduction

Microbial production of 2-FL is affected by a number of factors. One of them is to express heterologous α-1,2-fucosyltransferase in *E. coli* which catalyzes the transfer of L-fucose molecule from GDP-L-fucose to an acceptor such as lactose. However, the level of soluble α-1,2-fucosyltransferase (FucT2) from *H. pylori* expressed in recombinant *E. coli* is very low, which has disturbed efficient biosynthesis of 2-FL (Lee et al., 2012a; 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 the heterologous fucosyltransferase gene for enhancing soluble expression in *E. coli* (Albermann et al., 2001; Dumon et al., 2004; Engels & 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 partners such as GST and maltose binding protein (MBP) cause a high metabolic burden. Contrary to protein as a fusion partner, 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 an aspartate tag facilitated intracellular expression of heterologous proteins in *E. coli* (Kim et al., 2015). This suggests that an appropriate amino acid tag has a beneficial influence on soluble expression of FucT2 and thus 2-FL production. In this chapter, the effect of simple tagging of aspartate molecules at the N-terminal of FucT2 on expression of FucT2 and production of 2-FL in engineered *E. coli* was investigated.

## 3.3. Materials and methods

### 3.3.1. Strains and plasmids

All strains, plasmids, and oligonucleotides used in this study are listed in Table 3.1 and 3.2. *E. coli* TOP10 and *E. coli* BL21star(DE3) (Invitrogen, Carlsbad, CA, USA) were used for construction of plasmids and a host strain for 2-FL production, respectively. Plasmids BCGW and F were previously constructed to overexpress 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., 2009a; Lee et al., 2012a). To amplify the aspartate tagged *fucT2* genes, DNA primers were formulated with a forward primer and R_*Kpn*I_fucT2. For example, D3-fucT2 (FucT2 with three aspartate tag at the N-terminal) 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 by the same restriction enzymes and therefore the plasmids 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 restriction enzyme digestion and DNA sequencing.

### 3.3.2. 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 of 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 determined using a standard pH electrode (Mettler Toledo, USA) and controlled at 6.8 by addition of 28% $NH_4OH$. Agitation speed increased up to 1,200 rpm in order to prevent the deficiency of dissolved oxygen, and air flow rate was maintained at 2 vvm throughout the cultivation.

### 3.3.3. Measurement 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 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.

### 3.3.4. Analysis of expression patterns and purification of recombinant FucT2s

Total, soluble and insoluble protein fractions of the 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 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 was loaded onto the gel and visualized by staining the gels with Coomassie brilliant blue solution.

For purification of FucT2 and D3-FucT2, ΔL M15 strains harboring plasmid F-His or D3F-His were cultured in a flask containing 100 ml

LB broth, respectively. 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.

### 3.3.5. *In vitro* fucosylation activity assay

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 (pH7.5) 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 one 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.

## 3.4. Results

### 3.4.1. Effects of fusion of aspartate tags on 2-FL production

Fusion of simple amino acids such as aspartate (Asp, D) to a protein of interest has been 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 in batch fermentations of four engineered *E. coli* ΔL M15 BCGW strains expressing D3-FucT2, D4-FucT2, D5-FucT2 or D6-FucT2. Even though the patterns of cell growth 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 ΔL M15 BCGW-D3F (Table 3.3). In batch fermentation of the ΔL 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, ΔL M15 BCGW-F (Table 3.3). This might be due to change of the expression pattern and activity of the FucT2 variants by attachment of aspartate tags.

### 3.4.2. Analysis of expression and activity of the FucT2 variants

To elucidate if the 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. 3.1a, expression of D4-FucT2, D5-FucT2 and D6-FucT2 could not be confirmed. Most of the FucT2 was expressed as insoluble form in *E. coli* expressing FucT2, which is consistent with the previous study (Lee et al., 2012a). 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 level of soluble 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 that of FucT2 (Fig. 3.1b). 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 of 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. 3.2). To conclude, improved soluble expression of FucT2 by fusion of the three aspartate tag clearly enhanced 2-FL production relative to the control strain.

### 3.4.3. Production of 2-FL by fed-batch fermentation in a bioreactor

To verify the beneficial effects of the aspartate tag attached at the N-terminal of FucT2 on 2-FL production, fed-batch fermentation was performed by controlling the glycerol concentration while dumping lactose (Fig. 3.3). After IPTG induction, the cell growth pattern of the engineered *E. coli* ΔL 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 engineered *E. coli* ΔL M15 BCGW-F. The results of fed-batch fermentations are summarized in Table 3.4.

## 3.5. Discussion

It was reported that fusion of simple amino acid tags enhanced soluble expression of heterologous lipase in *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 soluble expression of FucT2 was improved by fusion of three aspartates. Moreover, in the glycerol pH-stat fed-batch fermentation of the engineered *E. coli* expressing D3 fused FucT2 increased 2-FL concentration by a 2.1 fold and yield by a 2.8 fold compared to the engineered *E. coli* expressing FucT2.

The charges of fusion partners are closely correlated with their soluble and functional expression of heterologous proteins (Choi et al., 2008). Consistently, anionic tags facilitate solubility of their fused proteins (Chen et al., 1998; Kweon et al., 2005; Zhang et al., 2004). The exact reason is unclear, but an adequate alteration of the N-terminal end of FucT2 could improve fucosylation efficiency. Interestingly, more than four aspartates inhibited FucT2 expression, indicating that three molecules of aspartate are the optimum amino acid tag for FucT2 expression.

Table 3.1. Strains and plasmids used in Chapter 3

| Strains/Plasmids | Relevant description | Reference |
|---|---|---|
| ΔL M15 | BL21star(DE3) *ΔlacZYA Tn7::lacZΔM15* | (Chin et al., 2015) |
| pETDuet-1 | Two T7 promoters, pBR322 replicon, $Amp^R$ | Novagen |
| pCOLADuet-1 | Two T7 promoters, ColA replicon, $Kan^R$ | Novagen |
| BCGW | pETDuet-1 + *manC-manB* (*Nco*I/*Sac*I) + *gmd-wcaG* (*Nde*I/*Kpn*I) | (Lee et al., 2009a) |
| F | pCOLADuet-1 + *fucT2* (*Nco*I/*Sac*I) | (Lee et al., 2012a) |
| D3F | pCOLADuet-1 + three aspartate-fucT2 (*Nde*I/*Kpn*I) | (Chin et al., 2015) |
| D4F | pCOLADuet-1 + four aspartate-fucT2 (*Nde*I/*Kpn*I) | (Chin et al., 2015) |
| D5F | pCOLADuet-1 + five aspartate-fucT2 (*Nde*I/*Kpn*I) | (Chin et al., 2015) |
| D6F | pCOLADuet-1 + six aspartate-fucT2 (*Nde*I/*Kpn*I) | (Chin et al., 2015) |
| F-His | pCOLADuet-1 + *fucT2* (*Nco*I/*Sac*I) + His-tag | (Chin et al., 2015) |
| D3F-His | pCOLADuet-1 + three aspartate-fucT2 (*Nde*I/*Kpn*I) + His-tag | (Chin et al., 2015) |

Table 3.2. Primers used in Chapter 3

| Name | Sequence |
|---|---|
| F_*Nde*I_fucT2 | GGAATTC*CATATG*GCTTTTAAGGTGGTGC |
| F_*Nde*I_D3-fucT2 | GGAATTC*CATATG***GATGATGAT**GCTTTTAA |
| 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***GATGATGATGATGATGAT**GCTTTTAA |
| R_*Kpn*I_fucT2 | G*GGTACC*ATTAAGCGTTATACTTTTGGGATTTTACCT |
| R_*Kpn*I_fucT2-His | GG*GGTACC*TTAGTGGTGATGATGGTGATGAGCGTTATACTTTTGGG |

The italic- and bold sequences present the recognition sites of specific restriction enzymes and aspartate tags, respectively.

Table 3.3. Summary of batch fermentations of engineered *E. coli* strains expressing aspartate fused FucT2s

| 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) |
|---|---|---|---|---|
| ΔL M15 BCGW-F[a] | 1.23 ± 0.13 | 0.16 ± 0.017 | 1.76 ± 0.263 | 0.091 ± 0.004 |
| ΔL M15 BCGW-D3F | 1.54 ± 0.11 | 0.34 ± 0.010 | 1.35 ± 0.026 | 0.252 ± 0.012 |
| ΔL M15 BCGW-D4F | 1.31 | N.D. | 1.38 | - |
| ΔL M15 BCGW-D5F | 1.28 | N.D. | 1.41 | - |
| ΔL M15 BCGW-D6F | 1.59 | N.D. | 1.30 | - |

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

[a] The result of batch fermentation of engineered *E. coli* ΔL M15 BCGW-F strain was cited in Chapter 2.

[b] Extracellular concentrations of lactose and 2-FL were determined and used for the calculation of lactose consumption and 2-FL production.

Table 3.4. 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) |
|---|---|---|---|---|
| ΔL M15 BCGW-F[c] | 73.1 | 2.6 | 0.063 | 0.043 |
| ΔL 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.

[c] The result of fed-batch fermentation of engineered *E. coli* ΔL M15 BCGW-F strain was cited in Chapter 2.

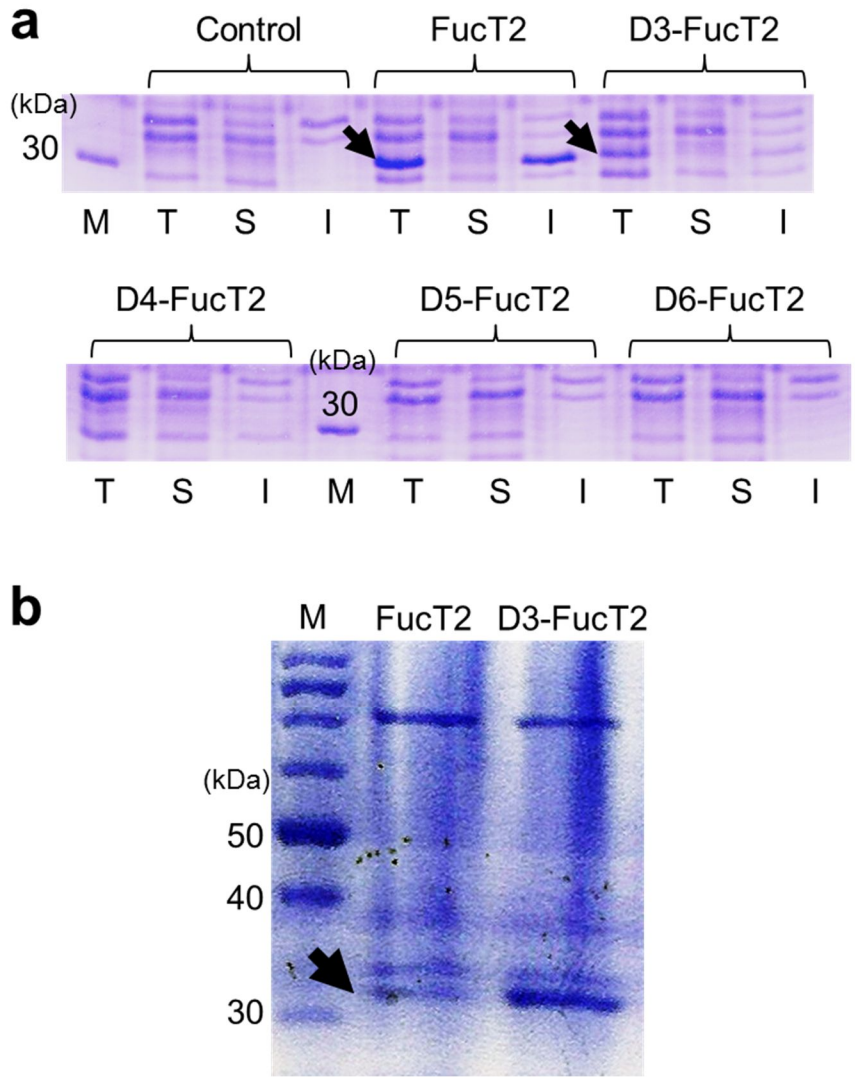



Fig. 3.1. SDS-PAGE analysis of crude extracts (a) and purified enzymes (b) to investigate the effects of aspartate tags on the expression patterns of FucT2 variants in engineered *E. coli* strains. The abbreviations were defined as follows: arrows; FucT2, T; total protein fraction, S; soluble protein fraction, I; insoluble protein fraction, M; molecular weight marker.

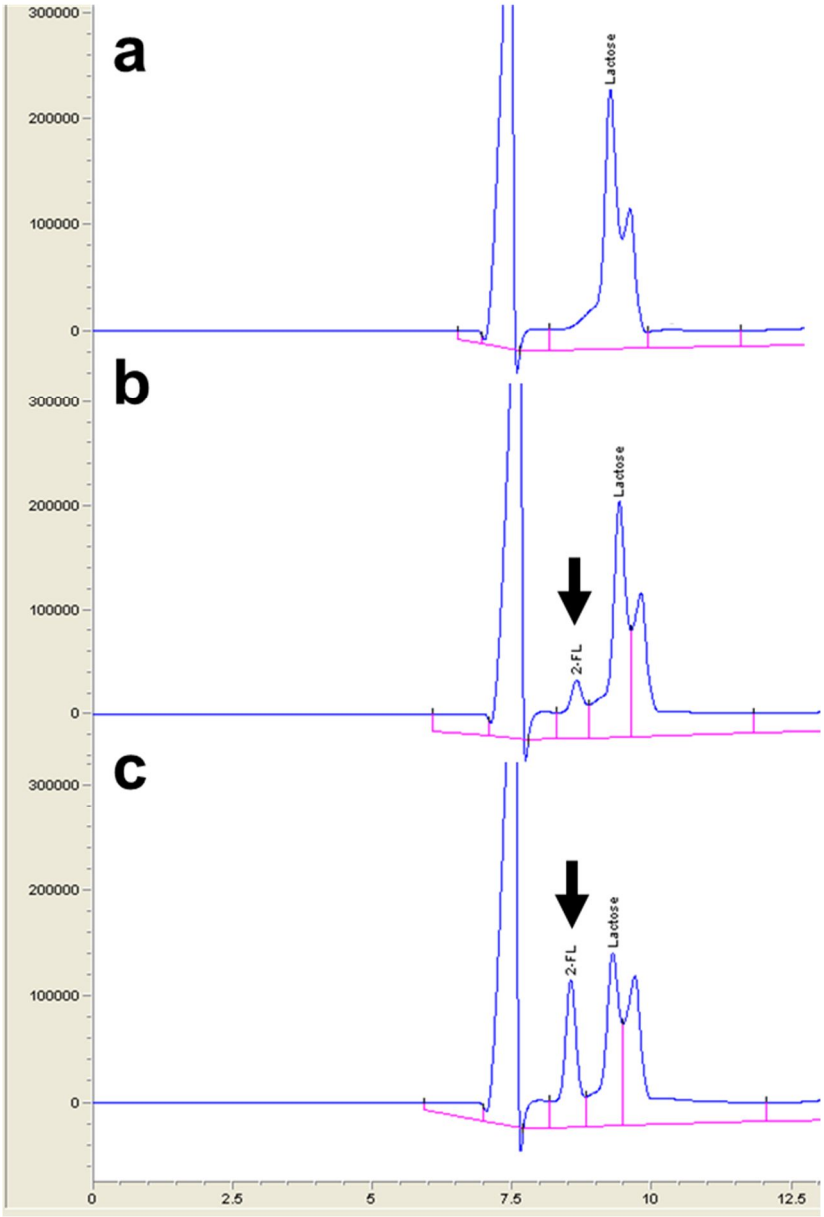



Fig. 3.2. HPLC analysis of *in vitro* enzymatic reaction mixtures with control (a), FucT2 (b) and D3-FucT2 (c). The arrow points the peaks of 2-FL.

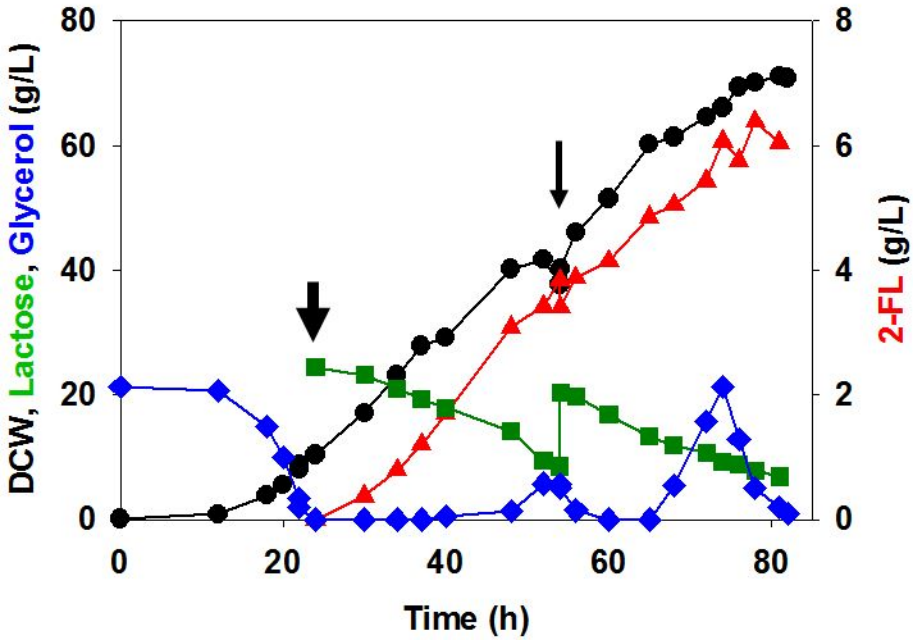



Fig. 3.3. Fed-batch fermentation of ΔL M15 harboring BCGW and D3F. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (thick arrow). 200 g/L lactose solution was further dumped after depletion of lactose (thin arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆.

# Chapter 4

# Enhanced production of 2-FL by expression of putative α-1,2-fucosyltransferase from *B. fragilis* and complete deletion of *lacZ* in engineered *E. coli*

## 4.1. Summary

In the present chapter, putative α-1,2-fucosyltransferase (WcfB) from *B. fragilis* was employed in replacement of FucT2 from *H. pylori* for efficient production of 2-FL in the engineered *E. coli* ΔL M15 strain with attenuated β-galactosidase. Though the expression of WcfB in the ΔL M15 strain led to a 4-fold enhancement in 2-FL concentration compared to that of ΔL M15 exspressing FucT2, however, unknown by-product also accumulated in the media along with 2-FL. As the unknown by-product is composed of galactose, a structural analogue of fucose, complete elimination of the residual β-galactosidase activity was suggested. Therefore, the β-galactosidase deleted *E. coli* strain (ΔL YA) was constructed by insertion of the *lac* operon without the *lacZ* gene to the chromosome of the *E. coli* ΔL strain (*lac* operon deleted strain). Finally, 2-FL titer was further increased up to 15.4 g/L with 2-FL yield of 0.858 g/g lactose and productivity of 0.530 g/L/h by fed-batch fermentation of the ΔL YA strain expressing WcfB and the enzymes involved in the *de novo* pathway for GDP-L-fucose biosynthesis. In addition, the feasibility of industrial production of 2-FL using this microbial system was demonstrated by performing fed-batch fermentation in a 75 L bioreactor. Due to the difficulty in maintenance of dissolved oxygen in large scale, 12.3 g/L of the final 2-FL

concentration was obtained. These results may permit mass production of 2-FL from glycerol and lactose using engineered *E. coli*.

## 4.2. Introduction

2-FL is one of the most important oligosaccharides in human milk which has received great attention because of its potential as nutraceutical and pharmaceutical materials (Castanys-Muñoz et al., 2013a). Microbial production of 2-FL was suggested to be influenced by a number of factors. One of them is fucosylation activity of α-1,2-fucosyltransferase. α-1,2-Fucosyltransferase present in both eukaryotic and prokaryotic organisms catalyzes the transfer of L-fucose molecule at an α-1,2 linkage from GDP-L-fucose to the galactose moiety of some acceptor molecules such as *N*-acetyllactosamine, lacto-*N*-biose and lactose in oligosaccharides, glycoproteins, and glycolipids (Ma et al., 2006; Oriol et al., 1999). α-1,2-Fucosyltransferases, belonging to the CAZY family 11 (http://afmb.cnrsmrs.fr/CAZY/fam/acc_GT.html) were shown to adopt the random bi bi mechanism for fucose transfer (Palcic et al., 1989). Several putative bacterial α-1,2-fucosyltransferases have been identified to date, including proteins involved in colanic acid synthesis in *E. coli* K-12, *Salmonella enterica* LT2, in O-antigen synthesis in *Yersinia enterocolitica* O8 (Reeves et al., 2006), the WbsJ in the enteropathogenic *E. coli* O128 strain (Li et al., 2008; Shao et al., 2003), WbnK and WbwK of the pathogenic *E. coli* O86 (Yi et al., 2005) as well as WbiQ from *E. coli* O127 (Pettit et al., 2010) and FucT2 (or FutC) from *H. pylori* (Wang et al., 1999; Wang et al., 2002). Among

these, the FucT2 from *H. pylori* has been mainly used for enzymatic- and microbial production of 2-FL since this enzyme has been functionally well-characterized (Wang et al., 1999; Wang et al., 2000; Wang et al., 2002). However, the level of soluble α-1,2-FucT2 from *H. pylori* expressed in recombinant *E. coli* is very low, which has limited 2-FL production (Lee et al., 2012a; Wang et al., 1999).

Scale up of fermentation processes is a critical problem for industrial applications. Fermentation scale up is aimed to manufacture large quantity of target products and if possible, with a simultaneous increase or at least consistency of yield and productivity. However, the change of geometric and physical conditions in larger scale fermentations leads to less favorable mixing behavior and therefore, would decrease reproducibility.

In this chapter, putative α-1,2-fucosyltransferase from *B. fragilis* was expressed instead of FucT2 from *H. pylori* in engineered *E. coli*. In addition, complete elimination of β-galactosidase activity was attempted to enhance 2-FL production further. Finally, fed-batch fermentation of the engineered *E. coli* strain ΔL YA harboring BCGW and W was carried out in a 75 L scale bioreactor to assess the possibility of industrial production of 2-FL.

## 4.3. Materials and methods

### 4.3.1. Strains and plasmids

All strains, plasmids, and oligonucleotides used in this study are listed in Table 4.1 and 4.2. *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. The integration of the *lac* operon without *lacZ* into the chromosome of the engineered *E. coli* was carried out using a Tn7-mediated site-specific transposition method as reported in previous research (McKenzie & Craig, 2006). For construction of plasmid pGlacYA, two DNA fragments were amplified from the genomic DNA of *E. coli* K-12 (ATCC10798) with two pairs of primers P1_M15 lac/P2_lacYA and P3_lacYA /P4_M15 lac. The two PCR products were ligated together into pGRG36 (digested by *Sma*I) by *in vitro* homologous recombination using In-Fusion HD Cloning Kit (TAKARA, Japan). After the transformation of pGlacYA plasmid into ΔL strain, transformants were selected at 30°C. The transformants were grown in non-selective media to ensure the chromosomal integration and the pGlacYA were eliminated by cultivation at 42°C. The insertion of *lac* operon without *lacZ* was confirmed by colony PCR using two primers, F_Tn7 and R_Tn7. Plasmid BCGW was constructed previously for overexpression

of the genes for GDP-L-fucose biosynthetic enzymes (ManB, ManC, Gmd and WcaG) (Lee et al., 2009a). To construct plasmid W, the *wcfB* gene was amplified from the genomic DNA of *B. fragilis* ATCC25285 by PCR using two primers F_*Nde*I_wcfB and R_*Kpn*I_wcfB. The PCR product cut by *Nde*I and *Kpn*I and ligated with pCOLADuet-1 digested by same restriction enzymes. All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

### 4.3.2. Culture conditions

Batch fermentations were carried out in a 500 mL baffled flask (Nalgene) containing 100 mL 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] 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 were added at a final concentration 0.1 mM and 20 g/L, respectively. Fed-batch fermentation was carried out in a 2.5 L bioreactor (Kobiotech, Incheon, Korea) containing 1.0 L of defined

medium 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 $MgSO_4.7H_2O$ 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 jar 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 by addition of 28% $NH_4OH$. Agitation speed elevated up to 1,200 rpm for prevention of the deficiency of dissolved oxygen, and air flow rate was maintained at 2 vvm during the fermentation.

### 4.3.3. Measurement 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.

### 4.3.4. 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, 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 an ultrasonic processor (Cole-Parmer, IL, USA). Cells were centrifuged for 10 minutes at 12,000 rpm and 4°C to separate soluble and insoluble fractions. Activity was determined by assaying soluble fractions using the β-Galactosidase Enzyme Assay System (Promega E2000). One unit of β-galactosidase was defined as the amount of an enzyme able to hydrolyze 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.

### 4.3.5. Identification of 2-FL produced by the engineered *E. coli* expressing WcfB

2-FL was readily purified and enriched by solid-phase extraction (SPE) using a porous graphitized carbon cartridge prior to mass spectrometry analysis. Enriched 2-FL was analyzed using a Bruker ultrafleXtreme MALDI-TOF/TOF system with 1,000Hz Smart beam Ⅱ laser. 2, 5-Dihydroxy-benzoic acid (DHB) was used as a matrix (5 mg/100 mL in 50% ACN:$H_2O$) and 0.01 M NaCl was added as a cation dopant to increase signal sensitivity. The sample was spotted on a stainless steel target plate, followed by the NaCl dopant and matrix. The spot was dried under vacuum prior to mass spectrometric analysis. To confirm the structure of 2-FL, MALDI-TOF/TOF MS via collision-induced dissociation (CID) was performed. Tandem MS spectra were gained at 1k eV collision energy with argon gas.

## 4.4. Results

### 4.4.1. Effects of expression of putative α-1,2-fucosyltransferase (WcfB) from *B. fragilis* on 2-FL production

The genes coding for novel α-1,2-fucosyltransferase has been cloned and tested for the feasibility of the production of 2-FL in flask cultures containing LB media with 15 g/L lactose (Kim, 2015). Eleven candidates were *wcfW* and *wcfB* from *B. fragilis* NCTC9343, *wbsJ* from *E. coli* O128:B12, SPO3391 from *Ruegeria pomeroyi* DSS-3, PGA1_c33070 from *Phaeobacter gallaeciensis* DSM 17395, Phep_1971 from *Pedobacter heparinus* DSM 2366, Pedsa_2797 from *P. saltans* DSM 12145, llmg_2349 from *Lactococcus lactis* subsp. *cremoris* MG1363, Dfer_0178 from *Dyadobacter fermentans* DSM 18053, HMPREF0351_11954 from *Enterococcus faecium* DO, Fut2 from *Homo sapiens*. Among the engineered *E. coli* strains transformed with the above candidates, 2-FL was produced in the culture of the strain transformed with pHwcfB, which harbored *wcfB* from *B. fragilis*.

For in-depth study, fed-batch fermentation of ΔL M15 BCGW-W was carried out in a 2.5 L bioreactor under the same condition as done for the ΔL M15 BCGW-F in Chapter 2 (Fig. 4.2). By consumption of glycerol, cell mass reached up to 56.2 g/L of final cell concentration. As a result, 10.3 g/L of 2-FL with a yield of 0.53 g 2-FL/g lactose and

productivity of 0.255 g/L/h were obtained at the end of fermentation. These values were 4.0, 8.4 and 5.9 fold higher than those of the ΔL M15 BCGW-F. Interestingly, the protein band corresponding to the size of WcfB was not observed in SDS-PAGE and no fucosylation activity was detected in *in vitro* fucosylation assay (data not shown).

To confirm biosynthesis of 2-FL in the engineered *E. coli* ΔL M15 BCGW-W, subsequent analysis was carried out with MALDI-TOF MS. As shown in Fig. 4.3a, the ions at 511.164 corresponding to 2-FL ($[Hex_2+Fuc_2+Na]^+$) was observed as a major oligosaccharide in the culture medium. Hexose series consisting of the degree of polymerization 5-11 were also present as minor oligomers of 2-FL production. Tandem mass spectrometry (MS/MS) using collision-induced dissociation (CID) was further performed to confirm the composition and to elucidate the structure of 2-FL. The representative MS/MS spectrum with the ion at *m/z* 511.164 was shown in Fig. 4.3b. CID yields the loss of a fucose at *m/z* 511.164, followed by the loss of a second fucose at *m/z* 365.085, and the loss of a hexose at *m/z* 203.032. Indeed, it was clearly confirmed the presence of 2-FL produced by engineered *E.coli* expressing WcfB. An interesting point is di-fucosyllactose was not detected in the engineered *E. coli* expressing WcfB as opposed to the engineered *E. coli* expressing FucT2 (Fig. 4.3a and c). It might be due to the difference in substrate specificity between

FucT2 and WcfB. Another point is that similar amount of the unknown by-product with 2-FL accumulated in the medium (Fig. 4.5a).

### 4.4.2. Complete deletion of *lacZ* for efficient production of 2-FL

As mentioned above, a significant amount of the unknown by-product was formed together with 2-FL. In the qualification analysis through tandem mass, it was revealed that the unknown by-product is a hexose trimer without fucose. Therefore, the possible candidates might be lactose + glucose, lactose + galactose and lactose + mannose. Among them, the lactose + galactose was the most potent candidate since BL21star(DE3) cannot metabolize galactose, an analogue of fucose. Thus, elimination of the residual β-galactosidase activity was suggested to remove the formation of galactose from lactose. The β-galactosidase deleted *E. coli* strain (ΔL YA) was constructed by insertion of the *lac* operon without the *lacZ* gene to the chromosome of the *E. coli* ΔL strain (*lac* operon deleted strain) (Fig. 4.1). The activity has not been detected at all in β-galactosidase activity assay of the ΔL YA strain (data not shown). To investigate the effects of complete deletion of *lacZ* on 2-FL production, batch fermentations of ΔL M15 and ΔL YA harboring plasmids BCGW and W were performed in flask containing defined medium (Fig. 4.4). As a result, the ΔL YA produced 1.33 g/L of

2-FL with a yield of 0.825 g/g lactose corresponding to 1.6- and 2.8 times higher than those of the ΔL M15 strain (Table 4.3).

To verify the advantageous effects of the complete deletion of *lacZ* on 2-FL production, fed-batch fermentation of the engineered *E. coli* ΔL YA BCGW-W was also performed. The growth pattern of the engineered *E. coli* ΔL YA BCGW-W was similar to the ΔL M15 BCGW-W, and its final dry cell weight reached 57.6 g/L. After IPTG induction and addition of lactose, 2-FL concentration increased sharply up to 15.4 g/L in 46 h culture with a yield of 0.858 g/g lactose and productivity of 0.530 g/L·h, which was 1.5-, 1.6- and 2.1 fold higher than those of the ΔL M15 BCGW-F strain (Table 4.4). Especially, the unknown by-product which was formed in the culture of ΔL M15 BCGW-W was significantly decreased in the culture of ΔL YA BCGW-W (Fig. 4.5b).

To prove the feasibility of industrial production of 2-FL using this engineered *E. coli* strain, large scale fermentation is needed. Before the large scale fermentation, the effect of agitation speed on 2-FL production was tested under agitation speed at 500 rpm since the agitation is limited in a large scale bioreactor. Though 2-FL yield was similar to that of the fermentation done at 1000 rpm, final 2-FL concentration decreased by 23% and productivity was declined by 70%

(Fig. 4.6b and Table 4.5). Therefore, agitation seemed to be one of the key important factors for 2-FL production using the engineered *E. coli*.

### 4.4.3. Large scale production of 2-FL

Fed-batch fermentation of the engineered *E. coli* strain ΔL YA BCGW-W was performed in a 75 L bioreactor to demonstrate the feasibility of mass production of 2-FL. The key objective was to reproduce 2-FL titer, productivity and yield as obtained in a laboratory scale bioreactor (2.5 L). The fermentation conditions such as media components, inoculum size, feeding strategy and pH were the same as those of the laboratory scale fermentor. However, temperature of the main culture before IPTG induction was changed to 37°C because the cells did not grow at 25°C (data not shown). The agitation speed and aeration were controlled manually because the dissolved oxygen (DO) is dependent on cell density. As shown in Fig. 4.7, the DO value dropped rapidly from 100% to 9.2% during the batch period (0 - 8 h) as dry cell weight increased from 0.4 g/L to 5.2 g/L. After exhaustion of 20 g/L glycerol initially added, the culture temperature was changed to 25°C and DO value was restored to 73%. The final dry cell weight obtained was similar to that in the 2.5 L scale fermentation under 500 rpm condition (Table 4.5). Finally, 12.3 g/L of 2-FL concentration was achieved with a yield of

0.591 g 2-FL/g lactose at the end of fed-batch fermentation.

## 4.5. Discussion

FucT2 from *H. pylori* has been mainly used for enzymatic- and microbial production of 2-FL since this enzyme has been functionally well-characterized. However, insoluble expression of this enzyme has limited biosynthesis of 2-FL. The insoluble expression might be due to an unusual structure of the FucT2 from *H. pylori* (Wang et al., 2000). Putative α-1,2-fucosyltransferase of *B. fragilis*, WcfB was selected to replace FucT2 in this chapter. By the expression of WcfB, 2-FL concentration and yield were improved by 4.0- and 8.4-fold. It is assumed that the higher *in vivo* fucosylation activity of WcfB than that of FucT2. However, fucosylation activity was not detected in *in vitro* activity assay although 2-FL was produced by engineered *E. coli* expressing WcfB. This result is consistent to the previous research which reported that the purified WcfB enzyme did not accept lactose as a substrate at all in an *in vitro* fucosylation activity assay (Albermann et al., 2001).

Complete elimination of the residual activity of β-galactosidase led not only to removal of unknown by-product, but also to enhancement of 2-FL production (increase 2-FL titer by 50%, yield by 62% and productivity by 100%). It might be due to diversion of the lactose flux from by-product formation and cell growth to 2-FL production. As described in Chapter 2, the small portion of lactose taken into the ΔL

M15 strain is hydrolyzed to glucose and galactose by partial β-galactosidase activity (Fig. 2.1). While the glucose is used for cell growth, the galactose is not assimilated since the genes involved in the galactose metabolism were deleted in the chromosome of BL21star(DE3) (Jeong et al., 2009; Xu et al., 2012). Thus, the galactose might be used in the formation of the unknown by-product and complete deletion of *lacZ* would eliminate the by-product.

Compared to 2.5 L scale fermentation, only 80% of the final concentration of 2-FL was obtained in the fermentation done in a 75 L bioreactor. It was reported that increase of vessel size reduced mixing quality and hence, not only reduce the overall oxygen transfer rate but also increase the anaerobic zone (Enfors et al., 2001). The detrimental effects caused by the irregular oxygen transfer and the subsequent repetitive activation and shut down of stress genes are believed to lead to a completely altered physiology with constant metabolic shifts, which ultimately reduce cell growth and productivity and increase by-product formation (Schmidt, 2005).

Table 4.1. List of strains and plasmids used in Chapter 4

| Name | Relevant description | Reference |
|---|---|---|
| Strains | | |
| ΔL | BL21star(DE3) *ΔlacZYA* | (Chin et al., 2015) |
| ΔL M15 | ΔL *Tn7::lacZΔM15YA* | (Chin et al., 2015) |
| ΔL YA | ΔL *Tn7::lacYA* | This study |
| Plasmids | | |
| pGRG36 | Tn7 insertion vector, pSC101 replicon, $Amp^R$ | (McKenzie & Craig, 2006) |
| pGlacYA | pGRG36+*lacYA* (*Sma*I) | This study |
| pETDuet-1 | Two T7 promoters, pBR322 replicon, $Amp^R$ | Novagen |
| pCOLADuet-1 | Two T7 promoters, ColA replicon, $Kan^R$ | Novagen |
| BCGW | pETDuet-1 + *manC-manB* (*Nco*I/*Sac*I) + *gmd-wcaG* (*Nde*I/*Kpn*I) | (Lee et al., 2009a) |
| W | pCOLADuet-1 + *wcfB* (*Nde*I / *Kpn*I) | This study |

Table 4.2. Primers used in Chapter 4

| Name | Sequence |
|---|---|
| P1_M15 lac | AATTAATCAGATCCCGGGACCATCGAATGGCGCAAAACCTTTC |
| P2_lacYA | TGGATTTCCTGTGTGAAATTGTTATCCGCTCACAATTCC |
| P3_lacYA | AATTTCACACAGGAAATCCATTATGTACTATTTAAAAAACACAAACTTTTGG |
| P4_M15 lac | GGCCGCTATTGACCCGGGGCTGTGGGTCAAAGAGGCATGATG |
| F_Tn7 | GATGCTGGTGGCGAAGCTGT |
| R_Tn7 | GATGACGGTTTGTCACATGGA |
| F_ *Nde*I_wcfB | GGAATTCATATGTTATATGTAATTTTACGTGGACGATTAGG |
| R_*Kpn*I_wcfB | GGGTACCTCACATATTCTTCTTTCTTTTCCATATTAATCGC |

The underlined sequences present the recognition sites of specific restriction enzymes.

Table 4.3. Comparison of results of batch fermentations of the engineered *E. coli* BL21star(DE3) strains expressing WcfB

| Strains | Maximum dry cell weight (g/L) | Maximum 2-FL concentration[b] (g/L) | Yield (mole 2-FL/mole lactose) | Productivity[a] (g/L·h) |
|---|---|---|---|---|
| ΔL M15 BCGW-W | 6.0 | 0.85 | 0.296 | 0.024 |
| ΔL YA BCGW-W | 5.9 | 1.33 | 0.825 | 0.038 |

[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.

Table 4.4. Comparison of results of fed-batch fermentations of the 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) |
|---|---|---|---|---|
| ΔL M15 BCGW-F[c] | 73.1 | 2.6 | 0.063 | 0.043 |
| ΔL M15 BCGW-W | 56.2 | 10.3 | 0.530 | 0.255 |
| ΔL YA BCGW-W | 57.6 | 15.4 | 0.858 | 0.530 |

[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.

[c] The result of fed-batch fermentation of engineered *E. coli* ΔL M15 BCGW-F strain was cited in Chapter 2.

Table 4.5. Comparison of results of fed-batch fermentations of the engineered *E. coli* ΔL YA BCGW-W under various conditions

| Conditions | Maximum dry cell weight (g/L) | Maximum 2-FL concentration[b] (g/L) | Yield (g 2-FL/g lactose) | Productivity[a] (g/L·h) |
|---|---|---|---|---|
| 1000 rpm (2.5 L) | 57.6 | 15.4 | 0.858 | 0.530 |
| 500 rpm (2.5 L) | 43.2 | 11.8 | 0.836 | 0.160 |
| 500 rpm (75 L) | 46.5 | 12.3 | 0.591 | 0.270 |

[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.

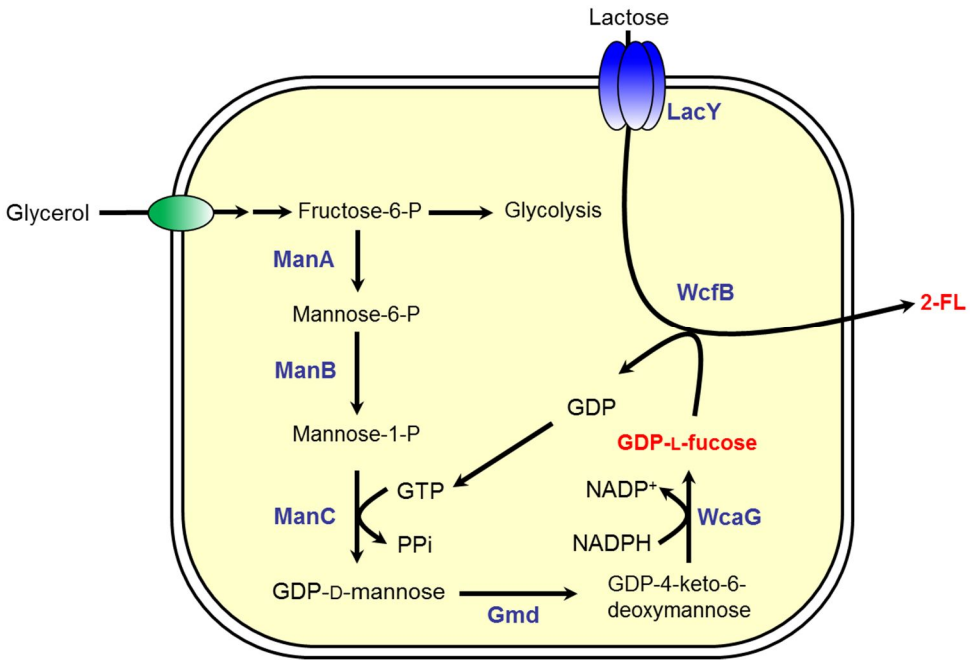



Fig. 4.1. The strategy for production of 2-FL in engineered *E. coli* ΔL YA BCGW-W strain. The names of proteins are abbreviated as follows; LacY, lactose permease; ManA, mannose 6-phosphate isomerase; ManB, phosphomannomutase; ManC, mannose 1-phosphate guanylyl-transferase; Gmd, GDP-D-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase; WcfB, α-1,2-fucosyltransferase. PPi and GTP denote diphosphate and guanosine 5'-triphosphate.

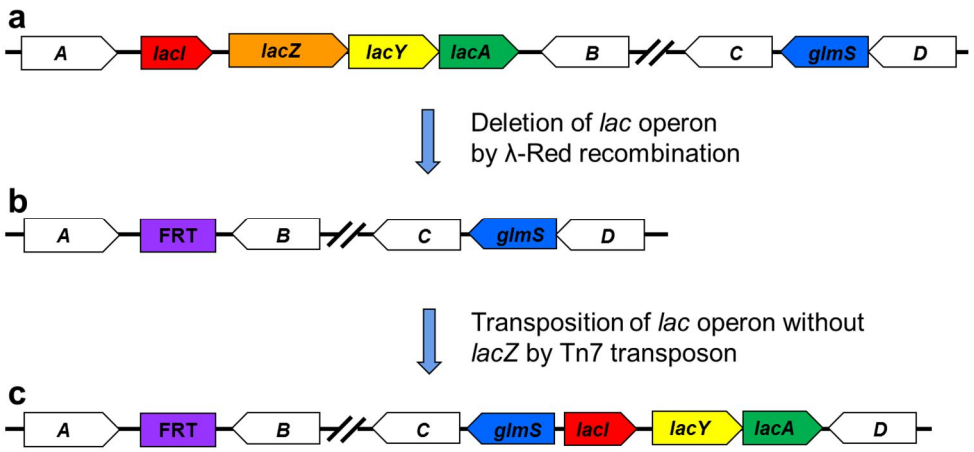



Fig. 4.2. Schematic of construction of the engineered *E. coli* strains (a) BL21star(DE3); (b) ΔL; (c) ΔL YA

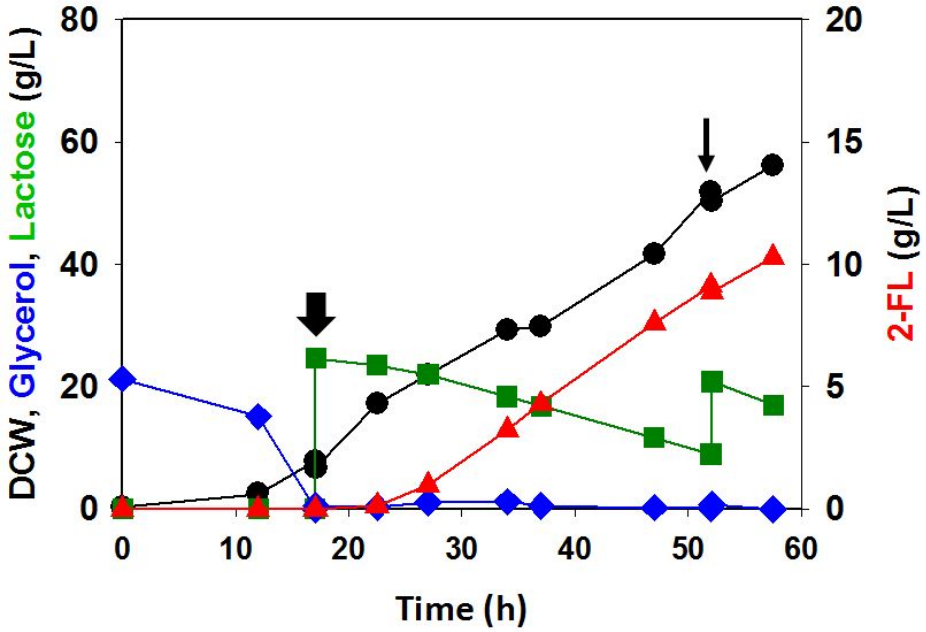



Fig. 4.3. Fed-batch fermentation of ΔL M15 BCGW-W. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (thick arrow). 200 g/L lactose solution was further dumped after depletion of lactose (thin arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆.

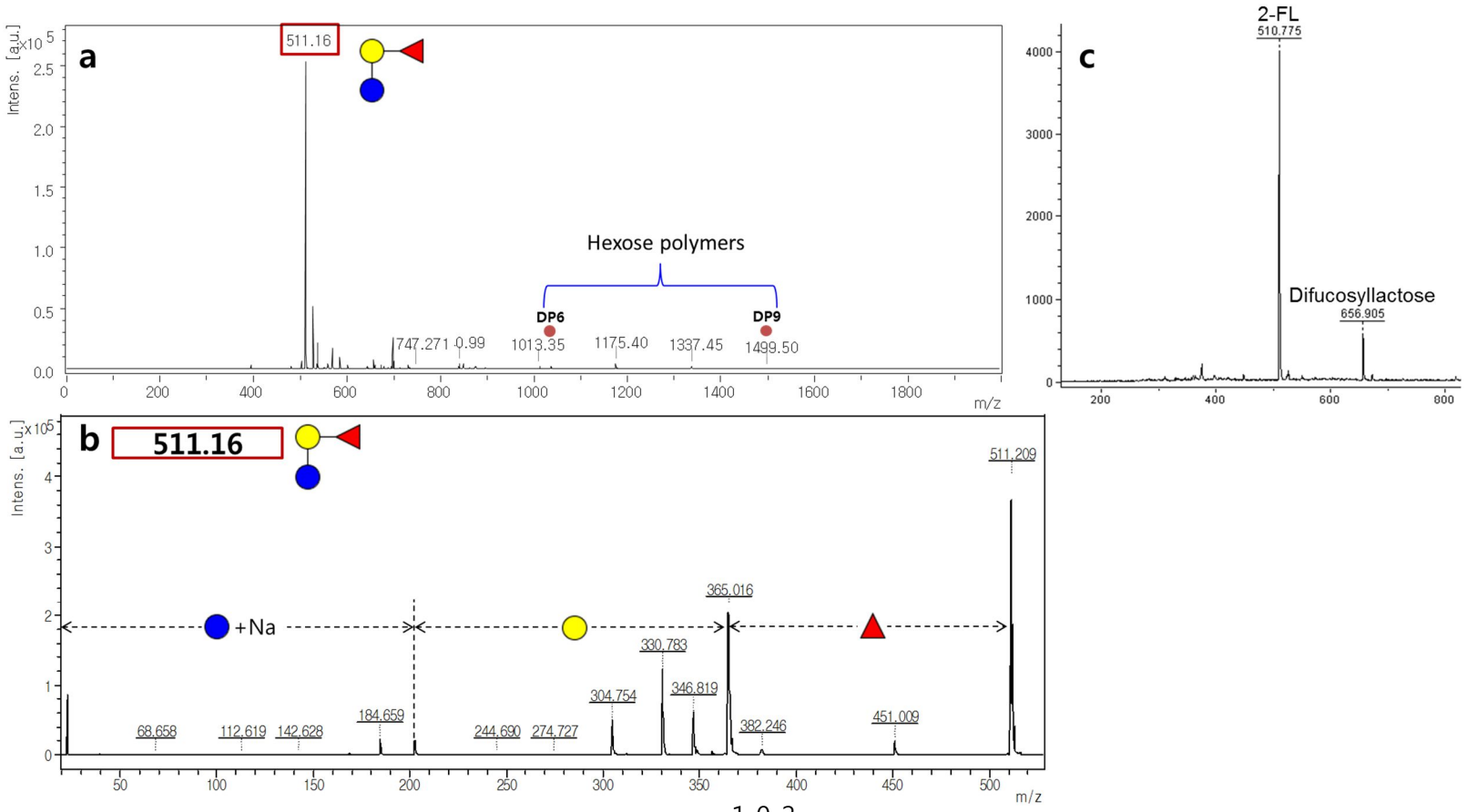

a
511.16
Hexose polymers
DP6
DP9
747.271 0.99
1013.35
1175.40
1337.45
1499.50
Intens. [a.u.]
m/z
b
511.16
+Na
511.209
365.016
330.783
346.819
304.754
184.659
68.658
112.619
142.628
244.690
274.727
382.246
451.009
c
2-FL
510.775
Difucosyllactose
656.905

Fig. 4.4. MALDI-TOF MS (a, c) and CID tandem MS (b) analysis of 2-FL production in the media. Culture broth of engineered *E. coli* harboring plasmids BCGW and W (a, b) and culture broth of engineered *E. coli* harboring BCGW and F. Symbols for the types of monosaccharaides (glucose, ●; galactose, ●; fucose, ▲)

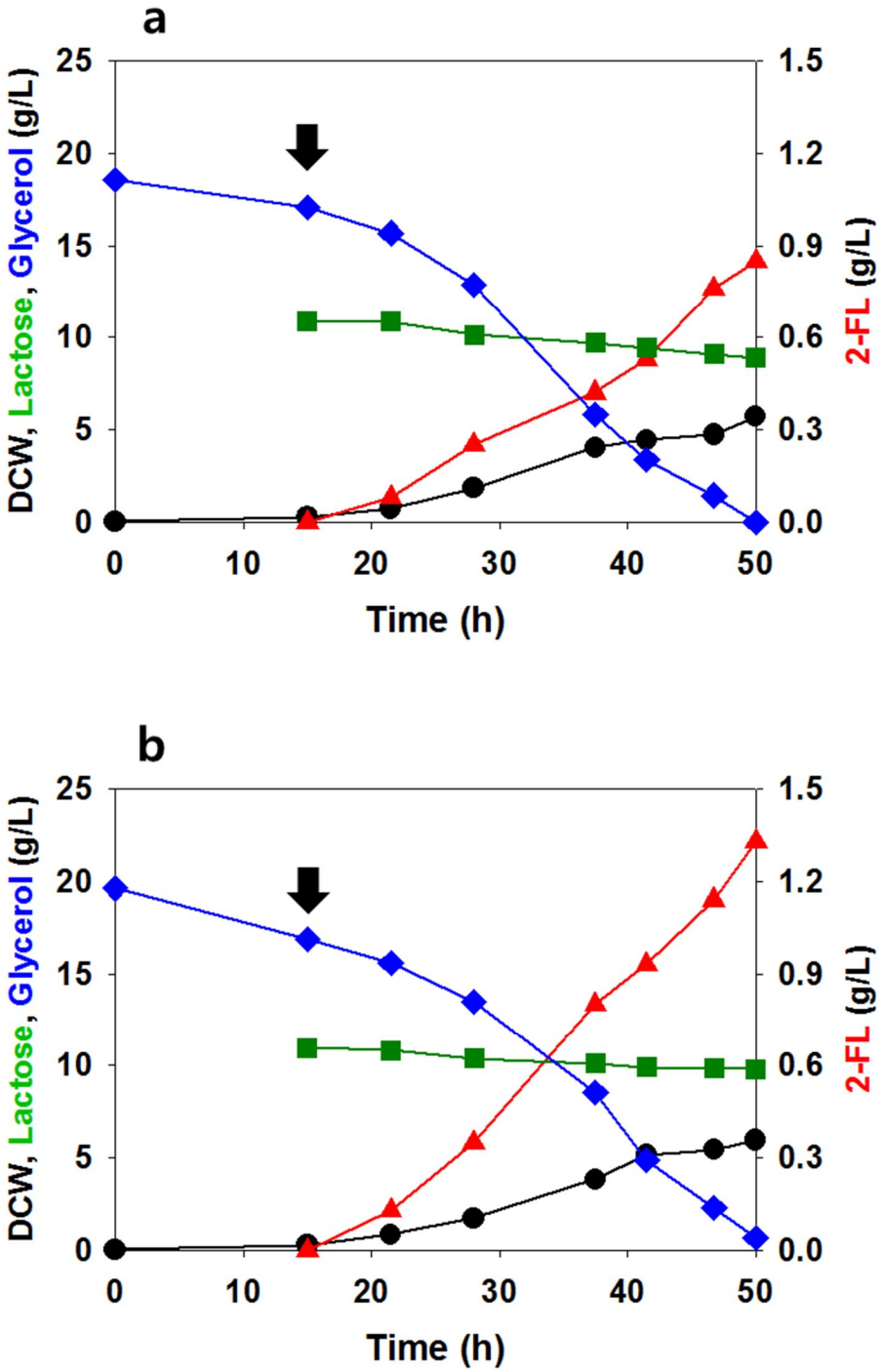

a
b
DCW, Lactose, Glycerol (g/L)
2-FL (g/L)
Time (h)
0
5
10
15
20
25
0.0
0.3
0.6
0.9
1.2
1.5
0
10
20
30
40
50

Fig. 4.5. Batch fermentation of engineered *E. coli* strains (a) ΔL M15 BCGW-W and (b) ΔL YA BCGW-W. After depletion of 20 g/L glycerol, IPTG and lactose were added (thick arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆.

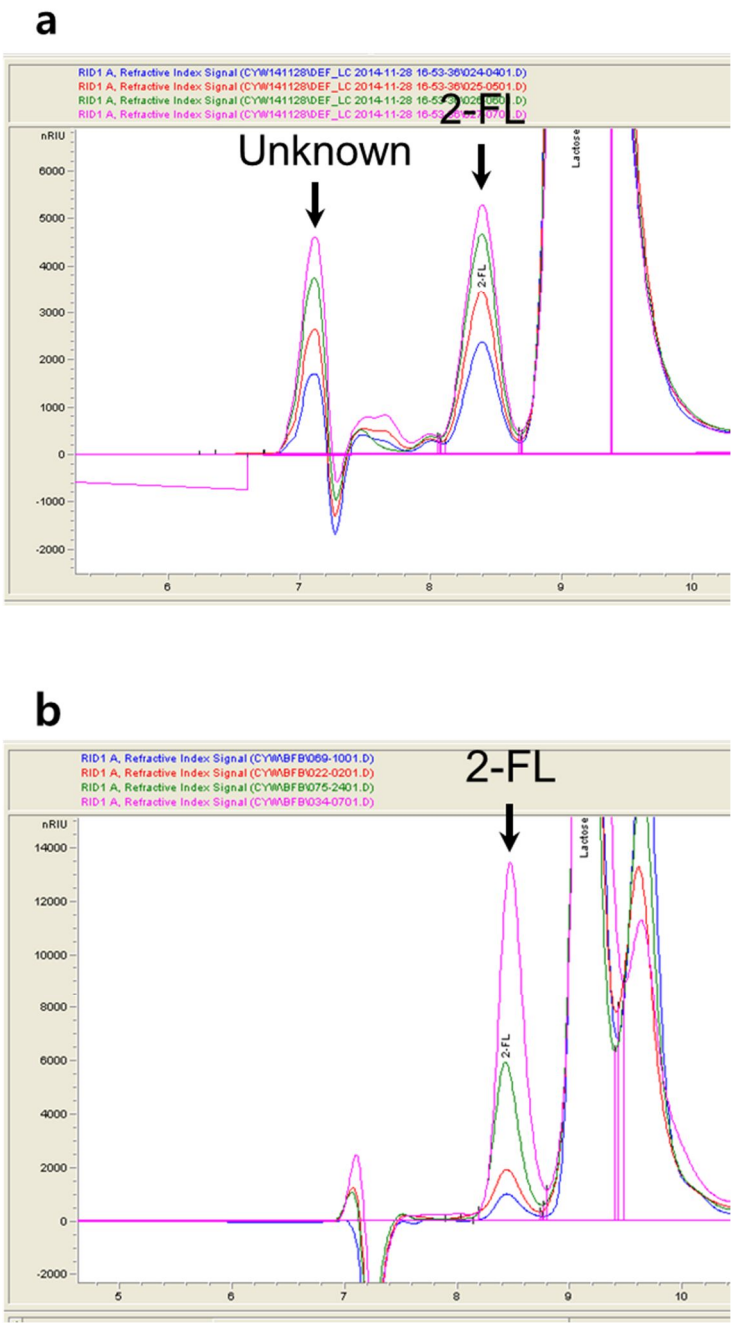
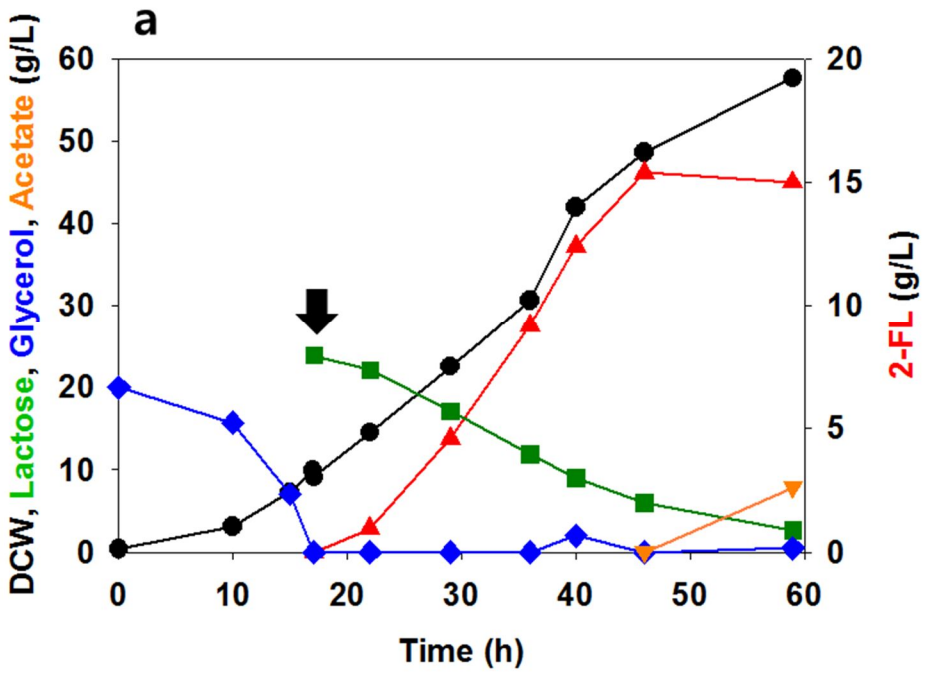



Fig. 4.6. HPLC analysis of supernatant of fed-batch fermentation of (a) ΔL M15 BCGW-W and (b) ΔL YA BCGW-W


a
DCW, Lactose, Glycerol, Acetate (g/L)
2-FL (g/L)
Time (h)
0
10
20
30
40
50
60
0
5
10
15
20

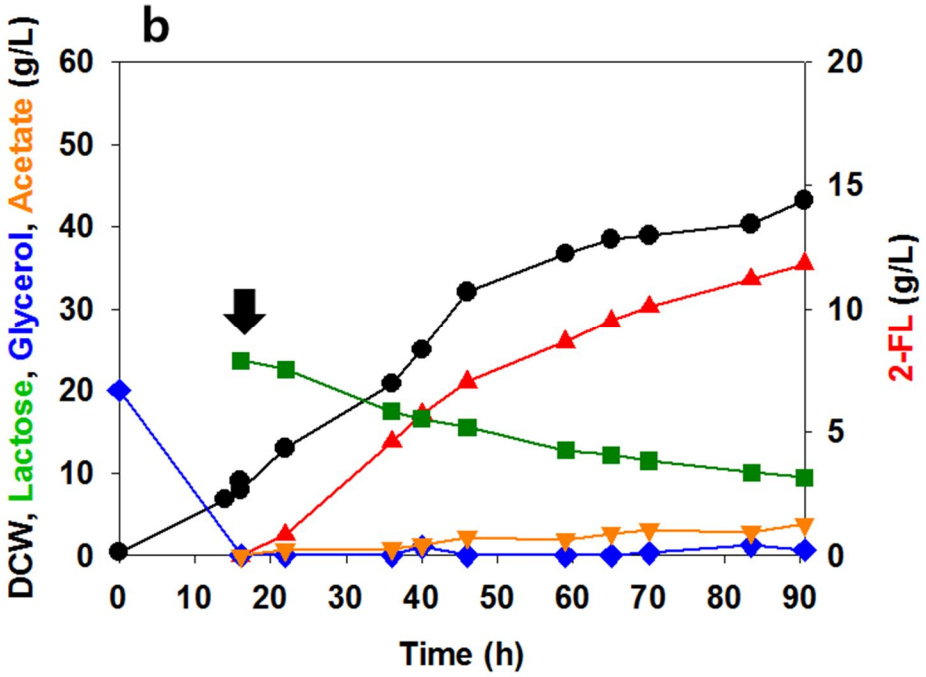

b
DCW, Lactose, Glycerol, Acetate (g/L)
2-FL (g/L)
Time (h)
0
10
20
30
40
50
60
70
80
90
0
5
10
15
20

Fig. 4.7. Fed-batch fermentations of engineered *E. coli* ΔL YA BCGW-W under agitation speed at (a) 1000 rpm and (b) 500 rpm. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (thick arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆; acetate, ▼.

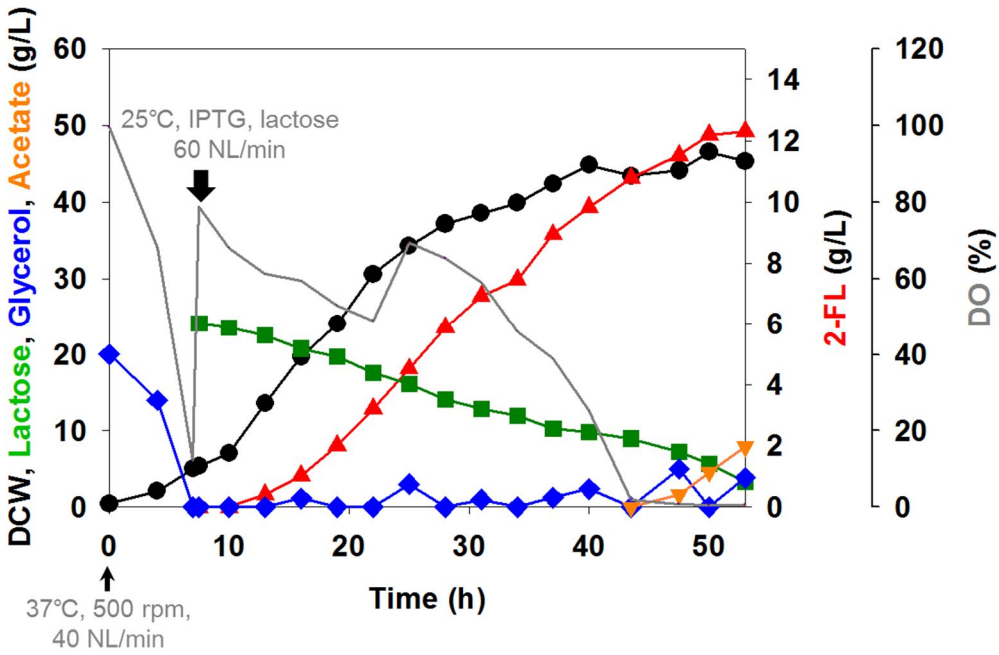



Fig. 4.8. Fed-batch fermentation of engineered *E. coli* ΔL YA BCGW-W done in 75 L bioreactor. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG and lactose were also added at the same time (thick arrow). In addition, temperature was shifted from 37°C to 25°C. Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆; acetate, ▼; dissolved oxygen (DO), ─.

# Chapter 5

# Production of 2-FL from fucose, glycerol and lactose via the *salvage* pathway of GDP-L-fucose biosynthesis

## 5.1. Summary

2-FL is one of the key oligosaccharides in human milk. In the present chapter, the *salvage* GDP-L-fucose biosynthetic pathway was employed in engineered *Escherichia coli* BL21star(DE3) for efficient production of 2-FL. Introduction of the *fkp* gene coding for fucokinase/GDP-L-fucose pyrophosphorylase (Fkp) from *B. fragilis* and the *fucT2* gene encoding α-1,2-fucosyltransferase from *H. pylori* allowed the engineered *E. coli* to produce 2-FL from fucose, lactose and glycerol. Since major lactose flux went to cell growth, marginal amounts of 2-FL was produced. Therefore, the attenuated and deleted mutants of β-galactosidase were employed and thereby, the 2-FL concentration, yield and productivity were significantly enhanced. In addition, the 2-FL yield and productivity were further improved by deletion of the *fucI-fucK* gene cluster coding for fucose isomerase (FucI) and fuculose kinase (FucK). Finally, fed-batch fermentation of engineered *E. coli* BL21star(DE3) deleting *lacZ* and *fucI-fucK*, and expressing *fkp* and *fucT2* resulted in 23.1 g/L of extracellular concentration of 2-FL concentration, 0.37 mole 2-FL/mole lactose, 0.36 mole 2-FL/mole fucose yield and 0.39 g/L/h productivity.

## 5.2. Introduction

2-FL is the one of main functional oligosaccharides in human milk, which is involved in various biological processes. In microbial production of 2-FL, GDP-L-fucose is a key precursor that can control the overall productivity and the yields of 2-FL (Lee et al., 2012a). GDP-L-fucose is able to be synthesized in the cytoplasm through the *de novo* pathway or the *salvage* pathway. The major pathway for biosynthesis of GDP-L-fucose is the *de novo* pathway which is present in both prokaryotes and eukaryotes and catalized by GDP-D-mannose 4,6-dehydratase (Gmd) and GDP-L-fucose synthase (WcaG) (Lee et al., 2009b). An alternative pathway of GDP-L-fucose biosynthesis is the *salvage* pathway that reutilizes L-fucose generating from the degradation of glycoproteins and glycolipids (Coffey et al., 1964). This pathway involves the phosphorylation of L-fucose by L-fucokinase (Fuk, EC 2.7.1.52) at the anomeric position to form L-fucose-1-phosphate with ATP as a cofactor (Butler & Serif, 1985; Park et al., 1998). And then, L-fucose-1-phosphate with GTP is condensed by L-fucose-1-phosphate guanylyltransferase (Fpgt, EC 2.7.7.30) to form GDP-L-fucose (Ishihara & Heath, 1968). The *salvage* pathway of GDP-L-fucose had considered only presented in eukaryotes until the discovery of L-fucokinase/GDP-L-fucose pyrophosphorylase (Fkp), a bifunctional enzyme isolated from *B. fragilis* 9343 (Coyne et al., 2005). A number

of attempts have been reported to produce GDP-L-fucose or fucosyllactose from fucose via the *salvage* pathway, however, the production titers were below the level of 1 g/L.

L-Fucose can be used as carbon source in *E. coli* through an inducible pathway by the sequential reactions of L-fucose isomerase (FucI), L-fuculose kinase (FucK), and L-fuculose 1-phosphate aldolase (FucA) (Chakrabarti et al., 1984). Therefore, blocking of the fucose metabolism seems to bring positive effects on 2-FL production from fucose.

In previous chapters, 2-FL has been produced from glycerol and lactose via the *de novo* pathway of GDP-L-fucose synthesis. To increase the product yield from lactose, the β-galactosidase activity has been minimized or eliminated by deletion of the endogenous *lac* operon of *E. coli* BL21star(DE3) and introduction of the engineered *lac* operon containing partially deleted *lacZ* or *lac* operon without *lacZ* into the chromosome of the *lac* operon deleted *E. coli* BL21star(DE3).

In this chapter, the *salvage* pathway of GDP-L-fucose, instead of the *de novo* pathway, has been introduced for 2-FL production from fucose, glycerol and lactose. It was done by expressing the Fkp from *B. fragilis* and the FucT2 from *H. pylori*. In addition, the lactose operon and the fucose metabolism have been modulated to increase the 2-FL yield.

## 5.3. Materials and methods

### 5.3.1. Strains and plasmids

All strains, plasmids, and oligonucleotides used in this study are listed in Table 5.1 and 5.2. *E. coli* TOP10 and BL21star(DE3) (Invitrogen, Carlsbad, CA, USA) were used for plasmid construction and a host strain for 2-FL production, respectively. To construct plasmid FF, the *fkp* gene was amplified from the genomic DNA of *B. fragilis* (ATCC25285) using two oligonucleotides (F_*Nde*I_fkp and R_*Pac*I-fkp) by the polymerase chain reaction (PCR). The PCR product digested with *Nde*I and *Pac*I was cloned into plasmid F (Lee et al., 2012a) cut by the same restriction enzymes.

To construct the engineered *E. coli* strains including ΔLF M15 and ΔLF YA, the *fucI-fucK* gene cluster was deleted in the chromosome of the engineered *E. coli* strains using the λ-red mediated recombination method (Datsenko & Wanner, 2000). A PCR fragment containing a kanamycin resistance gene and sequences homologous to the flanking regions of the *fucI-fucK* gene cluster was amplified from pKD13 using two primers, F_del_fucIK and R_del_fucIK. The 1.4 kb PCR product was transformed into ΔL M15 and ΔL YA harboring pKD46 by electroporation. Upon recombination and selection for resistant

colonies, the kanamycin-resistant gene was eliminated through transformation of pCP20. After overnight incubation at 42°C, the *fucI-fucK* gene cluster deleted mutant without the kanamycin resistance gene and pCP20 was selected. The deletion of the *fucI-fucK* gene cluster was verified by the colony PCR using two PCR primers of F_ch_ fucIK and R_ch_ fucIK.

### 5.3.2. Culture conditions

*E. coli* was grown in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) with appropriate antibiotics for genetic manipulation and seed culture. Batch fermentations were performed in a 500 mL baffled flask (Nalgene) containing 100 mL of a defined medium (Chin et al., 2015) with 20 g/L glycerol and 50 μg/mL kanamycin at 25°C. The agitation speed of 250 rpm was provided for aerobic condition. When optical density ($OD_{600}$) reached 0.8, isopropyl-*β*-D-thiogalactopyranoside (IPTG), lactose and fucose were added to a final concentration 0.1 mM, 10 g/L and 10 g/L, respectively. Fed-batch fermentations were carried out in a 2.5 L bioreactor (Kobiotech, Incheon, Korea) containing 1.0 L of a defined medium with 20 g/L glycerol and 50 μg/mL kanamycin at 25°C. After complete exhaustion of glycerol added initially, feeding solution containing 800 g/L glycerol

and 20 g/L $MgSO_4.7H_2O$ was added using the pH-stat mode. At the same time, IPTG, lactose as well as fucose were also added to a final concentration 0.1 mM, 25 g/L and 25 g/L for induction of the T7 promoter-mediated gene expression and production of 2-FL. For pH-stat feeding, an appropriate amount of 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 by addition of 28% $NH_4OH$. In order to prevent the deficiency of dissolved oxygen throughout the cultivation, agitation speed and aeration rate were maintained at 1,000 rpm and 2 vvm, respectively.

### 5.3.3. Measurement of cell and extracellular metabolite concentrations

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, fucose, glycerol 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.

### 5.3.4. Identification of 2-FL produced from fucose by the engineered *E. coli*

2-FL was readily purified and enriched by solid-phase extraction (SPE) using a porous graphitized carbon cartridge prior to mass spectrometry analysis. Enriched 2-FL was analyzed using a Bruker ultrafleXtreme MALDI-TOF/TOF system with 1,000Hz Smart beam Ⅱ laser. 2, 5-Dihydroxy-benzoic acid (DHB) was used as a matrix (5 mg/100 mL in 50% ACN:$H_2O$) and 0.01 M NaCl was added as a cation dopant to increase signal sensitivity. The sample was spotted on a stainless steel target plate, followed by the NaCl dopant and matrix. The spot was dried under vacuum prior to mass spectrometric analysis. MALDI-TOF/TOF MS via collision-induced dissociation (CID) was then

performed to confirm the structure of 2-FL. Tandem MS spectra were gained at 1k eV collision energy with argon gas.

### 5.3.5. Analysis of expression of Fkp and FucT2 in engineered *E. coli*

Total, soluble and insoluble protein fractions of engineered *E. coli* were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12 % polyacrylamide gel). After 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 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.

## 5.4. Results

### 5.4.1. Introduction of the *salvage* pathway of GDP-L-fucose into engineered *E. coli*

To produce 2-FL from fucose, lactose and glycerol, the *fkp* gene involved in the *salvage* pathway of GDP-L-fucose was introduced into BL21star(DE3) expressing α-1,2-fucosyltransferase (FucT2) (Fig. 5.1). As a result of batch fermentation of BL21star(DE3) harboring FF, lactose was consumed followed by glycerol, exhibiting typical diauxic growth. Although all of lactose added was consumed within 41 h, only 0.17 g/L of 2-FL was produced and the 2-FL yield from lactose (0.01 mole/mole) was much lower than 2-FL yield from fucose (0.14 mole/mole) (Fig 5.2a and Table 5.3). Considering the fact that the 2-FL yield from lactose (0.01 mole/mole) was much lower than 2-FL yield from fucose (0.14 mole/mole) (Table 5.3), most lactose might have been used for cell growth instead of 2-FL production, which is consistent with previous chapter (Table 2.4).

### 5.4.2. Identification of 2-FL produced by engineered *E. coli*

To confirm biosynthesis of 2-FL in the engineered *E. coli*, subsequent analysis was carried out with MALDI-TOF MS. Figure 5.3a shows the

representative MADLI-MS spectrum of 2-FL production in the positive ion mode. Oligosaccharide compositions were initially assigned according to accurate masses. The ions at *m/z* 657.222 and 511.164 corresponding to di-fucosyllactose ($[Hex_2+Fuc_1+Na]^+$) and mono-fucosyllactose ($[Hex_2+Fuc_2+Na]^+$), respectively were observed as major glycans in 2-FL production. Hexose series consisting of the degree of polymerization 5-11 were also present as minor oligomers of 2-FL production. Tandem mass spectrometry (MS/MS) using collision-induced dissociation (CID) was further performed to confirm the composition and to elucidate the structure of fucosyllactoses. The representative MS/MS spectrum of the ion at *m/z* 511.164 and *m/z* 657.222 were shown in Fig. 5.3b and 5.3c, respectively. Fig. 5.3b shows the product ion spectrum obtained from CID tandem MS of the 2-FL precursor ions at *m/z* 511.164 in the positive mode. The monosaccharide sequence was readily determined by consecutive loss of one fucose at *m/z* 365.114 and one hexose at *m/z* 203.061. And the CID of the isolated 3-FL at *m/z* 657.222 is shown in Fig. 5.3c. CID yields the loss of one fucose at *m/z* 511.164, followed by the loss of a second fucose at *m/z* 365.085, and the loss of a hexose at *m/z* 203.032. The structure assigned by tandem MS was inset in Fig. 5.3. Indeed, it was clearly confirmed the presence of mono-, di- fucosyllactose produced by engineered *E.coli* by mass spectrometry analysis.

### 5.4.3. Effect of modulation of *lac* operon on 2-FL production via the *salvage* GDP-L-fucose biosynthetic pathway

The effect of modulation of β-galactosidase activity on 2-FL production via the *salvage* pathway of GDP-L-fucose was investigated with three engineered *E. coli* strains (ΔL, ΔL M15 and ΔL YA) harboring FF in batch fermentations (Fig. 5.2). In contrast to the wild type BL21star(DE3), the *lac* operon deficient strain (ΔL) could not consume lactose at all and hence, could not produce 2-FL due to the lack of lactose permease (Fig. 5.2b). The ΔL M15 strain with minimized β-galactosidase activity (approximately 3% of wild type) was able to produce 0.93 g/L of 2-FL with the yield of 0.10 mole 2-FL/mole of lactose which was corresponding to a 5.5- and a 9.1-fold higher than those of BL21star(DE3) (Fig. 5.2c and Table 5.3). In the culture of the LacZ deletion mutant (ΔL YA), the yield from lactose increased by a 4-fold (0.43 mole/mole) albeit with 2-FL titer was similar (0.81 g/L) compared with the ΔL M15 strain (Fig. 5.2d and Table 5.3). As demonstrated in the previous chapters with the *de novo* pathway of GDP-L-fucose, blocking the lactose metabolism and retaining the lactose permeation might be also crucial for the bioconversion of lactose and fucose to 2-FL via the *salvage* pathway of GDP-L-fucose.

### 5.4.4. Deletion of the *fucI-fucK* gene cluster for efficient production of 2-FL

L-Fucose is metabolized through an inducible pathway in *E. coli* by the consecutive reactions of L-fucose isomerase (FucI), L-fuculose kinase (FucK), and L-fuculose 1-phosphate aldolase (FucA) (Chakrabarti et al., 1984). To redirect the fucose flux from cell growth to 2-FL production, the *fucI-fucK* gene cluster was deleted from the chromosome of LacZ-attenuation mutant (ΔL M15) and LacZ-deletion mutant (ΔL YA). Batch fermentation of the resulting strains (ΔLF M15 and ΔLF YA) harboring FF was carried out in flask culture under the same condition as the previous experiments (Fig. 5.1 and Table 5.1).

The engineered *E. coli* ΔLF M15 produced 2.08 g/L of 2-FL which is a 2.2-fold increase compare to the ΔL M15 strain. The 2-FL yield from fucose and the 2-FL yield from lactose were 0.63 mole 2-FL/mole fucose and 0.35 mole 2-FL/mole lactose, corresponding to a 3.5-fold and a 2.0-fold enhancement, respectively (Fig. 5.2e, Table 5.3). Similarly, deletion of the *fucI-fucK* gene cluster improved 2-FL production for the ΔL YA strain. Batch fermentation of the ΔLF YA harboring FF resulted in 1.9 g/L of 2-FL concentration, yield of 0.508 from lactose and yield of 0.677 from fucose, which were 230%, 17% and 93% higher than those obtained before the deletion of the *fucI-fucK*

gene cluster in host *E. coli* strain (Fig. 5.2f, Table 5.3). This might be due to the increase of the intracellular availability of fucose by eliminating the fucose assimilating enzymes such as fucose isomerase and fuculose kinase.

Deletion of *fucI-fucK* exerted a positive influence on the cell growth of the engineered *E. coli* as well. As shown in the Fig. 5.4a, the values of specific growth rate of the *fucI-fucK* deletion mutants (ΔLF M15 and ΔLF YA) were 14% to 78% higher than those of wild type or undeleted strains (ΔL M15 and ΔL YA). To examine the impact of the fucose catabolic pathway on the cell growth, *E. coli* ΔL YA and its *fucI-fucK* deletion mutant (ΔLF YA) were cultivated either with or without fucose in the minimal media. As shown in Fig. 5.4b, addition of fucose inhibited cell growth significantly. Interestingly, cell growth was restored by eliminating fucose catabolic metabolism through deletion of *fucI-fucK* showing a similar pattern of cell growth regardless of the presence of fucose in the growth medium.

### 5.4.5. Fed-batch fermentations of engineered *E. coli* strains expressing Fkp and FucT2

To achieve high cell density and high titer of 2-FL, fed-batch fermentation of the ΔL M15 strain harboring FF were carried out in a bioreactor. After complete depletion of glycerol added initially, feeding solution containing glycerol was fed by a pH-stat mode in order to supply a carbon source for cell growth (Fig. 5.5a). At the same time, production of 2-FL was started by IPTG induction as well as addition of lactose and fucose. After depletion of initial lactose in 55 h of culture, lactose and fucose were dumped to the fermentation broth in order to maintain 2-FL biosynthesis. Acetate accumulation was not observed throughout the fermentation. Through the assimilation of glycerol, cell growth was maintained to reach 59.0 g/L of final cell concentration. As a result, 20.6 g/L of 2-FL with a yield of 0.27 mole 2-FL/mole lactose, 0.26 mole 2-FL/mole fucose and productivity of 0.32 g/L/h were obtained at the end of fed-batch fermentation. The final concentration and productivity of 2-FL through the *salvage* pathway of GDP-fucose were 8.0 and 73.9-folds higher than those obtained from the engineered *E. coli* overexpressing the genes involved in the *de novo* synthesis of GDP-L-fucose (Gmd, WcaG, ManB and ManC) under the comparable conditions (Table 5.4).

To analyze the expression of Fkp and FucT2 during the fermentation period, SDS-PAGE of total, soluble and insoluble fractions of ΔL M15

harboring FF was performed (Fig. 5.6). Most of Fkp was expressed as a soluble form. Although most of FucT2 was expressed in inclusion bodies, 2-FL was produced after IPTG induction because a soluble and active form of FucT2 might be available as observed in previous studies (Chin et al., 2015; Lee et al., 2012a).

To evaluate the effect of complete elimination of the *lacZ* gene along with the *fucI-fucK* gene cluster on 2-FL production in a bioreactor, fed-batch fermentation of ΔLF YA harboring FF was performed in the same condition. As shown in Fig 5.5b, specific growth rate of the *fucI-fucK* lacking strain (ΔLF YA) was 34% higher than that of the ΔL M15 strain which is similar phenomenon observed in the batch fermentations of those strains. The final dry cell weight was 69.8 g/L. After addition of IPTG, lactose and fucose, 26 g/L lactose was consumed rapidly in about 47 h and more lactose was dumped. According to the consumption of lactose and fucose, 2-FL was produced efficiently and its maximum concentration was achieved at 23.1 g/L in culture medium. During the 2-FL production stage after 14 h culture, 2-FL yields of 0.37 mole/mole lactose, 0.36 mole/mole fucose and 2-FL productivity of 0.39 g/L/h were obtained finally. These values were 36%, 38% and 22% higher than those of the engineered *E. coli* ΔL M15 strain harboring FF (Table 5.4).

## 5.5. Discussion

Although direct supplementation of fucose for biosynthesis of GDP-L-fucose is favor in microbial production of 2-FL, the purified fucose is relatively expensive. However, the defatted residue of microalgal biomass could be hydrolyzed to several monosaccharides such as glucose, galactose, xylose, mannose, rhamnose, ribose and fucose (Brown, 1991). All of these sugars except for fucose were consumed by *S. cerevisiae* and *Streptomyces lividans* to produce ethanol and β-agarase (Park et al., 2015), which open the economically feasible production of 2-FL from the aqua-cultural wastes. Introduction of the *salvage* pathway of GDP-L-fucose from *B. fragilis* and α-1,2-fucosyltransferase from *H. pylori* allowed efficient production 2-FL from fucose, lactose and glycerol. Moreover, deletion of the *lacZ* and *fucI-fucK* genes led to an enhancement in the yield and productivity of 2-FL.

Considering the price of fucose, maximal conversion of fucose is crucial in 2-FL production through the *salvage* pathway of GDP-fucose. By elimination of the fucose metabolism, 2-FL yield from fucose significantly increased reaching 0.677 mole 2-FL/mole fucose (Table 5.3). It is noteworthy that the deletion of *fucI-fucK* led to an improvement of cell growth rate in the medium containing fucose when

glycerol was supplied as growth substrate (Fig. 6). It might be due to the L-lactaldehyde which is toxic to cell growth (Chakrabarti et al., 1984; Subedi et al., 2008; Zhu & Lin, 1989). L-Lactaldehyde is formed by fucose aldolase (FucA) during the fucose dissimilation in *E. coli* and the removal of the *fucI-fucK* alleviates the formation of L-lactaldehyde that could enhance cell growth rate.

The extracellular 2-FL concentration of engineered *E. coli* overexpressing Fkp and FucT2 increased up to 23.1 g/L which is a 8.9-fold greater than that of the engineered *E. coli* overexpressing the *de novo* GDP-L-fucose biosynthetic enzymes (Gmd, WcaG, ManB and ManC) and FucT2 (Table 5.4). Such an impressive enhancement of 2-FL could be possible by the efficient bioconversion of fucose to GDP-L-fucose through the *salvage* pathway as well as increased fucose and lactose availability by elimination of lactose and fucose metabolism.

Table 5.1. List of strains and plasmids used in Chapter 5

| Name | Relevant description | Reference |
|---|---|---|
| Strains | | |
| *E. coli* BL21star(DE3) | $F^-$, *ompT*, *hsdSB*($r_B^- m_B^-$), *gal*, *dcm rne131* (DE3) | Invitrogen (Carlsbad, CA, USA) |
| ΔL | BL21star(DE3) *ΔlacZYA* | (Chin et al., 2015) |
| ΔL M15 | ΔL *Tn7::lacZΔM15YA* | (Chin et al., 2015) |
| ΔL YA | ΔL *Tn7::lacYA* | Chapter 4 |
| ΔLF M15 | ΔL*ΔfucI-fucK Tn7::lacZΔM15YA* | This study |
| ΔLF YA | ΔL*ΔfucI-fucK Tn7::lacYA* | This study |
| Plasmids | | |
| pCOLADuet-1 | Two T7 promoters, ColA replicon, $Kan^R$ | Novagen |
| F | pCOLADuet-1 + *fucT2* (*Nco*I / *Sac*I) | (Lee et al., 2012a) |
| FF | pCOLADuet-1 + *fucT2* (*Nco*I / *Sac*I) + *fkp* (*Nde*I/*Pac*I) | This study |

Table 5.2. Primers used in Chapter 5

| Name | Sequence |
|---|---|
| F_*Nde*I_fkp | GGAATTC*CATATG*CAAAAACTACTATCTTTACCG |
| R_*Pac*I_fkp | C*TTAATTAA*TTATGATCGTGATACTTGGA |
| F_del_fucIK | CCTTTCTCCGCCTTAGCAGAGCGCCAATAAAGCGAGATGAGGAATCCTG GTGTAGGCTGGAGCTGCTTCG |
| R_del_fucIK | GCACTTTCAATAGTTCGGGAGAAATTAACGGCGAAATTGTTTTCAGCATT ATTCCGGGGATCCGTCGACC |
| F_ch_ fucIK | AAGCGAGATGAGGAATCCTG |
| R_ch_ fucIK | CGGCGAAATTGTTTTCAGCATT |

The italic sequences present the recognition sites of specific restriction enzymes.

The underlined nucleotides indicate the homologous recombination regions of the *fucI-fucK* gene cluster in the *E. coli* chromosome.

Table 5.3. Summary of batch fermentations of engineered *E. coli* strains harboring FF

| Strains | Final dry cell weight (g/L) | Lactose consumed[a] (g/L) | Fucose consumed[a] (g/L) | Maximum 2-FL concentration[a] (g/L) | Yield (mole 2-FL/mole lactose) | Yield (mole 2-FL/mole fucose) |
|---|---|---|---|---|---|---|
| WT | 4.35±0.14 | 10.57±0.06 | 0.40±0.06 | 0.17±0.05 | 0.011±0.003 | 0.137±0.022 |
| ΔL | 4.83±0.78 | 0 | 0.62±0.03 | N.D. | - | - |
| ΔL M15 | 4.48±0.46 | 6.56±0.16 | 1.00±0.16 | 0.93±0.24 | 0.100±0.028 | 0.310±0.033 |
| ΔL YA | 4.83±0.01 | 1.36±0.30 | 0.77±0.06 | 0.81±0.10 | 0.433±0.146 | 0.351±0.014 |
| ΔLF M15 | 5.36±0.45 | 4.16±0.33 | 1.11±0.03 | 2.08±0.18 | 0.351±0.003 | 0.631±0.072 |
| ΔLF YA | 5.20±0.13 | 2.59±0.49 | 0.95±0.27 | 1.85±0.10 | 0.508±0.069 | 0.677±0.156 |

[a] Extracellular concentrations of lactose, fucose and 2-FL were determined and used for the calculation of lactose and fucose consumption and 2-FL production.
All values were estimated based on the time of glycerol depletion.

Table 5.4. Comparison of results of fed-batch fermentations of engineered *E. coli* strains harboring FF

| Strains | Dry cell weight (g/L) | Maximum 2-FL concentration[c] (g/L) | Lactose consumed[c] (g/L) | Fucose consumed[c] (g/L) | Yield (mole 2-FL/ mole lactose) | Yield (mole 2-FL/ mole fucose) | Productivity[a] (g/L·h) |
|---|---|---|---|---|---|---|---|
| ΔL M15 BCGW-F[b] | 73.1 | 2.6 | 39.7 | - | 0.064 | - | 0.043 |
| ΔL M15 FF | 59.0 | 20.6 | 53.6 | 27.0 | 0.270 | 0.257 | 0.318 |
| ΔLF YA FF | 69.8 | 23.1 | 44.0 | 21.8 | 0.367 | 0.355 | 0.389 |

[a] 2-FL productivity was estimated during the 2-FL production period after IPTG induction

[b] The results of fed-batch fermentation of ΔL M15 harboring BCGW and F strain using glycerol and lactose was cited in Chin *et al.* (2015).

[c] Extracellular concentrations of lactose, fucose and 2-FL were determined and used for the calculation of lactose and fucose consumption and 2-FL production.

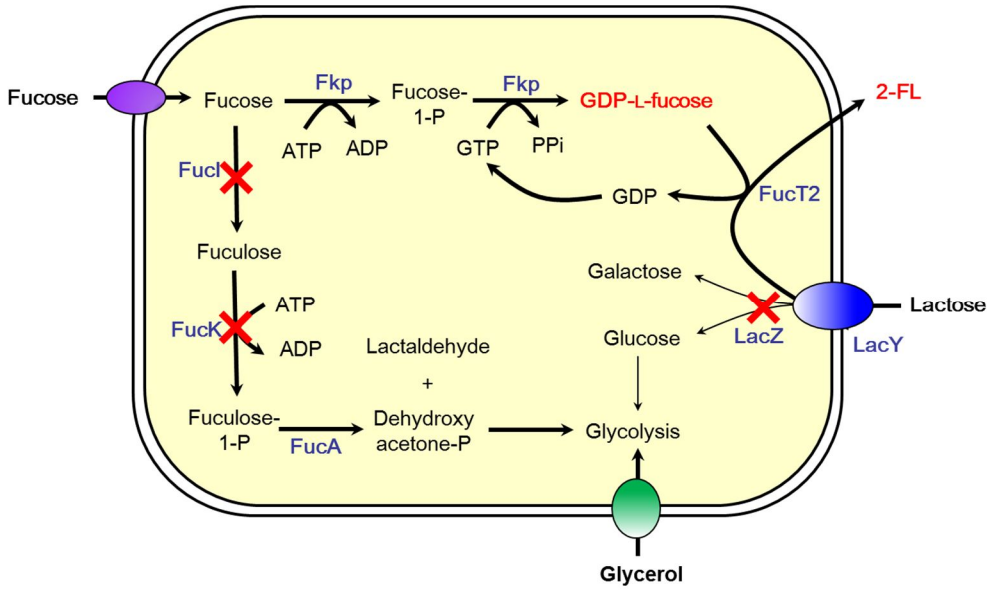
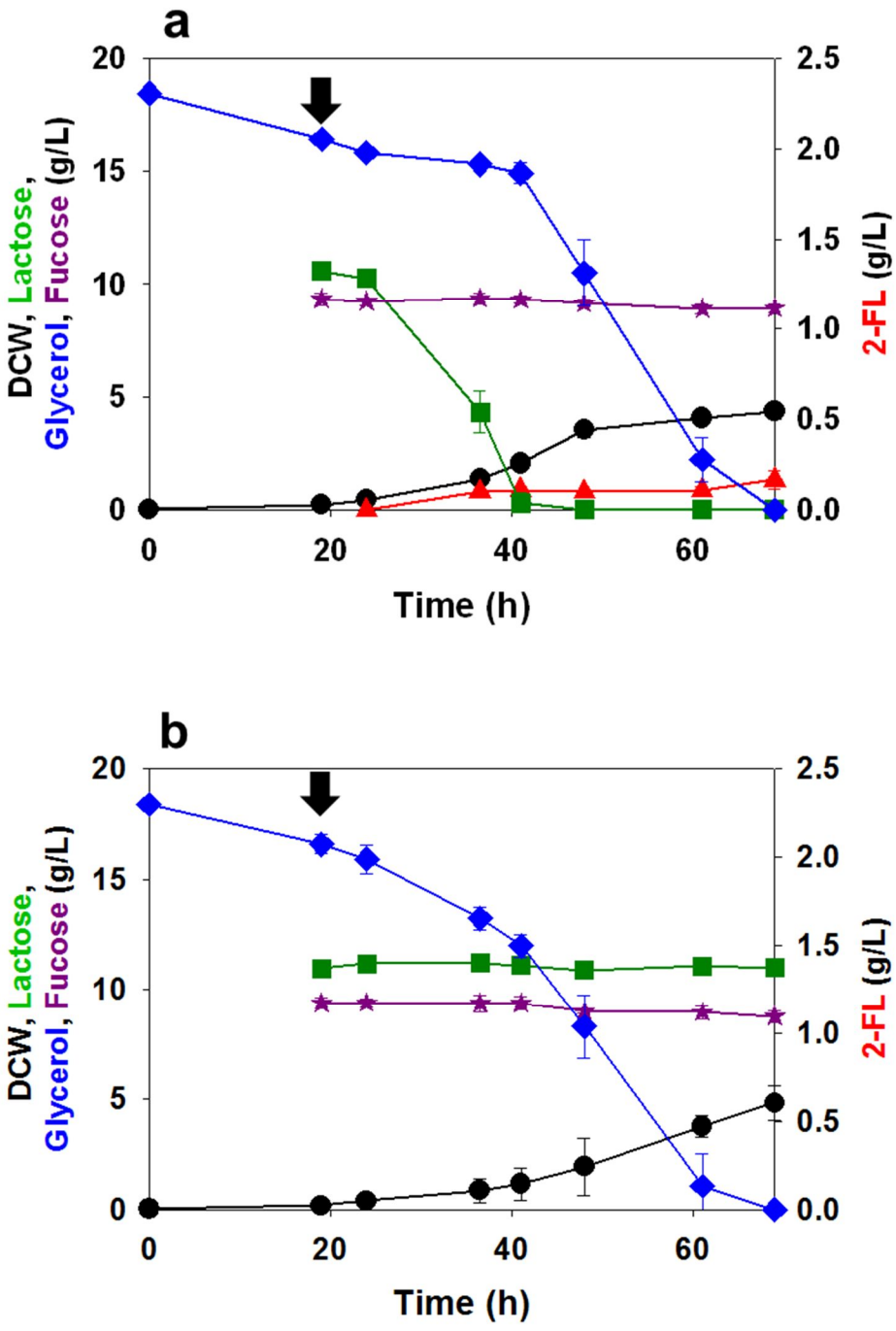
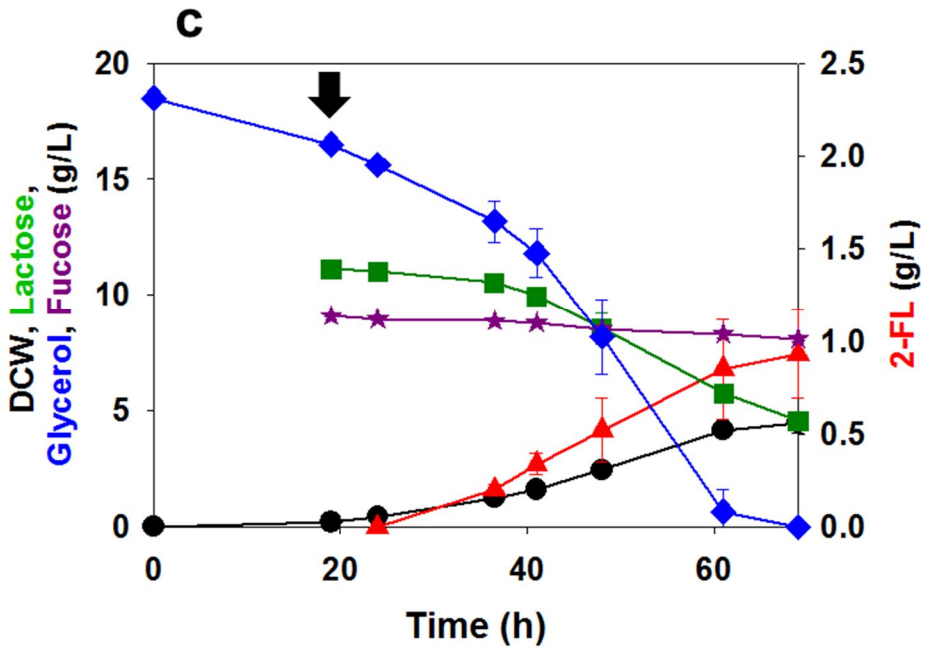
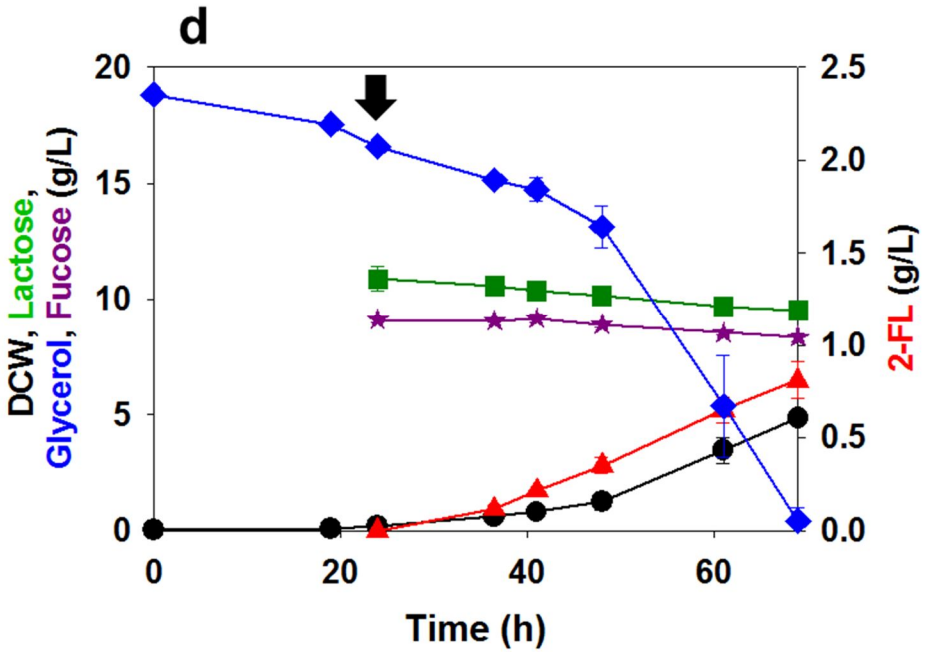
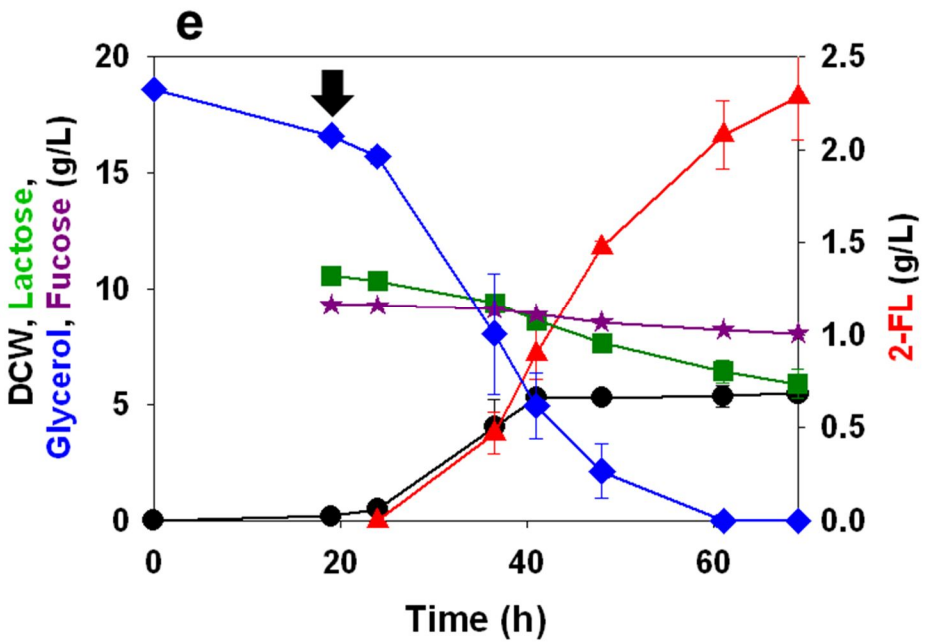



Fig. 5.1. Metabolic engineering of *E. coli* for the production of 2-FL via the *salvage* biosynthetic pathway of GDP-L-fucose. The overall strategy for the production of 2-FL is showed. The X indicated genes knocked out. Fkp and FucT2 were introduced by a plasmid. The names of proteins are abbreviated as follows; LacZ, β-galactosidase; LacY, lactose permease; FucT2, α-1,2-fucosyltransferase; Fkp, fucokinase/fucose-1-phosphate guanylyltransferase; FucI, fucose isomerase; FucK, fuculose kinase; FucA, fuculose-1-phosphate aldolase. PPi and GTP denote diphosphate and guanosine 5'-triphosphate.


a
b
DCW, Lactose,
Glycerol, Fucose (g/L)
2-FL (g/L)
Time (h)
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60


c
DCW, Lactose,
Glycerol, Fucose (g/L)
2-FL (g/L)
Time (h)
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60


d
DCW, Lactose,
Glycerol, Fucose (g/L)
2-FL (g/L)
Time (h)
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60


e
DCW, Lactose,
Glycerol, Fucose (g/L)
2-FL (g/L)
Time (h)
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60

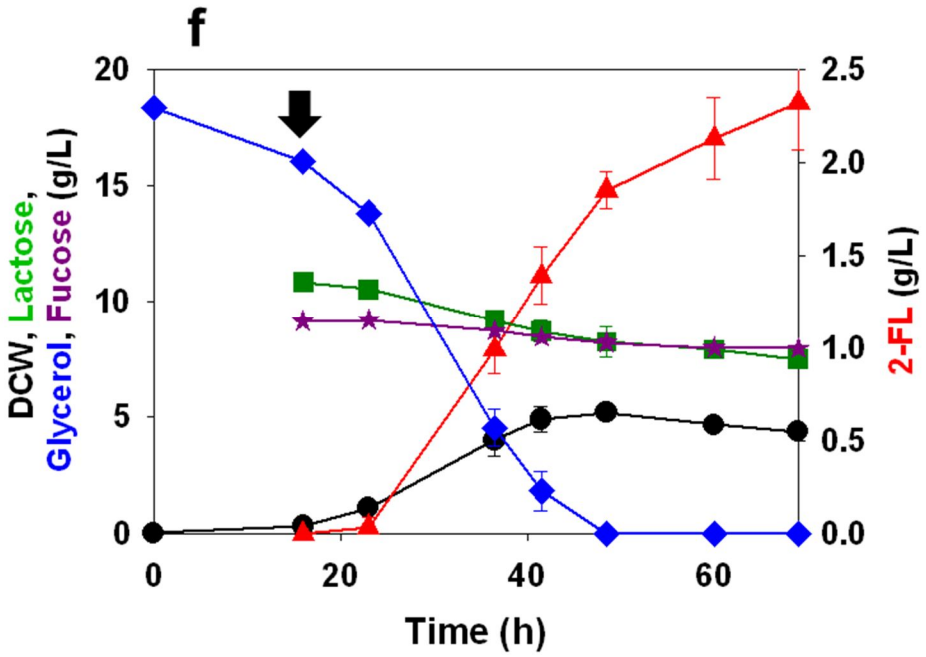

f
DCW, Lactose,
Glycerol, Fucose (g/L)
2-FL (g/L)
Time (h)
0
5
10
15
20
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60

Fig. 5.2. Batch fermentations of recombinant *E. coli* strains harboring FF [(a) BL21star(DE3); (b) ΔL; (c) ΔL M15; (d) ΔL YA; (e) ΔLF M15; (f) ΔLF YA]. When optical density ($OD_{600}$) reached 0.8, IPTG, lactose and fucose were added to a final concentration 0.1 mM, 10 g/L and 10 g/L, respectively (arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆; fucose, ★. Error bars represent standard deviations associated with three independent experiments.

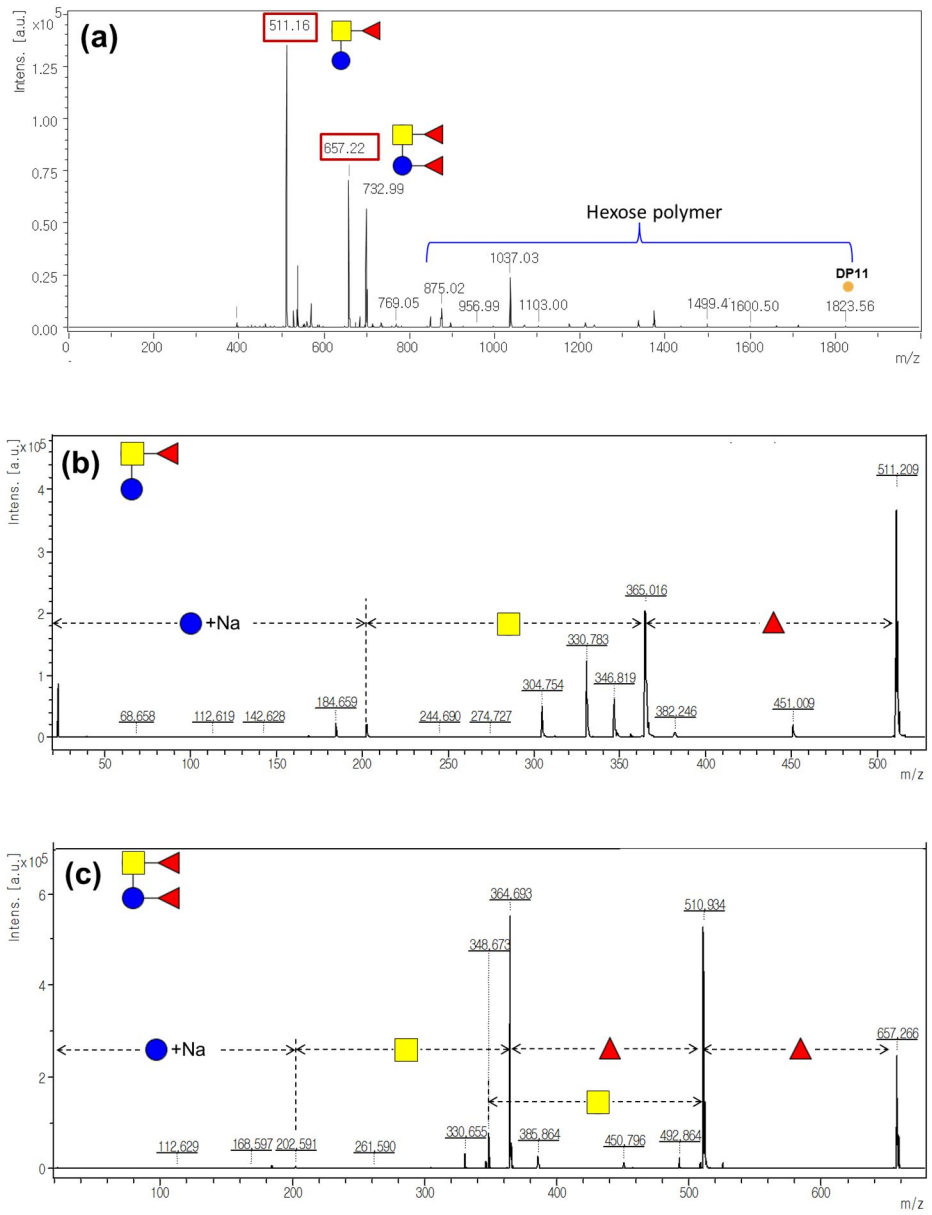



Fig. 5.3. MS spectrum of 2-FL production identified by MALDI-TOF MS (a). CID tandem MS spectrum of mono-FL ($[Hex_2+Fuc_1+Na]^+$) at *m/z* 511.164 (b) and di-FL ($[Hex_2+Fuc_2+Na]^+$) at *m/z* 657.222 (c). Symbols for the types of monosaccharaides (glucose, ●; galactose, ■; fucose, ▲)

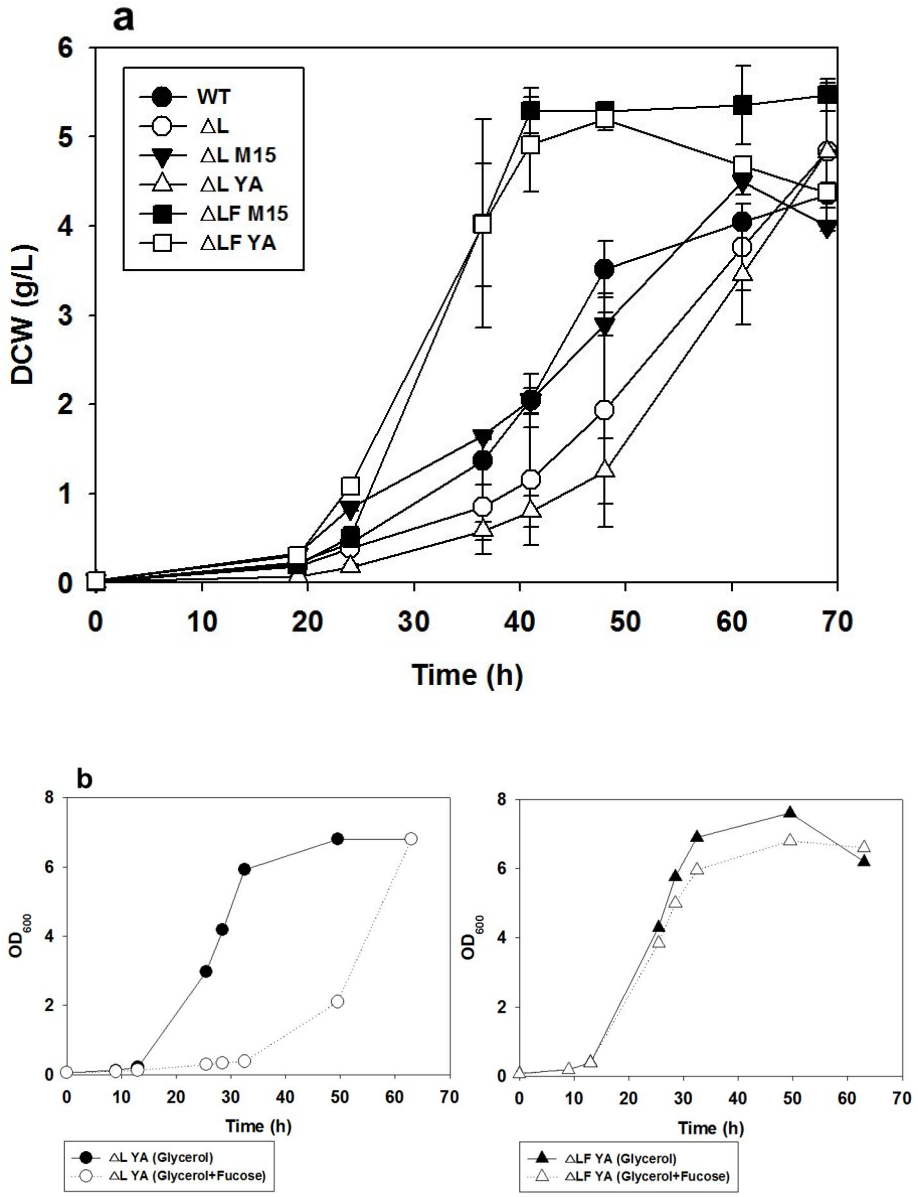
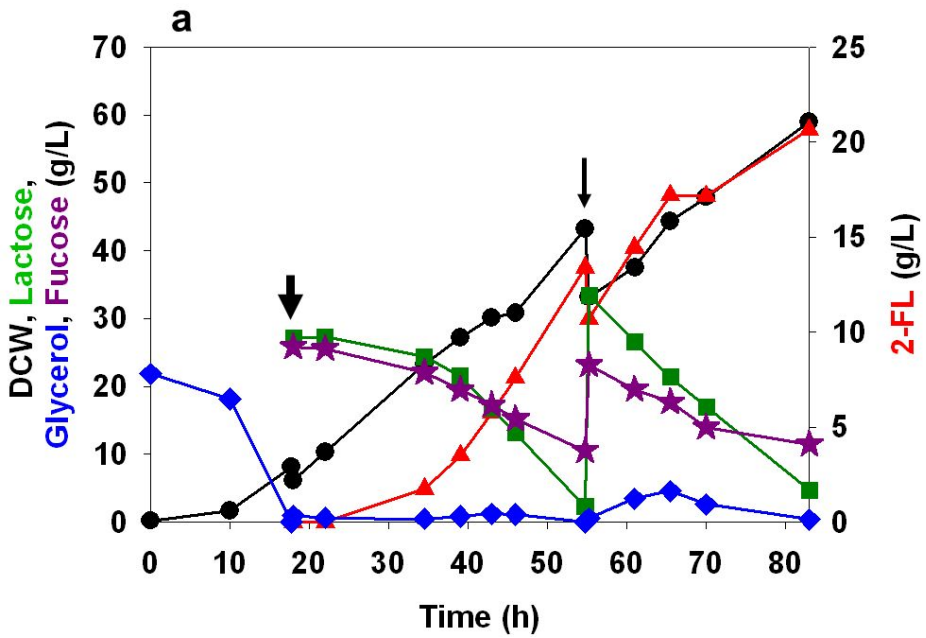



Fig. 5.4. Growth profiles of engineered *E. coli* strains with (a) or without (b) FF.


a
DCW, Lactose,
Glycerol, Fucose (g/L)
2-FL (g/L)
Time (h)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25

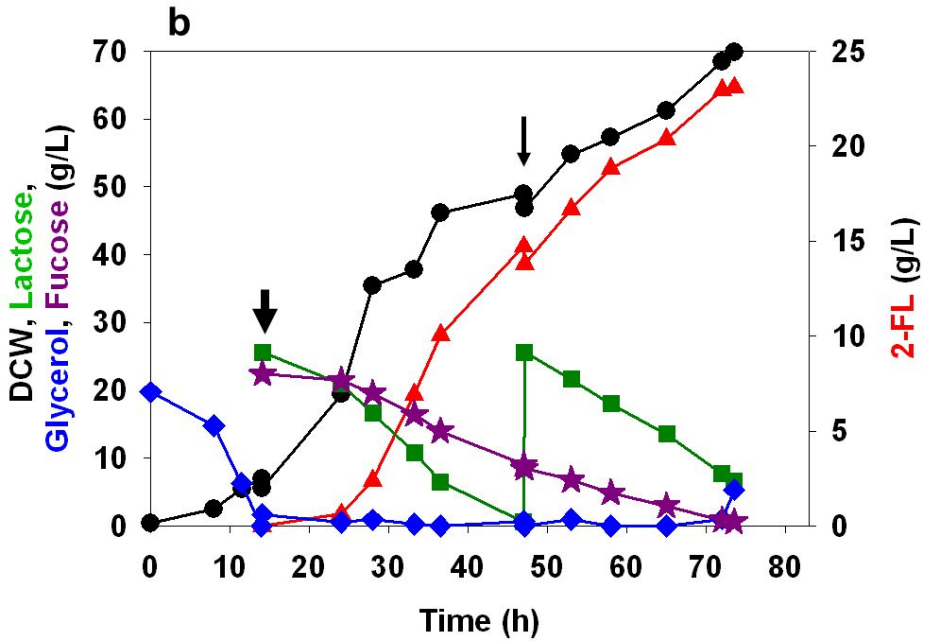

b
DCW, Lactose,
Glycerol, Fucose (g/L)
2-FL (g/L)
Time (h)
0
10
20
30
40
50
60
70
80
0
5
10
15
20
25

Fig. 5.5. Fed-batch fermentations of engineered *E. coli* strains harboring FF [(a) ΔL M15; (b) ΔLF YA]. After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG, lactose and fucose were also added at the same time (thick arrow). 200 g/L lactose and 200 g/L fucose solution were further dumped after depletion of lactose (thin arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆; fucose, ★.

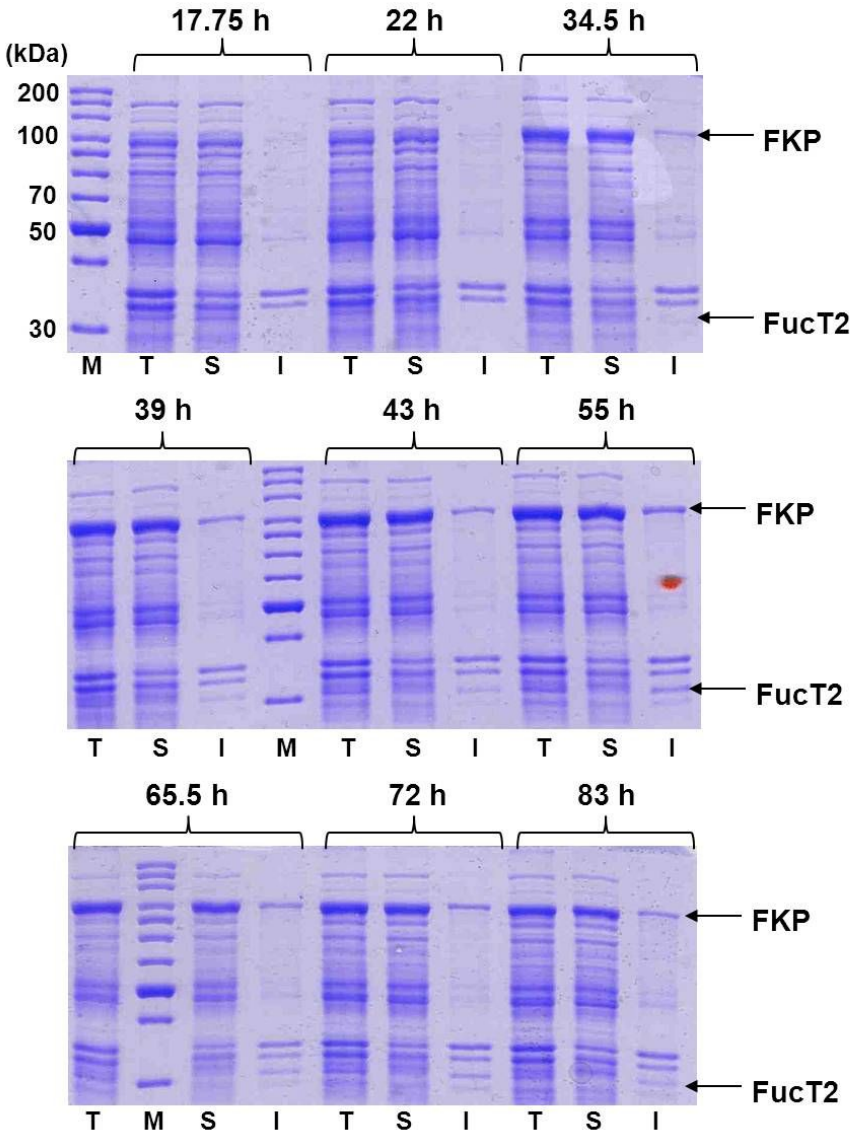



Fig. 5.6. SDS-PAGE analysis to investigate expression patterns during the fed-batch fermentation of engineered *E. coli* ΔL M15 harboring FF. The abbreviations were defined as follows: T; total protein fraction, S; soluble protein fraction, I; insoluble protein fraction, M; molecular weight marker.

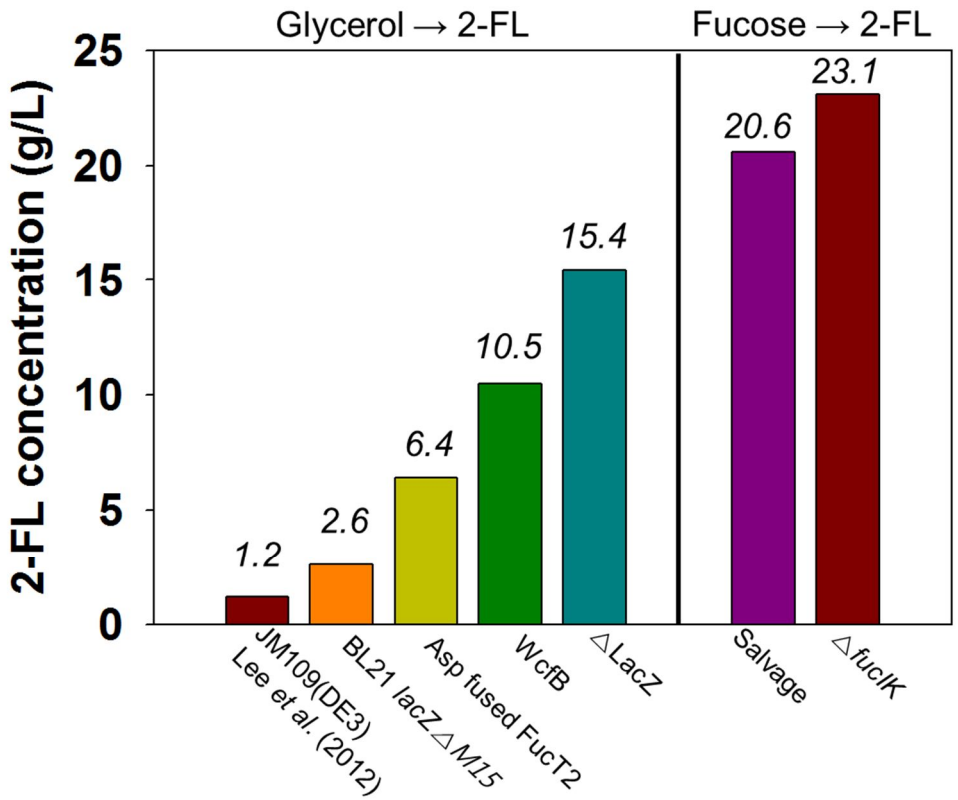



Fig. 5.7. Summary of 2-FL production in this dissertation.

# Chapter 6

# Conclusions

This dissertation has focused on development of microbial systems for efficient production of 2-FL via two kinds of metabolic pathways using engineered *E. coli*. To accomplish this purpose, the limiting factor affecting microbial synthesis of 2-FL such as intracellular lactose availability, activity of fucosyltransferase and supply of GDP-L-fucose were modulated.

Firstly, the β-galactosidase activity of *E. coli* BL21star(DE3) was attenuated by deletion of the whole endogenous *lac* operon and introduction of the modified *lac* operon containing *lacZΔM15*. Ninety-seven percent decreased β-galactosidase activity in engineered *E. coli* improved 2-FL production yield by a three-fold. In fed-batch fermentation using the glycerol pH-stat feeding, 2.6 g/L 2-FL was produced by the engineered *E. coli* expressing enzymes involved in the *de novo* pathway of GDP-L-fucose (ManB, ManC, Gmd and WcaG) and FucT2 from *H. pylori*.

Secondly, the effects of attachment of three to six aspartate molecules at the N-terminal of FucT2 from *H. pylori* on expression of the enzyme and 2-FL production were investigated. As a result, the level of soluble expression increased only in the case of three aspartate tagging. Fucosylation activity was also improved and thus, enhanced 2-FL concentration by a 2.5 fold compared to the control strain was obtained

in fed-batch fermentation of engineered *E. coli* ΔL YA expressing enzymes involved in the *de novo* pathway of GDP-L-fucose and three aspartate tagged fused FucT2.

Thirdly, putative α-1,2-fucosyltransferase (WcfB) from *B. fragilis* was employed in replacement of FucT2 from *H. pylori* for efficient production of 2-FL. A 4 fold enhancement in 2-FL concentration was achieved by expressing WcfB. Moreover, 2-FL titer was further increased up to 15.4 g/L by complete elimination of β-galactosidase activity of the engineered *E. coli* expressing enzymes involved in the *de novo* pathway of GDP-L-fucose and WcfB in fed-batch fermentation.

Finally, an engineered *E. coli* strain which is able to produce 2-FL from fucose, lactose and glycerol was constructed by introducing the *fkp* gene involved in the *salvage* pathway of GDP-L-fucose and coexpressing α-1,2-fucosyltransferase. Through the deletion of the *fucI-fucK* gene cluster involved in fucose assimilation, 23.1 g/L of final concentration of 2-FL was obtained in a fed-batch fermentation.

The microbial systems developed in this research, especially 2-FL production from glycerol and lactose would meet requirements for industrial applications. Substitution of *E. coli* as a 2-FL producer with GRAS microorganisms such as *Corynebacterium* and lactic acid bacteria might be accelerating industrial applications of 2-FL.

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Baumgärtner, F., Seitz, L., Sprenger, G.A., Albermann, C. (2013b) Construction of *Escherichia coli* strains with chromosomally integrated expression cassettes for the synthesis of 2'-fucosyllactose. Microbial Cell Factories, 12(1), 40.

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**Appendix:** Production of 3'-fucosyllactose (3-FL) by expressing α-1,3-fucosyltransferase via the *salvage* pathway of GDP-L-fucose biosynthesis

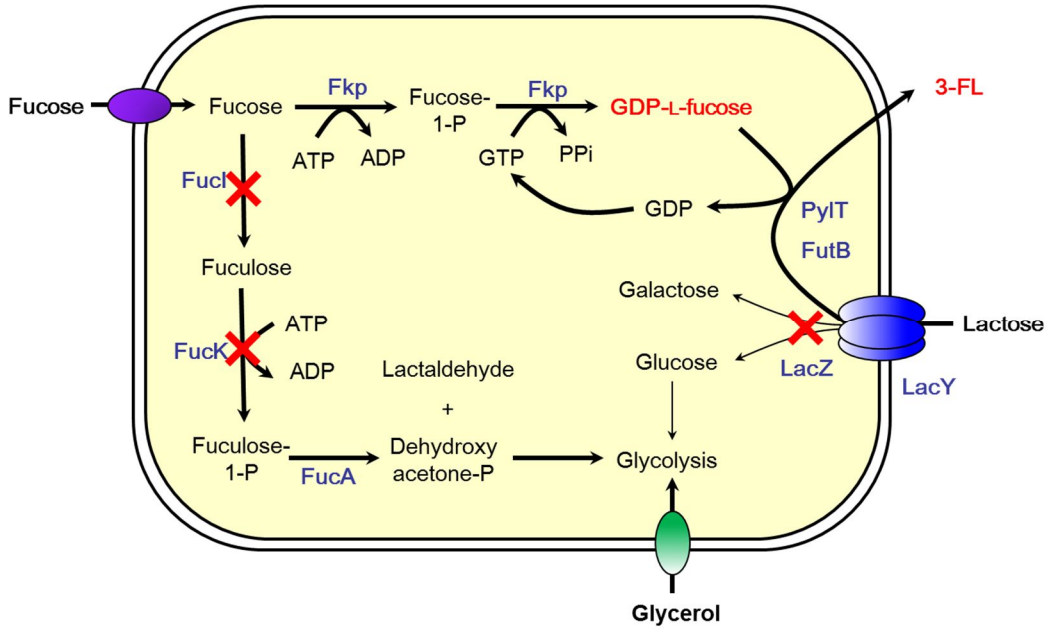
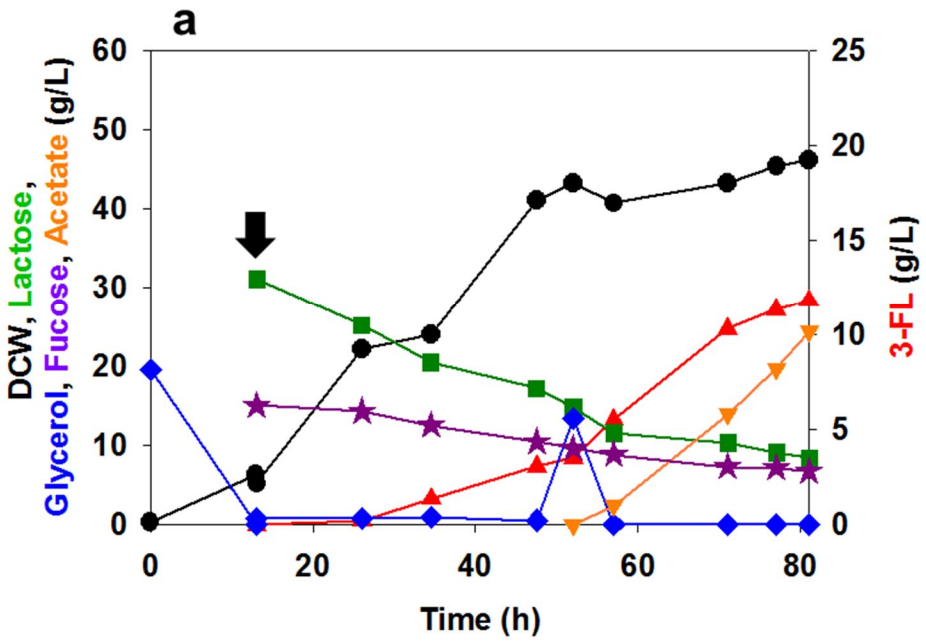



Fig. 1. The strategy for the production of 3-FL via the *salvage* pathway of GDP-L-fucose. The X indicated genes knocked out. Fkp and FucT2 were introduced by a plasmid. The names of proteins are abbreviated as follows; LacZ, β-galactosidase; LacY, lactose permease; PylT/FutB, α-1,3-fucosyltransferases from *H. pylori*; Fkp, fucokinase/fucose-1-phosphate guanylyltransferase; FucI, fucose isomerase; FucK, fuculose kinase; FucA, fuculose-1-phosphate aldolase. PPi and GTP denote diphosphate and guanosine 5'-triphosphate.


a
DCW, Lactose,
Glycerol, Fucose, Acetate (g/L)
3-FL (g/L)
Time (h)
0
10
20
30
40
50
60
0
5
10
15
20
25
0
20
40
60
80

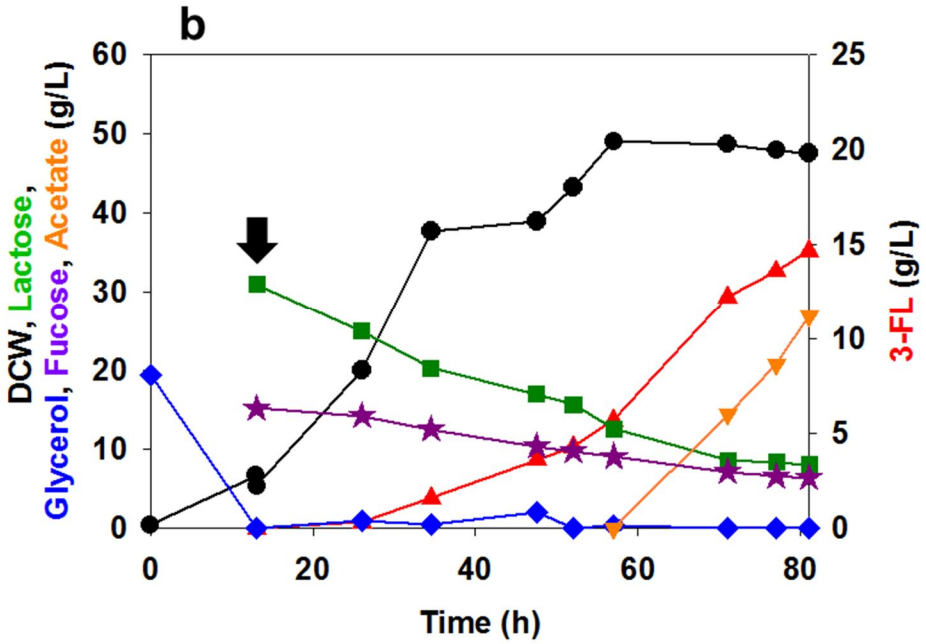

b
DCW, Lactose,
Glycerol, Fucose, Acetate (g/L)
3-FL (g/L)
Time (h)
0
10
20
30
40
50
60
0
5
10
15
20
25
0
20
40
60
80

Fig. 2. Fed-batch fermentations of engineered *E. coli* ΔLF YA harboring pHfkpfutB (a) and pHfkppylT (b). After depletion of 20 g/L glycerol, glycerol pH-stat was started. IPTG, lactose and fucose were also added at the same time (Arrow). Symbols are denoted as follows: dry cell weight, ●; lactose, ■; 2-FL, ▲; glycerol, ◆; fucose, ★; acetate, ▼.

Table 1. Comparison of summarized results of fed-batch fermentations of engineered *E. coli* strains to produce 3-FL

| Strains | Maximum dry cell weight (g/L) | Lactose consumed[a] (g/L) | Fucose consumed[a] (g/L) | Maximum 3-FL conc.[a] (g/L) | Yield (mole 3-FL/mole lactose) | Yield (mole 3-FL/mole fucose) | Productivity[b] (g/L·h) |
|---|---|---|---|---|---|---|---|
| ΔLF YA fkpfutB | 46.1 | 22.6 | 8.3 | 11.8 | 0.366 | 0.479 | 0.17 |
| ΔLF YA fkppylT | 49.0 | 22.9 | 8.8 | 14.7 | 0.448 | 0.558 | 0.22 |

[a] Extracellular concentrations of lactose, fucose and 2-FL were determined and used for the calculation of lactose and fucose consumption and 2-FL production.

[b] 2-FL productivity was estimated during the 2-FL production period after IPTG induction.

# 국문 초록

다른 포유류의 젖과는 달리 인간의 모유에는 독특한 구조를 가지는 올리고당이 상당히 높은 농도(5~15 g/L)로 존재한다. 이러한 모유 올리고당은 prebiotic 효과, 병원균에 의한 감염방지, 면역체계 조절, 염증반응 억제 등의 다양한 생물학적 활성을 갖는 것으로 알려져 있다. 지금까지 밝혀진 약 200여 종의 모유 올리고당 중에서도 특히, 2'-푸코실락토오스 (fucosyllactose, 2-FL)는 그 함량이 가장 높기 때문에 기능성 식품소재 또는 의약품으로의 이용가능성으로 주목 받고 있다. 미생물을 이용한 2-FL의 생산은 화학적 또는 효소적 생산방법에 비해 경제성이 우수하므로 최근 많이 연구되고 있다.

미생물을 이용한 2-FL의 생합성은 세포 내에서의 락토오스의 이용도, 푸코스전이효소의 활성도, GDP-L-fucose의 공급과 같은 인자들에 영향을 받는데, 본 연구에서는 이러한 인자들에 초점을 맞추어 대장균 BL21star(DE3)를 이용한 효율적인 2-FL의 생산시스템을 구축하고자 하였다. 먼저, 세포생장에 이용되는 락토오스의 탄소흐름을 2-FL생산쪽으로 전환시키고자 대장균 염색체 상의 락오페론 전체를 파쇄하고, lacZ△M15를 포함하는 락오페론을 도입시켰고, 그 결과 베타-갈락토시다아제의 활성도가 야생형 대비 3% 수준으로

감소하였다. 이렇게 구축한 ΔL M15 균주에 GDP-L-fucose생합성 효소들과 *H. pylori*유래의 α-1,2-fucosyl-transferase인 FucT2를 과발현 시켜서 대조군에 비해 약 3배 정도 향상된 2-FL의 생산수율을 얻을 수 있었다. 또한 FucT2의 N-말단에 아스파테이트 태그를 부착하여 기존에 대부분이 불용성으로 발현되던 FucT2의 가용성 발현을 향상시켰고, 이러한 돌연변이 FucT2를 발현시킨 재조합 대장균의 유가식 배양을 통해 6.4 g/L의 2-FL을 생산하였는데, 이는 야생형 FucT2를 발현시킨 재조합 대장균보다 약 2.5배 증가된 결과이다.

FucT2를 대신해 선별된 *B. fragilis* 유래의 WcfB를 도입한 재조합 대장균의 유가식 배양을 통해 2-FL 생산 농도를 약 4배 향상시켰다. 또한 남아있는 베타 갈락토시다아제의 활성도를 완전히 제거시켜 구축한 재조합 대장균 ΔL YA에 GDP-L-fucose생합성 효소들과 WcfB를 과발현시켜 유가식 배양을 실시한 결과, 글리세롤과 락토오스로부터 최종 15.4 g/L의 2-FL을 0.858 g/g 락토오스의 수율로 생산할 수 있었고, 이때의 생산성은 시간당 0.53 g/L였다.

한편, 재생경로(*salvage* pathway)는 앞서 이용하였던 신생경로(*de novo* pathway)에 비해 경로가 짧고, 관여하는 효소 및 보효소가 적으므로 fucose의 공여체인 GDP-L-fucose를 보다 효율적으로 공급할 수 있다. 따라서 재생경로를 이용하여 푸코스, 락토오스, 글리세롤로부터 2-FL을 생산하고자 *B. fragilis* 유래의 *fkp*와 *H. pylori*유래의 *fucT2*를 대장균에

도입하였다. 또한 락토오스 및 푸코스 대비 2-FL의 생산수율을 향상시키고자, 락토오스 대사와 푸코스 대사경로를 조절한 재조합 대장균들을 구축하였다. 최종적으로 *lacZ*, *fucI*, *fucK* 유전자가 파쇄되고, Fkp와 FucT2가 과발현된 재조합 대장균의 유가식 배양을 통해 23.1 g/L의 2-FL을 락토오스 대비수율 0.37 mole/mole, 푸코스 대비수율 0.36 mole/mole로 생산할 수 있었다.

본 연구는 대량생산이 어려운 2-FL을 재조합 대장균을 이용하여 상업적인 수준으로 생산할 수 있는 시스템을 개발하였다는 점에서 의의를 가진다.

주요어: 2'-푸코실락토오스, 재조합 대장균, GDP-L-푸코스, 락오페론, 푸코스전이효소, 아스파테이트 태그, 푸코스

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