A Thesis for the Degree of Master of Science

Enhanced production of 2'-fucosyllactose by introducing fucosyllactose exporter and optimizing biosynthetic pathway of GDP-L-fucose in engineered *Corynebacterium glutamicum*

대사공학적으로 설계된 코리네박테리움 글루타미쿰에서 푸코실락토오스 수송체 도입과 GDP-L-fucose 경로의 최적화를 통한 2'-푸코실락토오스 생산 증대

By

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Department of Agricultural Biotechnology
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Advisor : Professor Jin-Ho Seo

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science

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農 學 碩 士 學 位 論 文

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指導敎授 徐鎭浩

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李道行

李道行의 農學碩士學位論文을 認准함
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ABSTRACT

Human milk contains a considerable amount of oligosaccharides (about 15 g/L), unlike other mammalian milks. Human milk oligosaccharides (HMOs) consist of 200-300 kinds of oligosaccharides. In particular, 80% of HMOs are fucosylated oligosaccharides, and 2'-fucosyllactose (2'-FL) is the most abundant oligosaccharide among HMOs. 2'-FL is a trisaccharide composed of lactose and fucose. Fucose binds to the galactose of lactose through α1-2 linkage. 2'-FL has many physiological functions such as prebiotic effects, inhibition of intestinal adhesion of pathogens and enterotoxin secreted from pathogens and alleviation of the inflammatory responses.

Corynebacterium glutamicum, which is approved as GRAS (Generally Recognized As Safe), was used to produce 2'-FL in previous researches. In order to biosynthesize 2'-FL, GDP-$_L$-fucose and lactose are required. The wild-type *C. glutamicum* lacks the GDP-$_L$-fucose biosynthetic pathway, so the genes for biosynthesis of GDP-$_L$-fucose were introduced. In addition, the lactose permease gene from *Escherichia coli* was introduced since the wild type *C. glutamicum* cannot import lactose and the codon-optimized α-1,2 fucosyltransferase gene from *Helicobacter pylori* was introduced for fucosylation. Using the above-mentioned metabolically engineered strain, 11.5 g/L of 2'-FL was produced in fed-batch fermentation.

In this study, several attempts have been made to enhance 2'-FL production. First, the ABC transporter permease gene from *Bifidobacterium infantis* was introduced to export intracellular 2'-FL.

As a result, it showed a 38% improvement in 2'-FL secretion per gram of cells than the strain constructed previously. The concentration of 2'-FL in the medium was 830 mg/L, which was 52% higher than that of the strain constructed in previous research.

Next, the GDP-$_L$-fucose biosynthetic pathway was optimized to enhance the metabolic flux to GDP-$_L$-fucose. In the system for synthesizing GDP-$_L$-fucose using glucose only, the metabolic flux mostly directed to glycolysis for cell growth. In order to solve this problem, the phosphofructokinase A (*pfkA*) gene was disrupted by the double crossover method using a pK19mobsacB vector, and the ΔP strain that blocked the first major pathway of the glycolysis (Fructose 6-phosphate → Fructose 1,6-bisphosphate) was constructed. By using a mixture of glucose and fructose as a carbon source, a two track system was constructed in which glucose was used for synthesis of GDP-$_L$-fucose and fructose for cell growth. As a result, 2'-FL was produced at a concentration of 1.22 g/L in flask fermentation, and 12.6 g/L in fed-batch fermentation.

However, cell growth was delayed in the middle of fermentation, and the consumption rate of glucose and fructose continuously changed, making it difficult to maintain a constant concentration during fermentation. To solve this problem, glucose was supplied intermittently while supplying fructose to maintain a constant concentration. As a result, 2.05 g/L of 2'-FL was produced in flask fermentation. But, in fed-batch fermentation, cell growth was delayed for a long time when glucose was supplied. The cell adaptation process was added at the pre-culture step in which the cells to be inoculated into

the main culture were prepared. After establishing an environment similar to that of the main culture, the cells were cultured up to the mid-log phase and inoculated into the main culture. As a result, the delay of cell growth was solved, and 21.5 g/L of 2'-FL was produced in fed-batch fermentation. This result is 87% higher than the amount of 2'-FL produced in the previous research.

Finally, by changing the culture temperature from 30℃ to 37℃ in the latter stage of the fermentation, the fluidity of the cell membrane was increased to allow the intracellular 2'-FL to go out of the cells. As a result, 2.61 g/L of 2'-FL was produced in flask fermentation, which is 379% higher than that the amount of 2'-FL produced in the previous research.

In this study, a microbial bioprocess was developed to produce 2'-FL with high titer and productivity. It is believed that the bioprocess developed in this study would provide a technical framework for industrial production of 2'-FL.

Keywords: Metabolic engineering, 2'-fucosyllactose, GDP-L-fucose, 2'-fucosyllactose exporter, pK19mobsacB, phosphofructokinase A, fed-batch fermentation, *Corynebacterium glutamicum*

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CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1. Human milk

Human milk is secreted from the female mammary gland in the second half of pregnancy and after delivery. It is considered the best diet for newborn nutrition. In addition to providing all the nutrients needed for growth and development to the baby, human milk contains a variety of bioactive factors that promote healthy colonization of the neonatal intestine, prevent infections and support the immune system's maturation (Jantscher-Krenn and Bode 2012).

By the 1950s, breastfeeding had been considered to be practiced by the uneducated and those of lower classes. The practice was considered old-fashioned for those who could not afford infant formula. In contrast, infant formula was considered superior to breastfeeding (Nathoo and Ostry 2009). However, as the functionality of human milk has been reported since 1960s, breastfeeding has resumed in Canada and the US, especially among more educated, affluent women (Nathoo and Ostry 2009). Currently the World Health Organization (WHO) recommends exclusive breastfeeding for six months after birth.

Human milk is composed of 3~5% fat, 0.8~0.9% protein, 6.9~7.2% carbohydrate, and 0.2% mineral salts and other constituents (Jenness 1979). These roughly classified components are subdivided into many useful ingredients that offer health benefits as well as primary nutrient sources. These health benefits include prebiotic effects, prevention of infection by pathogens, modulation of immune responses, reduction of inflammatory processes, neurological development, and enhancement

of vaccine responses (Lanting, Huisman et al. 1994, Severin and Wenshui 2005, Boehm and Stahl 2007, Hahn-Zoric, Fulconis et al. 2008, Jantscher-Krenn and Bode 2012).

The composition of human milk and bovine milk is shown in Table 1. There is a significant difference. Oligosaccharides content of human milk is much higher than that of bovine milk. The high concentration of oligosaccharides is the most distinctive feature of human milk. The oligosaccharides contained in human milk are involved in many physiological functions.

Table 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

2. Human milk oligosaccharides (HMOs)

The oligosaccharides contained in human milk are called Human Milk Oligosaccharides (HMOs). They are the third most abundant ingredient in human milk, followed by lactose and fat. Based on numerous researches, it is generally agreed that this key ingredient represents 5-15 g/L of mature milk and approximately 22 g/L of colostrum (Newburg 1997, Coppa, Pierani et al. 1999, Kunz, Rudloff et al. 2000, Rivero-Urgell and Santamaria-Orleans 2001, Bode 2012).

To date, more than two hundred different HMOs have been identified and structurally characterized. Actually, approximately 200 different kinds of HMOs have been discovered in human milk (Ninonuevo, Park et al. 2006, Bode 2012, Jantscher-Krenn and Bode 2012). The composition of HMOs is very complex. The physiological functions of oligosaccharides are closely related to their structure. Because HMOs are not digested in the small intestine of infants, they keep their structure in there, which explains why there are so many different HMOs (Miller and McVeagh 2007). Basically, HMOs are composed of the five monosaccharides; D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc), and sialic acid [*N*-acetylneuraminic acid (NeuAc)] with lactose (Lac) core at the reducing end (Bode 2012, Jantscher-Krenn and Bode 2012). Biosynthesis of HMOs begins at the lactose core. Lactose can be elongated by an enzymatic attachment of GlcNAc residues linked in β1-3 or β1-6 linkage to the Gal residue followed by further addition of Gal in the β1-3 (lacto-*N*-biose) or β1-4 bond (*N*-acetyllactosamine) (Fig. 1A). Further modifications are derived from attachments of lactosamine, fucose,

and/or NeuAc residues at different positions of the core region and the core elongation chain (Kunz, Rudloff et al. 2000, McVeagh and Miller 2008, Bode 2012). Elongation with lacto-*N*-biose terminates the chain, while *N*-acetyllactosamine can be extended by the addition of one of the two disaccharides. The β1-6 linkage between two disaccharide units introduces a chain branch. Branched structures are referred as *iso*-HMO; linear structures without branches as *para*-HMO (Fig. 1B). Lactose or the elongated oligosaccharide chain can be fucosylated at α1-2, α1-3 or α1-4 linkage and/or sialylated at α2-3 or α2-6 linkage (Fig. 1C–E). Also, some HMOs have several isomeric forms, such as lacto-*N*-fucopentaose (LNFP, Fig. 1D) or sialyllacto-*N*-tetraose (LST, Fig. 1E).

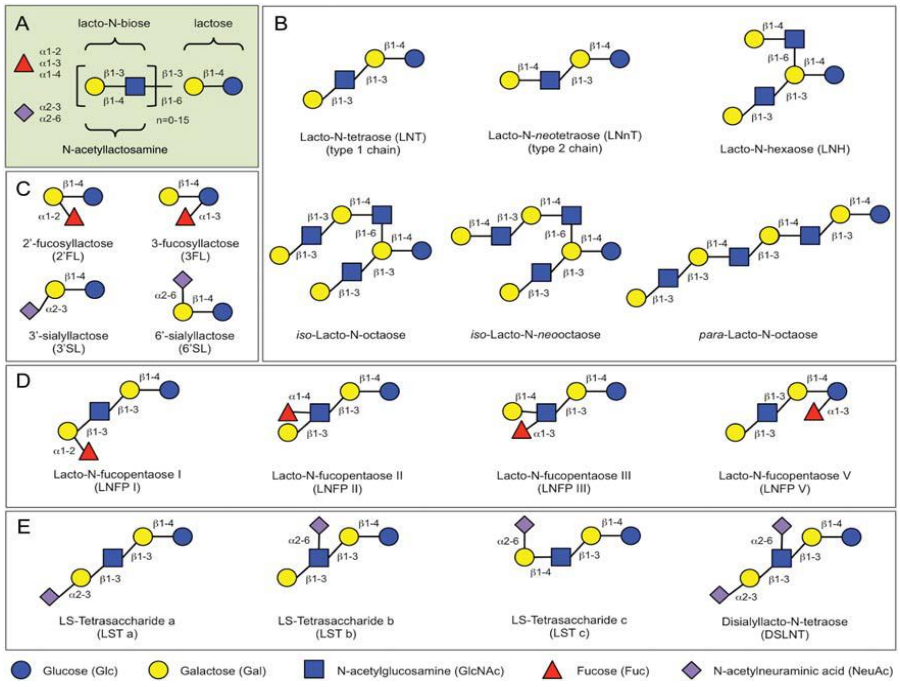


Figure 1. Typical HMO structures. (A) HMOs follow a basic structural blueprint. (Monosaccharide key is shown at the bottom of the figure.) (B) Lactose can be fucosylated or sialylated in different linkages to generate trisaccharides. (C) Lactose can be elongated by addition of either lacto-*N*-biose (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. (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).

3. 2’-Fucosyllactose (2’-FL)

3.1. Fucosyloligosaccharides

As mentioned above, about 200 kinds of HMOs have been found in human milk. Most HMOs are fucosylated, Fucosyloligosaccharides. 50~80% of the HMOs are fucosylated and 10~20% are sialylated (Kunz, Rudloff et al. 2000, Ninonuevo, Park et al. 2006, Bode 2012). They contain fucose and 3-8 sugars in size, in some cases up to 32 sugars. Fucosyloligosaccharides are attracting attention as their functions. They are used as a growth factor for *Bifidobacterium* or *Lactobacillus* and also they act as soluble analogues of cell surface receptors, so preventing infants from infection of enteric pathogens and binding of toxins (Morrow, Ruiz-Palacios et al. 2004, Newburg, Ruiz-Palacios et al. 2005).

Among HMOs, 2’-fucosyllactose (2’-FL) is the most abundant fucosyloligosaccharide in human milk and it has most of the physiological properties of HMOs (Table 2) (Chaturvedi, Warren et al. 2001, Castanys-Muñoz, Martin et al. 2013, Smilowitz, O’Sullivan et al. 2013).

2’-FL is a trisaccharide composed of lactose and fucose (Fig. 2). Fucose binds to the galactose of lactose through α1-2 linkage. So, 2’-FL is referred as L-fucopyranosyl-(1→2)-D-galactopyranosyl-(1→ 4)-D-glucose. In infants, 2’-FL is digested by fucosidase of *Bifidobacterium* and playing a role as soluble prebiotic fiber. Furthermore, it could modulate immune response by balancing Th1 and Th2 cells and protect infants from the risk of infection by pathogens. It is reported that 2’-FL inhibits adhesion of *Campylobacter jejuni*,

Pseudomonas aeruginosa, enterotoxin of *Escherichia coli*, *Calcivirus* etc. Additionally, it helps to reduce inflammatory processes by preventing adhesion of leukocyte and reducing extravasation at endothelial cells (Castanys-Muñoz, Martin et al. 2013). Therefore, it can be said that 2'-FL is a key component of HMOs. However, unfortunately, about 20% of women around the world cannot synthesize 2'-FL because of genetic defects (Castanys-Muñoz, Martin et al. 2013). For these reasons, movements to utilize 2'-FL as a functional food and therapeutic material are emerging and also it has generated the need for larger amounts of 2'-FL (Han, Kim et al. 2012).

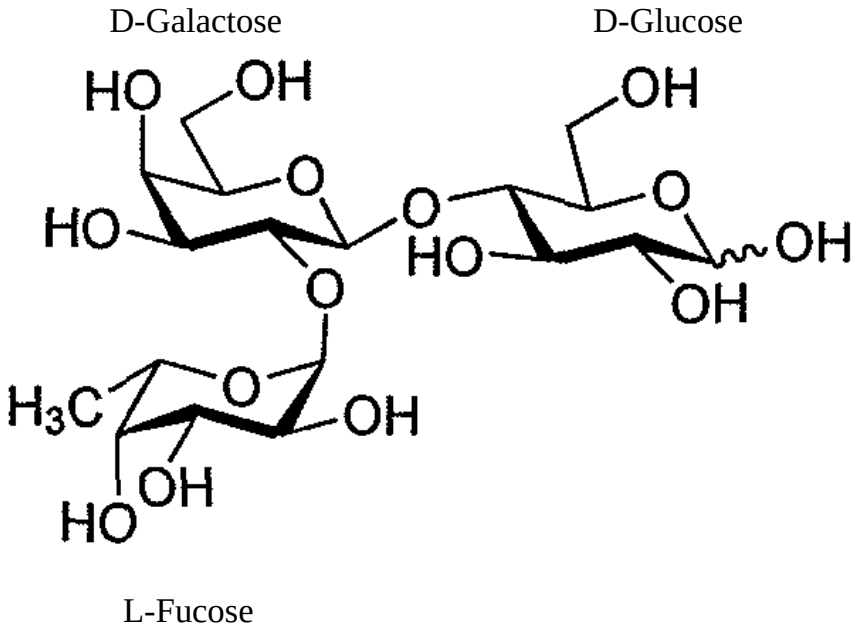


Figure 2. Structure of 2'-fucosyllactose (2'-FL)

Table 2. Contents of major carbohydrates in human milk (Smilowitz, O'Sullivan 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	$170 \times 10^3 \pm 7.30 \times 10^3$

3.2. Methods for 2'-FL production

There are several methods for 2'-FL production industrially, chemical synthesis, enzymatic synthesis and whole cell synthesis. First, the chemical synthesis has been carried out for a long time (Gokhale, Hindsgaul et al. 1990, Kameyama, Ishida et al. 1991, Kretzschmar and Stahl 1998), however, this method is uneconomical and consumes much time. In addition, it requires multiple protection and deprotection steps (Gokhale, Hindsgaul et al. 1990, Kameyama, Ishida et al. 1991, Kretzschmar and Stahl 1998). These problems are major drawbacks of this method for industrial applications.

The second method for 2'-FL production is enzymatic synthesis (Albermann, Piepersberg et al. 2001). This method may be more efficient due to the high stereoselectivity of an enzyme, α-1, 2-fucosyltransferase. Furthermore, by-products are hardly produced. However, the guanosine 5'-diphospho-β-$_L$-fucose (GDP-$_L$-fucose) used as a fucose donor is very expensive, and the cost of enzyme purification and cofactor involved in 2'-FL synthesis are very high. These make large-scale production difficult.

The last method is whole-cell synthesis, using microorganisms. It does not require enzyme isolation. Also, there is no need to prepare expensive substrates, GDP-$_L$-fucose and the cofactors involved in GDP-$_L$-fucose biosynthesis such as nicotinamide dinucleotide phosphate (NADPH) and guanosine triphosphate (GTP) (Lee, Pathanibul et al. 2012). Therefore, this method is considered to be an appropriate strategy for large-scale 2'-FL production. In this thesis, 2'-FL was produced by using this method, microbial fermentation.

3.3. Biosynthesis of GDP-$_L$-fucose

In order to produce 2'-FL, GDP-$_L$-fucose, the activated sugar nucleotide, is a key material used as a fucose donor (Fig. 3). There are two different metabolic pathways for biosynthesis of GDP-$_L$-fucose; the salvage pathway and *de novo* pathway. For the salvage pathway, L-fucose kinase (EC 2.7.1.52) phosphorylates L-fucose by consuming ATP. Then, L-fucose-1-phosphate guanylyltransferase (EC 2.7.7.30) synthesizes GDP-$_L$-fucose by combining L-fucose-2-phosphate with GTP (Becker and Lowe 2003).

For the *de novo* pathway, GDP-$_L$-fucose is synthesized as shown in Fig. 4. Fructose-6-phosphate, an intermediate of glycolysis, is converted to mannose-1-phosphate by mannose-6-phosphate isomerase (ManA, E.C. 5.3.1.8) and phosphomannomutase (ManB, E.C. 5.4.2.8), and then mannose-1-phosphate is combined with GTP by mannose-1-phosphate guanyltransferase (ManC, E.C. 2.7.7.22) to form GDP-$_D$-mannose. GDP-$_D$-mannose is converted to GDP-$_L$-fucose by two enzymes, GDP-$_D$-mannose-4,6-dehydratase (Gmd, E.C. 4.2.1.27) and GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase (WcaG, EC 1.1.1.271). First, a water molecule is removed GDP-$_D$-mannose by Gmd. Next, WcaG catalyzes the reduction reaction of the keto group at the C_4 position of GDP-4-keto-6-deoxymannose to synthesize GDP-$_L$-fucose. This reaction requires NADPH as a cofactor offering reducing power (Albermann, Distler et al. 2000, Becker and Lowe 2003, Jang, Lee et al. 2010).

Although GDP-$_L$-fucose is synthesized more simply through the salvage pathway, the cost of fucose used as a precursor for GDP-$_L$-

fucose is very high. So, it is uneconomically for large-scale production of 2'-FL by the salvage pathway. In contrast, the *de novo* pathway consists of multiple steps, but the starting material is economically appropriate. In the previous research, the *de novo* pathway was constructed in *Corynebacterium glutamicum* (Fig. 4) (Chin, Park et al. 2013).

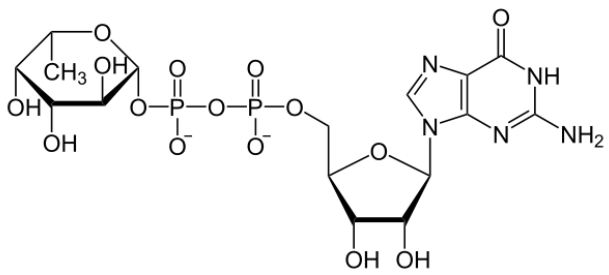


Figure 3. Structure of guanosine 5'- diphospho-β-L-fucose (GDP-L-fucose)

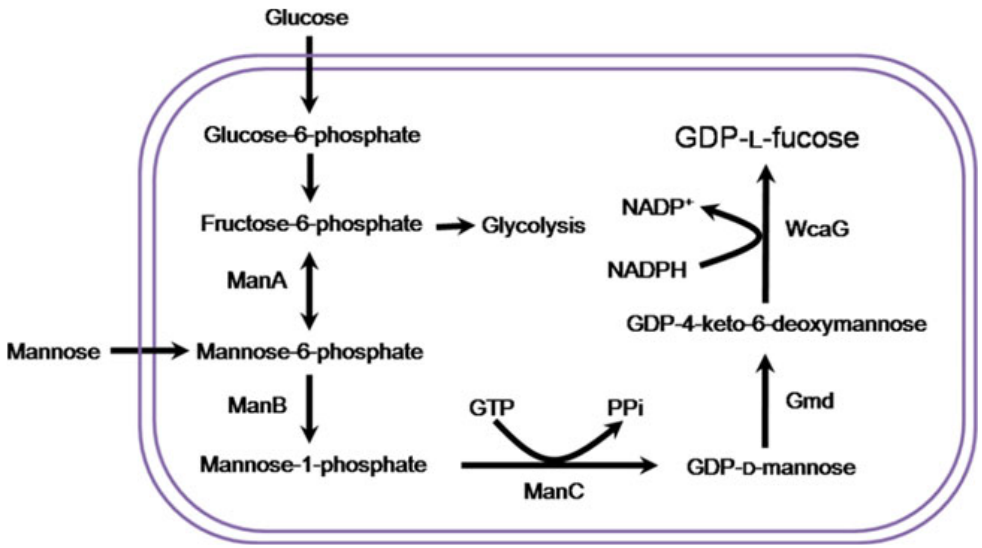


Figure 4. *De novo* biosynthetic pathway of GDP-$_{L}$-fucose.

ManA, mannose-6-phosphate isomerase; ManB, phosphomannomutase; ManC, GTP-mannose-1-phosphate guanylyltransferase; Gmd, GDP-$_{D}$-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase (Chin, Park et al. 2013).

3.4. Fucosyltransferse

2'-FL is synthesized by fucosylation of lactose by α-1, 2-fucosyltransferase. This enzyme catalyzes the transfer of fucose from GDP-$_L$-fucose to the galactose of lactose.

Fucosyltransferase is an enzyme group that transfers L-fucose of GDP-$_L$-fucose to various oligosaccharide acceptors (Breton, Oriol et al. 1998). Fucosyltransferase is a type of glycosyltransferases because α-fucosylated products are formed from a β-fucosylated sugar nucleotide, GDP-$_L$-fucose (Zhang, Lau et al. 2010). Based on the types of acceptors and the regional specificity of the fucosides formed by the reaction of fucosyltransferase, fucosyltransferases are classified as α-1, 2-, α-1, 3 and/or α-1, 4-, α-1, 6- and O-fucosyltrnasferases (Ma, Simala-Grant et al. 2006).

Among them, α-1, 2-fucosyltransferases are found in eukaryotes and prokaryotes. Fucosyltransferase is thought to be involved in tissue development, angiogenesis, fertilization, cell adhesion, inflammation and tumor metastasis in eukaryotes (Ma, Simala-Grant et al. 2006, Miyoshi 2008). In prokaryotes, fucosyltransferase are associated with the synthesis of lipopolysaccharides (LPS) and exopolysaccharides (EPS) which are involved in molecular mimicry, adhesion, colonization and modulation of host immune responses (Ma, Simala-Grant et al. 2006).

α-1, 2-fucosyltransferases is a key enzyme in 2'-FL production. As mentioned above, it is involved in the last transition reaction for 2'-FL synthesis that is transferring the fucose of GDP-$_L$-fucose to the galactose of lactose forming α-1,2 glycosidic linkage. To produce 2'-

FL, α-1, 2-fucosyltransferase from *Helicobacter pylori* has been mainly used (Albermann, Piepersberg et al. 2001, Drouillard, Driguez et al. 2006, Lee, Pathanibul et al. 2012, Baumgärtner, Seitz et al. 2013). In the previous research, α-1, 2-fucosyltransferase from *H. pylori* was introduced into *C. glutamicum* for 2'-FL production (Jo, Thesis. 2016).

4. *Corynebacterium glutamicum*

4.1. Characterization of *Corynebacterium glutamicum*

In the middle of 1950s, a bacterium was isolated which accumulate L-glutamic acid extracellularly. Originally, this bacterium was named *Micrococcus glutamicus* (KINOSHITA, UDAKA et al. 1957). In 2000, it was renamed as *Corynebacterium glutamicum* according to the taxonomy (Kumagai 2000). Since discovered several decades ago, *C. glutamicum* has played a key role in producing amino acids and nucleotides in an industrial scale. Amino acids such as L-valine, L-histidine, L-phenylalanine, L-tryptophan, L-glutamate and L-lysine (Ikeda 2003) and nucleotides such as 5'-inosinic acid (IMP), 5'-guanylic acid (GMP), 5'-xanthylic acid (XMP) and others have been produced in an industrial scale or have been attempted to produce.

C. glutamicum is an aerobic or facultative anaerobic, Gram-positive, non-spore forming bacterium. Commonly it has a rod-shape, somewhat irregular ("coryneform") morphology (Fig .5) (Eggeling and Bott 2005). At first, many random mutations and screening tests were required to make superior strains. These methods are time-consuming and do not give the reason for improvement. Recently, many genetic engineering tools have been developed for *C. glutamicum*. In the 1980s, host-vector systems for coryneform bacteria were developed to allow the development of strains in a more rational manner (Katsumata, Ozaki et al. 1984, SANTAMARiA, GIL et al. 1984, Kiyoshi, Kazuhiko et al. 1985, Yoshihama, Higashiro et al. 1985). In the 1990s, various tools for genetic engineering of the coryneform bacteria were developed (Haynes and Britz 1989, Schäfer, Kalinowski et al. 1990, Schwarzer and Pühler

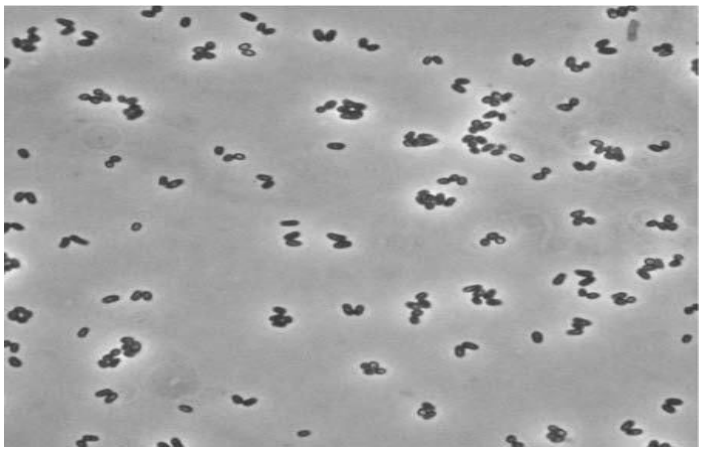
1991, Ikeda and Katsumata 1998). Furthermore, the complete genome of *C. glutamicum* ATCC 13032 has been determined by two independent research teams: the Japanese Kyowa Hakko Co. & Kitasato Univ. team and German Degussa Co. & Bielefeld Univ. team identified 3,309,401 and 3,282,708 base pairs.

4.2. *C. glutamicum* as a 2'-FL producer

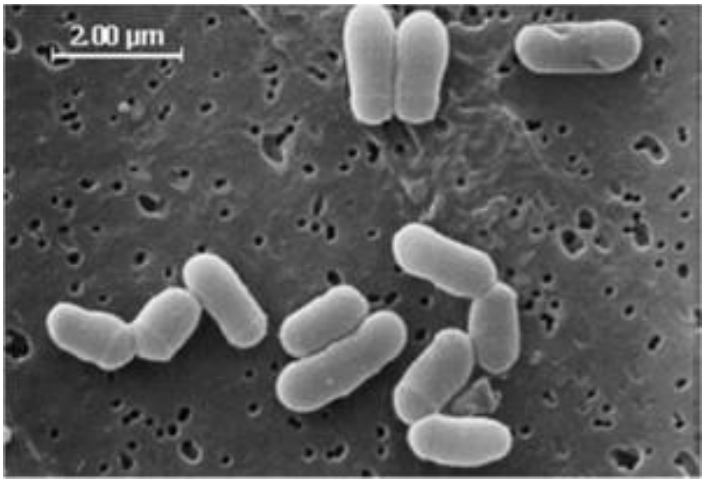
C. glutamicum has a high NADPH regeneration capacity. The ratio of carbon flux into the pentose phosphate pathway (PPP) is higher in *C. glutamicum* than other microorganisms when glucose is used as a sole carbon source (Marx, de Graaf et al. 1996, Eggeling and Bott 2005). A wild-type stain *C. glutamicum* ATCC 13032 has a large NADPH potential of over 80% during growth. That is the key feature for efficient amino acid production in mutants derived from this parent strain for decades (Eggeling and Bott 2005). Moreover, the carbon flux ratio to the PPP is significantly increased by the increased cell requirement of NADPH. *C. glutamicum* is also used for the fermentative production of nucleotides of interest as a flavor enhancing additive for foods (Komata 1976). Actually, mutant strains of *C. glutamicum* which secrete IMP, XMP and GMP were developed (Aharonowitz and Demain 1978). Above all, *C. glutamicum* is classified as a 'Generally Recognized As Safe' (GRAS) microorganism. Therefore, it is believed that *C. glutamicum* has sufficient potential to be an ideal host not only for production of amino acids or nucleotides, but also for the production of food additives or therapeutic materials

such as 2'-FL.

As mentioned above, in order to produce 2'-FL in microbial cells, GDP-$_{L}$-fucose and lactose are required. However, wild-type *C. glutamicum* does not have the GDP-$_{L}$-fucose biosynthetic pathway, so it cannot synthesize GDP-$_{L}$-fucose. Thus, in the previous researches, the strain capable of biosynthesizing GDP-$_{L}$-fucose was developed (Chin, Park et al. 2013). Also, wild-type *C. glutamicum* cannot metabolize lactose, so there is no lactose permease. However, in order to produce 2'-FL, it is necessary to import lactose into the cell, and therefore the lactose permease gene from *Escherichia coli* K-12 was introduced as the *lacYA* operon in which the β-galactosidase gene, *lacZ* was removed (Chin, Seo et al. 2016). Additionally, the codon-optimized α-1, 2-fucosyltransferase gene (CO*fucT2*) for *C. glutamicum* was introduced for fucosylation (Fig. 6) (Jo, Thesis. 2016).



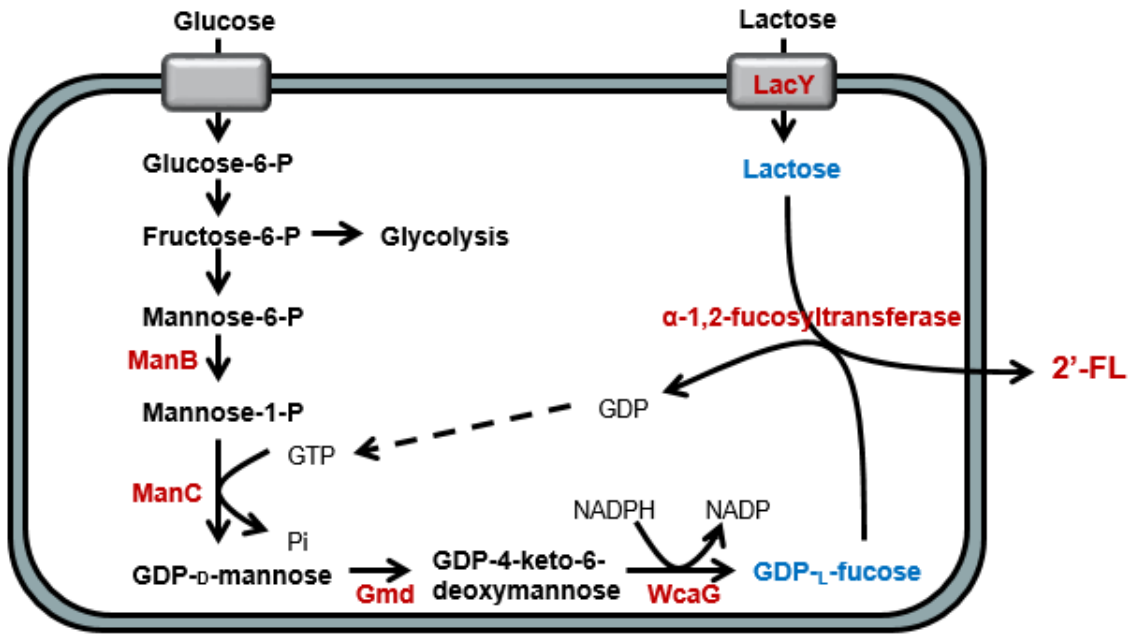
(A)



(B)

Figure 5. *Corynebacterium glutamicum*. (A) Phase-contrast micrograph of *C. glutamicum* cells grown on complex medium. Note frequent V-type arrangement of cell pairs, due to "snapping division." (B) Same cells placed on a nucleopore membrane and viewed by scanning electron microscopy (Eggeling and Bott 2005).

Figure 6. Biosynthesis pathway of 2'-FL from glucose and lactose in engineered *C. glutamicum* (Jo, Thesis. 2016).



ManB : Phosphomannomutase
ManC : GTP-mannose-1-phosphate guanylyltransferase
Gmd : GDP-D-mannose-4,6-dehydratase from *E. coli* K-12
WcaG : GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase from *E. coli* K-12
LacY : Lactose permease from *E. coli* K-12

5. Research objectives

This thesis was focused on the enhancement of 2'-FL production in engineered *C. glutamicum* by improving the strain in a metabolic engineering way. The specific objectives of this research were described as follows.

(1) To introduce the 2'-FL exporter from *Bifidobacterium infantis* to *C. glutamicum* for improved secretion of intracellular 2'-FL.

(2) To optimize biosynthetic pathway of GDP-$_{L}$-fucose by modulating glycolysis.

(3) To mass-produce 2'-FL in engineered *C. glutamicum* by carrying out fed-batch fermentation.

(4) To enhance 2'-FL production by exporting more intracellular 2'-FL.

II. MATERIALS AND METHODS

1. Reagents and Enzymes

All experiments were carried out using chemicals of reagent grade. Lactose, ethidium bromide, isoniazid, protocatechuic acid, biotin, cupric sulfate, sulfuric acid and antifoam 204 were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Glucose, ammonium sulfate, urea, potassium phosphate monobasic, potassium phosphate dibasic, magnesium sulfate heptahydrate, ferrous sulfate, sodium chloride, sodium hydroxide, ammonia water and hydrochloric acid were purchased from Duksan (Ansan, Korea). Kanamycin monosulfate, IPTG and MOPS were purchased from Duchefa (Haarlem, The Netherlands). Fructose, calcium chloride, zinc sulfate, manganese(II) sulfate and Nickel(II) chloride were purchased from Junsei Chemical (Tokyo, Japan). Brain heart infusion, bacto-tryptone, yeast extract and bacto-agar were purchased from Difco (Detroit, MI., USA).

Restriction enzymes and calf intestinal alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, MA, USA). T4 ligation mix and In-Fusion® HD cloning kit were purchased from Takara (Otsu, Japan).

2. Strains and Plasmids

2.1. Strains

E. coli Top10 (Invitrogen, Carlsbad, CA, USA) was used for construction of plasmid DNA. *C. glutamicum* ATCC 13032 (KACC,

Suwon, Korea) was used as host strain for 2'-FL production. To construct the phosphofructokinase A gene (*pfkA*) knock-out strain (ΔP), the 348 bp within *pfkA* was deleted by a double crossover method using a pK19mobsacB vector (Schäfer, Tauch et al. 1994).

The wild type and recombinant strains were incubated on Brain-heart infusion (BHI, Difco) containing appropriate antibiotics and stocked in a deep freezer at -80°C suspended in 15% glycerol.

2.2. Plasmids

Plasmids pVWEx2 and pEKEx2 were donated kindly by Prof. J. B. Park at Ewha Womans University. They were used as the backbone vectors for the expression of heterologous genes or overexpression of innate genes.

Plasmid pVBCLE harbors the *lacYA* operon from *E. coli* and the *manB, manC* genes from *C. glutamicum* and the *blon_2204-2203* cluster from *Bifidobacterium infantis* under the *tac* promoter. Plasmid pEGWTT(CO) was previously constructed. It harbors the *gmd-wcaG* gene from *E. coli* under *tac* promoter and the codon-optimized α-1,2-fucosyltransferase gene (CO*fucT2*) from *H. pylori* under *tac* promoter. CO*fucT2* is transcribed monocistronically by addition of the *tac* promoter (Jo, Thesis. 2016).

Plasmid pK19mobsacB was donated kindly by Prof. K. J. Jeong at Korea Advanced Institute of Science and Technology (KAIST). It was used as a vector for gene knock-out on the chromosome.

Plasmid pK19-Δ*pfkA* was constructed to disrupt *pfkA* on chromosome. It carries 311 bp from the start codon of *pfkA* and 382 bp from the

middle of *pfkA* to the stop codon.

Plasmids pVmBC and pEGW were previously constructed for overexpression of the genes for GDP-L-fucose biosynthesis (Chin, Park et al. 2013). All constructs were confirmed by restriction enzyme digestion and DNA sequencing.

Table 3. List of strains and plasmids used in this study

Strains/Plasmids	Relevant description	Reference
Strains		
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)
C. glutamicum	Wild-type strain, ATCC 13032	(ABE, TAKAYAMA et al. 1967)
BCGW TTL(CO)	*C. glutamicum* ATCC 13032 harboring pVBCL and pEGWTT(CO)	(Jo, Thesis. 2016)
BCGW TTLE(CO)	*C. glutamicum* ATCC 13032 harboring pVBCLE and pEGWTT(CO)	This study
ΔP	*C. glutamicum* ATCC 13032 Δ*pfkA*	This study
ΔP BCGW TTL(CO)	*C. glutamicum* ATCC 13032 Δ*pfkA* harboring pVBCL and pEGWTT(CO)	This study
ΔP BCGW TTLE(CO)	*C. glutamicum* ATCC 13032 Δ*pfkA* harboring pVBCLE and pEGWTT(CO)	This study
Plasmids		
pEKEx2	Km^R; *C. glutamicum/E. coli* shuttle vector for regulated gene expression (P_{tac}, *lacIq*, pBL1, *oriVC.g.*, *oriVE.c.*)	(Eikmanns, Kleinertz et al. 1991)
pVWEx2	Tc^R; *C. glutamicum/E. coli* shuttle vector for regulated gene expression (P_{tac}, *lacIq*, pHM1519, *oriVC.g.*, *oriVE.c.*)	(Wendisch and Jülich 1997)
pK19mobsacB	Mobilizable vector, Km^R	(Schäfer, Tauch et al. 1994)

pVmBC	pVWEx2 + *manB* + *manC*	(Chin, Park et al. 2013)
pEGW	pEKEx2 + *gmd-wcaG*	(Chin, Park et al. 2013)
pVBCL	pVmBC + *lacYA*	(Jo, Thesis. 2016)
pVBCLE	pVBCL + *blon_2204-2203*	This study
pEGWTT(CO)	pEGWT(CO) + *tac* promoter (before CO*fucT2*)	(Jo, Thesis. 2016)
pK19-Δ*pfkA*	pK19mobsacB + (311 bp from the start codon of *pfkA* – 382 bp from the middle of *pfkA* to stop codon)	This study

Table 4. List of primers used in this study

Name	Sequence
F_inf_*Spe*I_RBS_Exp	TCGTCTGATCAGTAG **ACTAGT** *AAGGAGATATACA* ATGACAAATGCAACGGCGC
R_inf_*SpeI*_Exp	CGGGGATCCGG **ACTAGT** TCACTGCTTGACAGAGCCG
F1_Exp_seq	CGGTGTGAACTCAACGCAG
F2_Exp_seq	ATTCTGCACTGATCGGCATTC
R_Exp_seq	TCATCCGCCAAAACAGCC
F1_*Bam*HI_*pfkA*_dis	CG**GGATCC** ATGGAAGACATGCGAATTGCTACT
R1_*pfkA*_dis.ovl	CCGCAACGACGATAATG ATTGGGATAAGGGCATCGATGC
F2_*pfkA*_dis.ovl	GATGCCCTTATCCCAAT CATTATCGTCGTTGCGGAAGG
R2_*Eco*RI_*pfkA*_dis	CG**GAATTC** CTATCCAAACATTGCCTGGGC

The italic sequences present the RBS (ribosome binding site) and spacer.
The bold sequences present the recognition sites of specific restriction enzymes.
The underlined sequences are overlapped regions to construct the defected *pfkA* fragment for pK19-Δ*pfkA*

Figure 7. Genetic maps of plasmids pVBCLE and pEGWTT(CO)

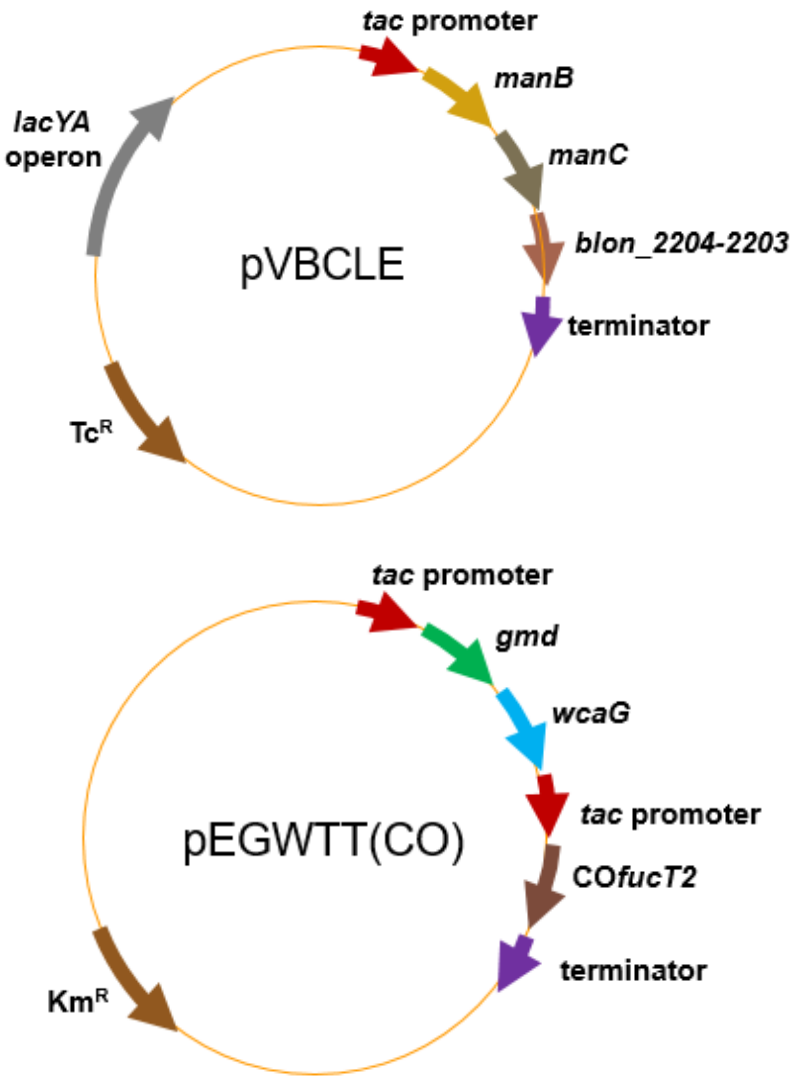
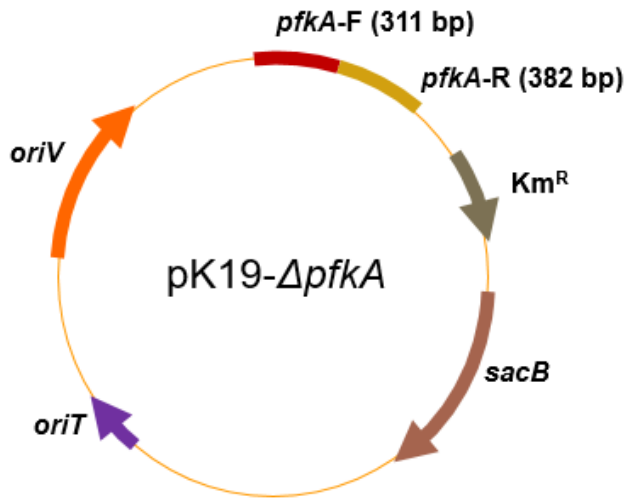


Figure 8. Genetic maps of plasmids pK19-Δ*pfkA*



3. DNA Manipulation and Transformation

3.1. Preparation of DNA

Mini-scale preparation of plasmid DNA was carried out by using DNA-spin™ Plasmid DNA Purification Kit from iNtRON (Sungnam, Korea). Preparation of *C. glutamicum* and *B. infantis* chromosomal DNAs for PCR template was carried out by using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany). As *C. glutamicum* and *B. infantis* are Gram-positive bacteria, buffer for enzymatic lysis composed of 20 mM Tris·HCl (pH 8.0), 2 mM EDTA, 1.2% Triton X-100, 20 mg/mL lysozyme was used. PCR amplified or enzyme treated DNA was purified by using respectively the QIAquick® Gel Extraction / PCR purification Kit from QIAGEN (Düsseldorf, Germany).

3.2. Polymerase Chain Reaction (PCR)

PCRs were performed with an Applied Biosystems Veriti 96 well Thermal Cycler (Lincoln, CA, USA). PCRs for cloning of genes were performed in 50 μL of PrimeStar™ dyemix solution from Takara (Otsu, Japan) containing 20 pM each of forward and reverse primers (Table 4), and 1 μL of the genomic DNA which is a template of cloning. After heating the reaction tubes for 5 min at 95℃, 30 cycles of PCR amplification were performed as follows: 10 sec at 98℃, 5 sec at 55℃ and 1 min per 1 kb DNA at 72℃, followed by 7 min at 72℃ during the last cycle.

3.3. Digestion and ligation of DNA

Restriction enzymes *Spe*I, *BamH*I and *Eco*RI and calf intestinal

alkaline phosphatase (CIP) were purchased from New England Biolabs (Beverly, USA). Plasmid pVBCL was digested with *Spe*I. Plasmid pK19mobsacB was digested with *Bam*HI and *Eco*RI. The Ligation Mix and In-Fusion® HD cloning kit obtained from Takara (Otsu, Japan) were used for ligation of PCR products and plasmids.

3.4. Transformation of *E. coli*

Transformation of *E. coli* was carried out as described by Sambrook (Sambrook and Russell, 1989). *E. coli* Top10 was cultured in 5 mL LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) for 12 hours. 0.5 mL of the culture was transferred to fresh 50 mL LB medium and cultured until OD_{600} reached 0.5. Cells harvested by centrifugation at 6,000 rpm for 5 min at 4°C were resuspended in 5 mL of cold 100 mM $CaCl_2$ solution containing 15% (v/v) glycerol. Resuspended cells were aliquoted to 100 μL, mixed with DNA, and kept on ice for 30 min. They were subjected to heat-shock at 42°C for 45 sec, and 1 mL of LB medium was added to the test tubes and incubated at 37°C for 1 hour to allow the bacteria to express the antibiotic resistance. Transformed cells were spread on LB agar plates with an appropriate concentration of antibiotics, kanamycin or tetracycline.

3.5. Electroporation of *C. glutamicum*

The modified protocol for preparation of electrocompetent *C. glutamicum* referred to Handbook of *Corynebacterium glutamicum* and van der Rest *et al*. (van der Rest, Lange et al. 1999, Eggeling and Bott 2005). Briefly, incubated at 30°C, overnight cultures of *C. glutamicum*

was inoculated in 100 mL BHIS (37 g/L BHI, 91 g/L sorbitol) medium in a 500 mL baffled flask containing isoniazid, glycine and tween80. Then, incubated at 30°C, 250 rpm cultured until OD_{600} reached 1.75. The culture dispensed into 50 mL falcon tubes and harvested by centrifugation at 3,000 rpm for 20 min. After removing the supernatant, cell pellet was resuspended with 20 mL TG buffer (1 mM Tris·HCl (pH 7.5), 104.4 g/L glycerol) and centrifuged again. After repeating this step, cell pellet was resuspended with 20 mL of 10% (v/v) glycerol as done before. Finally the cells were resuspended in 1 mL 10% (v/v) glycerol and dispensed 150 μL aliquots in cooled Eppendorf tubes and stored at -70°C. 10 μL of plasmid DNA was added into an electrocompetent cell and transferred the mixture into a pre-chilled electroporation cuvette (Bio-Rad, Hercules, CA, USA) with a gap width of 2 mm. The electroporation is performed at 2,500 V, 25 μF and 200 Ω in MicroPulser™ Electroporation apparatus (Bio-Rad, Hercules, CA, USA). After the electric shock, the transformant was transferred immediately into 1 mL BHIS medium pre-warmed at 46°C and incubated for 6 min at 46°C without shaking to carry out the heat-shock process. Then, the transformant was incubated for 1 hour at 30°C, 250rpm for regeneration of cells. An appropriate volume of the transformants were spread on a BHIS agar plate containing appropriate antibiotics and incubated the plates at 30°C for 2 days.

4. Gene disruption progress

4.1. Construction of gene disruption vector

To construct the *pfkA* knock-out vector pK19-Δ*pfkA*, 311 bp from the start codon of *pfkA* (called F region) and 382 bp from the middle of *pfkA* to the stop codon (called R region) were respectively amplified with primer pairs, F1_*Bam*HI_*pfkA*_dis and R1_*pfkA*_dis.ovl / F2_*pfkA*_dis.ovl and R2_*Eco*RI_*pfk*A_dis (Table 4). The PCR products were used as the templates for overlapping PCR and the second PCR was carried out by using primers, F1_*Bam*HI_*pfkA*_dis and R2_*Eco*RI_*pfk*A_dis. The obtained PCR products were digested with *Bam*HI and *Eco*RI, and plasmid pK19mobsacB was also digested with the same restriction enzymes. Then, the PCR products were cloned into pK19mobsacB to construct pK19-Δ*pfkA*.

4.2. Screening of gene disrupted strain

Disruption of *pfkA* was carried out by the double crossover method (Schäfer, Tauch et al. 1994). To disrupt *pfkA*, plasmid pK19-Δ*pfkA* was introduced into *C. glutamicum* by electroporation and the transformants spread on a BHIS agar plate with 25 μg/mL kanamycin were incubated for 2-3 days at 30℃. The cells formed colonies in Km medium had Km-resistance, and thus they were the plasmid-integrated clones. Then, the Km-resistant cells were cultured in LB medium overnight, and they were properly diluted and spread on a 10% (w/v) sucrose LBG (LB, 0.5% sodium acetate, 5 g/L glucose) agar plate to pop out the integrated plasmid. After incubation for about 2 days, the cells formed colonies in sucrose medium were found, and they did not have *sacB*. Thus, they

were the cells without the integrated plasmid. With the isolated clones, the desired *pfkA* disruption was checked by colony PCR with the primer pairs, F1_*Bam*HI_*pfkA*_dis and R2_*Eco*RI_*pfk*A_dis.

5. Media and Culture conditions

5.1. Media

Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing appropriate antibiotics (50 μg/mL kanamycin, 15 μg/mL tetracycline) was used for cultivation of *E. coli* strains. Brain heart infusion (BHI) (Difco, USA) containing with appropriate antibiotics (25 μg/mL kanamycin, 5 μg/mL tetracycline) was used for incubation of *C. glutamicum.*

The minimal medium used for *C. glutamicum* was CGXII, consisting of (per liter) 20 g of $(NH4)_2SO_4$, 5 g of urea, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 42 g of 3-morpholinopropanesulfo nic acid, 10 mg of $CaCl_2$, 10 mg of $FeSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot H_2O$, 1 mg of $ZnSO_4 \cdot 7H_2O$, 0.2 mg of $CuSO_4$, 0.02 mg of $NiCl_2 \cdot 6H_2O$, 0.2 mg of biotin (pH 7.0), and 0.03 mg of protocatechuic acid (Eggeling and Bott 2005).

5.2. Culture conditions

For the inoculation of recombinant *C. glutamicum*, a frozen stock was transferred to a test-tube containing 5 mL of BHI medium and incubated overnight at 30°C and 250 rpm in a shaking incubator (Vision, Korea). For recombinant *C. glutamicum* containing a single vector (pEKEx2 derived plasmid) 25 μg/mL kanamycin was added and for the dual vector system (pEKEx2 and pVWEx2 derived plasmids) 25 μg/mL kanamycin and 5 μg/mL tetracycline were added.

For the flask fermentation, 1 mL of cell culture broth grown overnight was inoculated in a 500 mL baffled flask (NALGENE, USA) with 100

mL CGXII (4% glucose, or in case of Δ*pfkA* strain, 1.2% glucose and 2.8% fructose mixture or 4% fructose) media and grown at 30°C and 250 rpm. The appropriate antibiotics were supplemented. As an optical density reached OD_{600} of 0.8, isopropyl-β-$_D$-l-thiogalactopyranoside (IPTG) was added to a final concentration 1.0 mM for induction of gene expression, and also lactose was added to a final concentration 10 g/L for 2'-FL production.

Fed-batch fermentation was performed in a bioreactor of 2.5 L (Kobiotech, Korea) with 1 L initial working volume of CGXII medium containing 4% glucose and antibiotics of the same concentration as flask culture. In case of Δ*pfkA* strain, CGXII medium containing 1.2% glucose and 2.8% fructose mixture or 4% fructose was used. The 100 mL pre-culture was prepared with in a 500 mL baffled flask with 100 mL BHI. In case of Δ*pfkA* strain, the pre-culture was prepared with the same method with flask fermentation. And then, the culture solution was transferred to the bioreactor, giving an initial OD_{600} of approximately 1 or 2 (Δ*pfkA* strain). Aeration rate and agitation speed were in between 2 ~ 2.5 vvm of air supply and 1,000 rpm, respectively. The pH was automatically controlled at 7.0 by addition of 28% ammonia water and 2N HCl. To keep the cell growth and a basal level of carbon source after depletion of 4% sugar initially added, feeding solution was fed at a continuous feeding rate of 5.7 g/L/h on average. The feeding solution was composed of 800 g/L glucose, or in case of Δ*pfkA* strain, 240 g/L glucose and 560 g/L fructose or 800 g/L fructose. When the initial carbon source was consumed completely, 1.0 mM IPTG was added for induction of the gene expression regulated by the

tac promoter. Also, 20 g/L lactose was added as an acceptor for α-1,2-fucosyltransferase. Especially, in case of the optimized fed-batch fermentation of Δ*pfkA* strain, when the pre-culture broth was inoculated into the bioreactor containing CGXII medium with 4% fructose, 1 mM IPTG, 20 g/L lactose and 20 g/L glucose were simultaneously added. To keep the cell growth and to produce 2'-FL, 800 g/L fructose feeding solution was fed at a continuous feeding rate of 2.8 g/L/h on average and glucose was added intermittently to maintain concentrations of 10-20 g/L.

6. Fermentation analysis

6.1. Dry cell weight

Cell growth was monitored by measuring the optical density of culture broth. Absorbance at 600 nm was measured using a spectrophotometer (OPTIZEN POP, MECASYS, Korea) after culture broth samples were properly diluted to keep optical density between 0.1 and 0.5. Optical density was converted to dry cell weight by using the following conversion equation:

$$\text{Dry cell mass (g/L)} = 0.30 \times OD_{600}$$

6.2. Quantification of metabolites concentrations

Concentrations of glucose, fructose, lactose, lactate and 2'-FL were measured by a high performance liquid chromatography (1200 series, Agilent, Santa Clara, CA, USA) with a Rezex ROA-organic acid H^+ Column (Phenomenex, USA) heated at 60°C. A mobile phase of 5 mM H_2SO_4 was used at a flow rate of 0.6 mL/min. Detection was made with a reflective index detector.

6.3. Measurement of the amount of intracellular 2'-FL

In case of flask fermentation, 1% triton X-100 was added to the culture broth after fermentation to measure the amount of intracellular 2'-FL. Then, the cells were cultured overnight and the supernatant of the culture broth was taken to measure the total amount of 2'-FL. The amount of intracellular 2'-FL was quantified by the difference between

the total amount of 2’-FL and the amount of extracellular 2’-FL.

For fed-batch fermentation, 0.5 mL of the culture broth was taken to measure the amount of intracellular 2’-FL. After centrifugation at 13,200 rpm for 10 min, the supernatant was removed and the cell pellet was resuspended with 0.5 mL of DDW. Then, in order to break the cells, it was boiled in boiling water for 5 min. To quantify the amount of intracellular 2’-FL, the supernatant after centrifugation and filtration was taken.

III. RESULTS AND DISCUSSIONS

1. Introduction of 2'-FL exporter from *Bifidobacterium infantis*

1.1. Construction of the strain expressing 2'-FL exporter

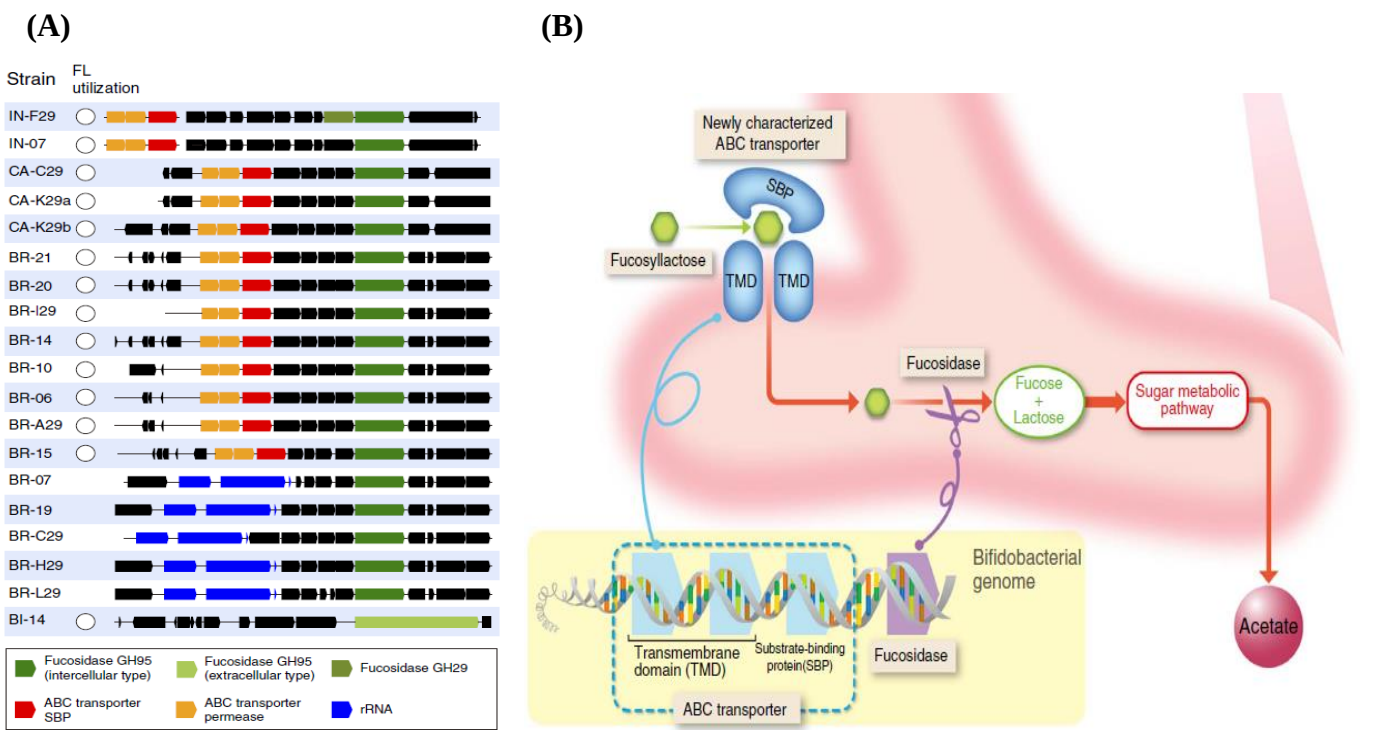
To produce 2'-FL in engineered *C. glutamicum*, the strain capable of biosynthesizing GDP-$_L$-fucose was constructed (Chin, Park et al. 2013). Next, plasmids pVBCL and pEGWTT(CO) were constructed for expression of the lactose permease gene from *E. coli* and α-1,2-fucosyltransferase gene from *H. pylori*. The strain BCGW TTL(CO) harboring plasmids pVBCL and pEGWTT(CO) was constructed for 2'-FL production from glucose and lactose. This strain produced 0.547 g/L of 2'-FL in flask fermentation (Jo, Thesis. 2016). The 2'-FL detected in the supernatant of culture broth was secreted from cells into the medium, and the amount of intracellular 2'-FL was thought to be considerably high. Therefore, to enhance the production of 2'-FL by secreting the intracellular 2'-FL, the 2'-FL exporter was explored.

Bifidobacteria can utilize fucosyllactoses (FLs) in a selfish manner. In other words, Bifidobacteria can utilize FLs by importing them into a cell. This physiological characteristic of Bifidobacteria is because they have a common FL-utilization gene cluster. The gene cluster is composed of the two ABC transporter permease genes, ABC transporter substrate-binding protein (SBP) gene and fucosidase gene (Fig. 9) (Matsuki, Yahagi et al. 2016). The ABC transporter permeases have transmembrane domain, so ABC transporter permeases are thought to be a 2'-FL transporter. Since 2'-FL was continuously

produced in the cell, it seems that 2'-FL might be exported through the ABC transporter permeases more smoothly by facilitated diffusion.

To introduce the ABC transporter permeases to *C. glutamicum*, the *blon_2204-2203* cluster from *B. infantis* able to utilize FLs in a selfish manner was chosen. The *blon_2204-2203* cluster from *B. infantis* genomic DNA was amplified with primer pairs, F_inf_*Spe*I_RBS_Exp and R_inf_*Spe*I_Exp (Table 4). The amplified *blon_2204-2203* cluster was digested with *Spe*I and ligated into plasmid pVBCL. The constructed plasmid pVBCLE was identified by the restriction enzyme (*Spe*I) digestion and DNA sequencing (SolGent, Daejeon, Korea). To construct the 2'-FL production strain, *C. glutamicum* ATCC 13032 was transformed with plasmids pVBCLE and pEGWTT(CO) by electroporation. The transformants were isolated on the BHIS agar plate containing kanamycin and tetracycline and identified by colony PCR.

Figure 9. FL utilization in bifidobacteria. (A) Identification of bifidobacterial genes responsible for HMO utilization. (B) Molecular mechanisms of FL utilization by bifidobacteria (Matsuki, Yahagi et al. 2016).



1.2. Flask fermentation of the strain expressing 2'-FL exporter

As mentioned above, for production of 2'-FL in engineered *C. glutamicum*, the strain BCGW TTLE(CO) harboring plasmids pVBCLE and pEGWTT(CO) was constructed. In order to compare the fermentation ability of BCGW TTL(CO): harboring plasmids pVBCL and pEGWTT(CO) and BCGW TTLE(CO), flask fermentation was performed in CGXII medium containing 40 g/L glucose. As an optical density reached OD_{600} of 0.8, IPTG was added to a final concentration 1.0 mM for induction of gene expression, and also lactose was added to a final concentration 1, 2, 5 and 10 g/L respectively for 2'-FL production. During 72 hours of fermentation, BCGW TTL(CO) produced 2'-FL of 0.46 g/L, 0.58 g/L, 0.6 g/L and 0.6 g/L under the respective lactose concentration (Fig. 10, Table 5). In comparison, during the same time, BCGW TTLE(CO) produced 2'-FL of 0.51 g/L, 0.56 g/L, 0.66 g/L and 0.83 g/L under the respective lactose concentration (Fig. 11, Table 5). Overall, BCGW TTLE(CO) produced more 2'-FL. Especially, under the 10 g/L lactose condition, 2'-FL titer was higher by 38% than that of BCGW TTL(CO). As the amount of extracellular 2'-FL was increased, it seems that the introduced ABC transporter permeases from *B. infantis* work as a 2'-FL exporter. Indeed, if the ABC transporter permeases worked as s 2'-FL exporter, the amount of intracellular 2'-FL in BCGW TTLE(CO) would decrease.

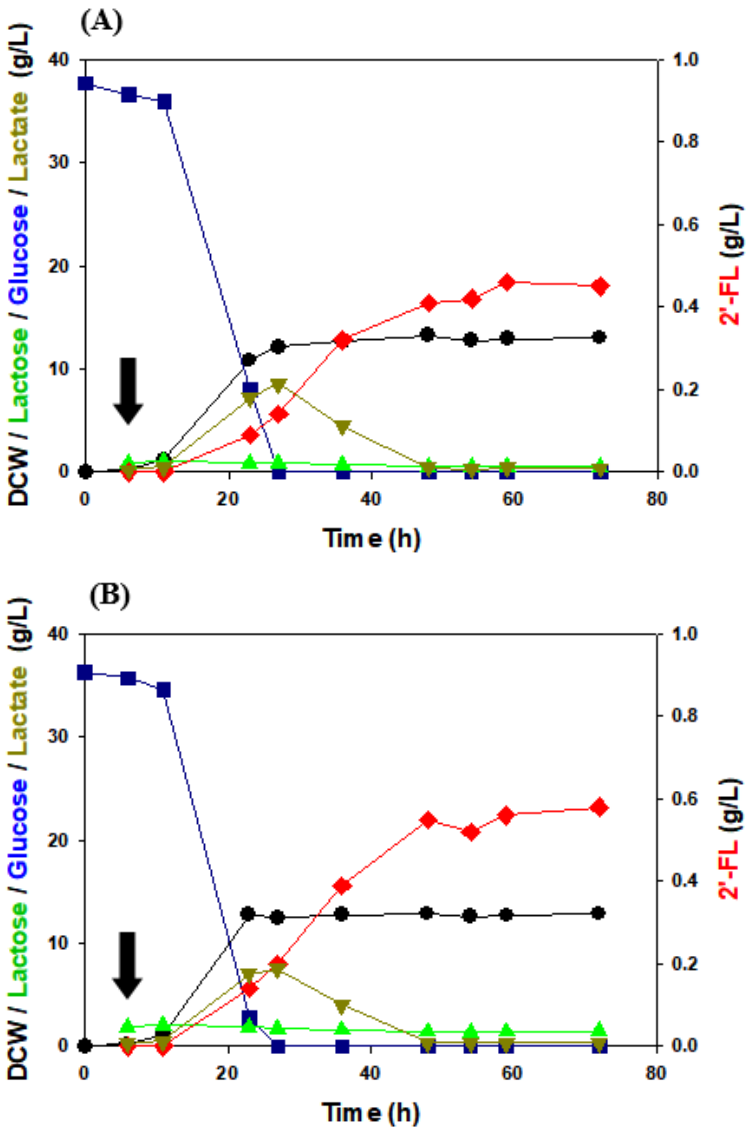
In order to measure the amount of intracellular 2'-FL, cells needed to be broken. To break the cells, 1% triton X-100 was added to the culture broth after fermentation. The cells were cultured overnight and the total

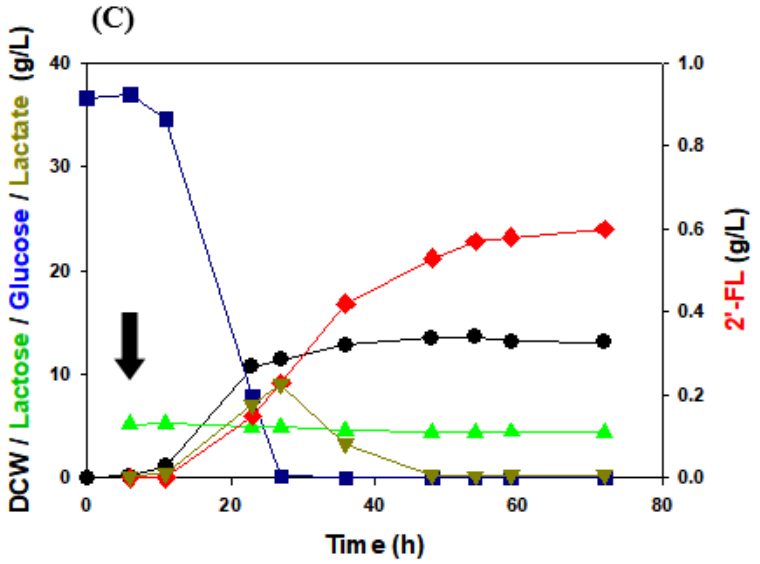
2’-FL was obtained from the supernatant. The amount of intracellular 2’-FL was estimated by the difference between total 2’-FL and extracellular 2’-FL. The estimated amount of intracellular 2’-FL was the same as that measured by breaking cells by boiling as described above (data not shown).

The amount of intracellular 2’-FL produced in BCGW TTL(CO) and BCGW TTLE(CO) was measured. As a result, the amount of intracellular 2’-FL decreased in BCGW TTLE(CO) compared to BCGW TTL(CO) for all fermentations performed under the various lactose conditions. Conversely, the amount of extracellular 2’-FL increased in BCGW TTLE(CO) compared to BCGW TTL(CO) in all fermentations (Fig. 12). The total amount of 2'-FL was similar in both strains or higher in BCGW TTL(CO) than in BCGW TTLE(CO). The experiments results suggested that expression of 2’-FL exporter from *B. infantis* did not affect 2’-FL production capacity of cells, but enhanced the secretion of the intracellular 2’-FL and enhanced the amount of extracellular 2’-FL. The expression of 2’-FL exporter can be helpful to collect the produced 2’-FL.

Figure 10. Flask fermentation of BCGW TTL(CO). (A) 1 g/L lactose (B) 2 g/L 1actose (C) 5 g/L lactose (D) 10 g/L lactose. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL




(C)
DCW / Lactose / Glucose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80

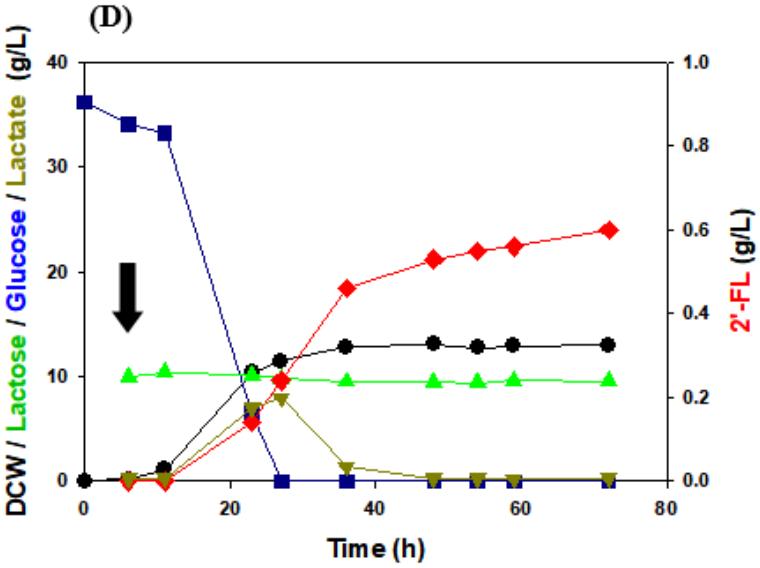
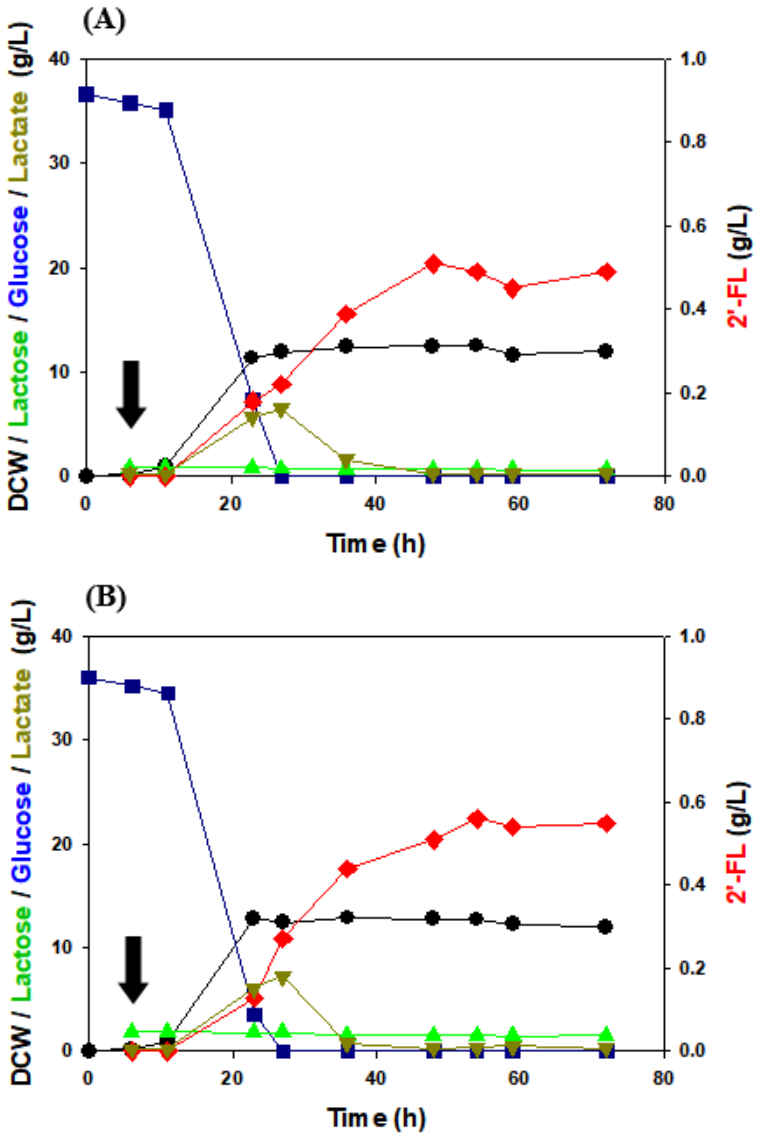
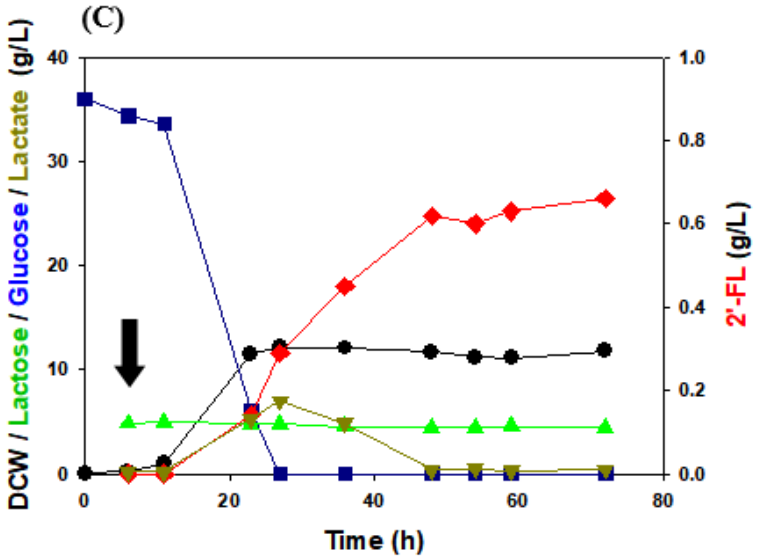

(D)
DCW / Lactose / Glucose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80

Figure 11. Flask fermentation of BCGW TTLE(CO). (A) 1 g/L lactose (B) 2 g/L 1actose (C) 5 g/L lactose (D) 10 g/L lactose. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL




(C)
DCW / Lactose / Glucose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80

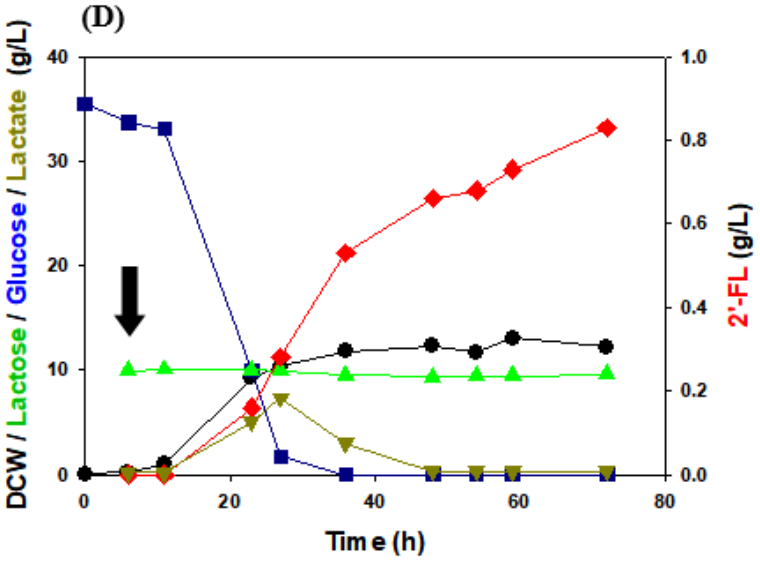
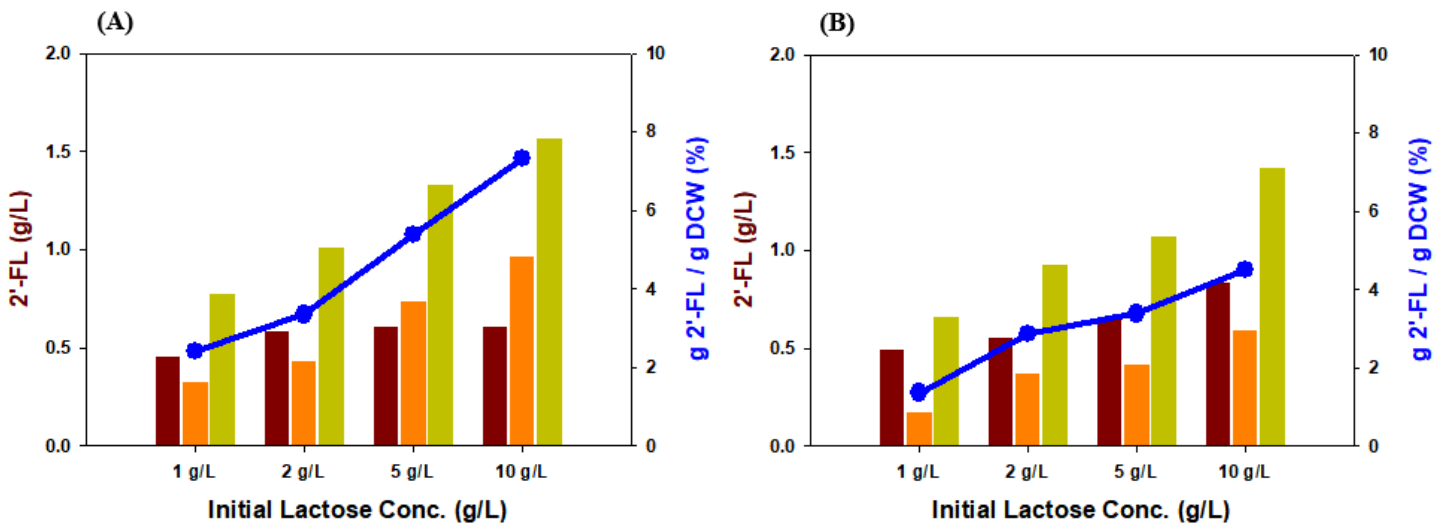

(D)
DCW / Lactose / Glucose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80

Table 5. Summary of flask fermentation under 10 g/L lactose of BCGW TTL(CO) and BCGW TTLE(CO)

Strains	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Yield (mole 2'-FL/mole lactose)	*Productivity (mg/L/h)
BCGW TTL(CO)	13.1	0.60	0.76	8.33
BCGW TTLE(CO)	13.1	0.83	0.98	11.5

*2'-FL yield and productivity were calculated based on total fermentation time.

Figure 12. Intracellular 2'-FL of (A) BCGW TTL(CO) and (B) BCGW TTLE(CO)
Colors: Brown (first bar), Extracellular 2'-FL; Orange (second bar), Intracellular 2'-FL;
Yellow green (third bar), Total 2'-FL, Blue dot, g intracellular 2'-FL / g DCW (%)

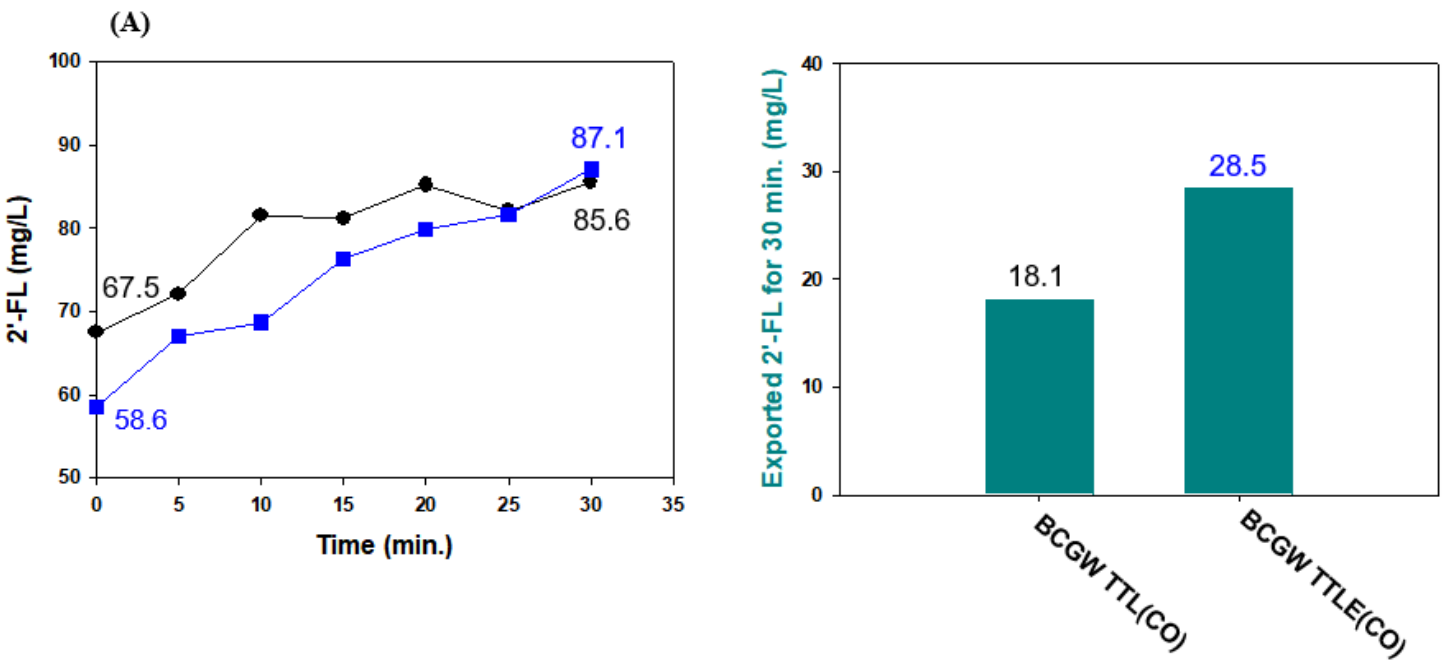


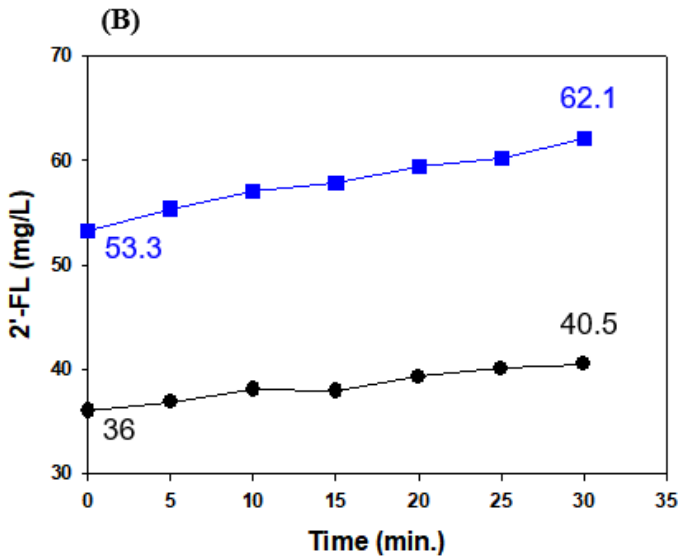
1.3. Measurement of 2'-FL secretion ability

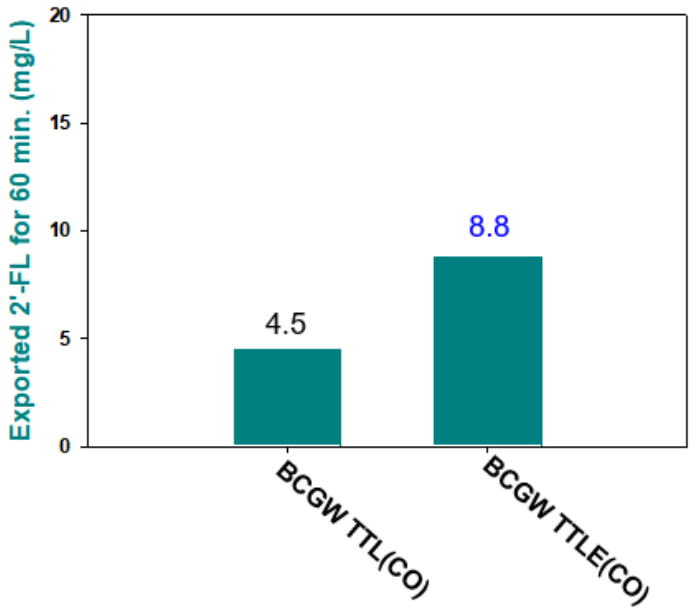
In order to demonstrate the ability of the ABC transporter permeases from *B. infantis* as 2'-FL exporter, the following experiment was performed additionally. During flask fermentation, at 20 hours after IPTG induction, a culture broth sample corresponding to 0.3 g dry cell weight (DCW) was taken. DCW was estimated as described in MATERIALS AND METHODS. Cells were harvested after centrifugation and resuspended in 10 mL of fresh medium. 2'-FL in the culture medium was measured every 5 min while incubating at 30℃ and 250 rpm. As a result, a larger amount of 2'-FL was detected in BCGW TTLE(CO) than in BCGW TTL(CO) during the same time (Fig. 13). The 2'-FL detected in the culture medium can be regarded as the intracellular 2'-FL originally present in the cells. Due to the difference in intracellular and extracellular concentrations of 2'-FL, the intracellular 2'-FL seems to be secreted out of the cells by facilitated diffusion. The experiment result suggested that the introduction of 2'-FL exporter from *B. infantis* increased the chance that the intracellular 2'-FL can go out of the cells by providing passages for 2'-FL. The same experiment was performed at 40 hours after IPTG induction, and a larger amount of 2'-FL was also confirmed in BCGW TTLE(CO). Likewise, a larger amount of 2'-FL was detected in BCGW TTLE(CO) than in BCGW TTL(CO).

Figure 13. Measurement of 2'-FL secretion ability of BCGW TTL(CO) and BCGW TTLE(CO). (A) At 20 hours after IPTG induction (B) At 40 hours after IPTG induction. In case of (B), the sample corresponding to 0.15 g DCW was taken.

Symbols: ●, BCGW TTL(CO); ■, BCGW TTLE(CO)




(B)
2'-FL (mg/L)
Time (min.)
62.1
53.3
40.5
36


Exported 2'-FL for 60 min. (mg/L)
4.5
8.8
BCGW TTL(CO)
BCGW TTLE(CO)

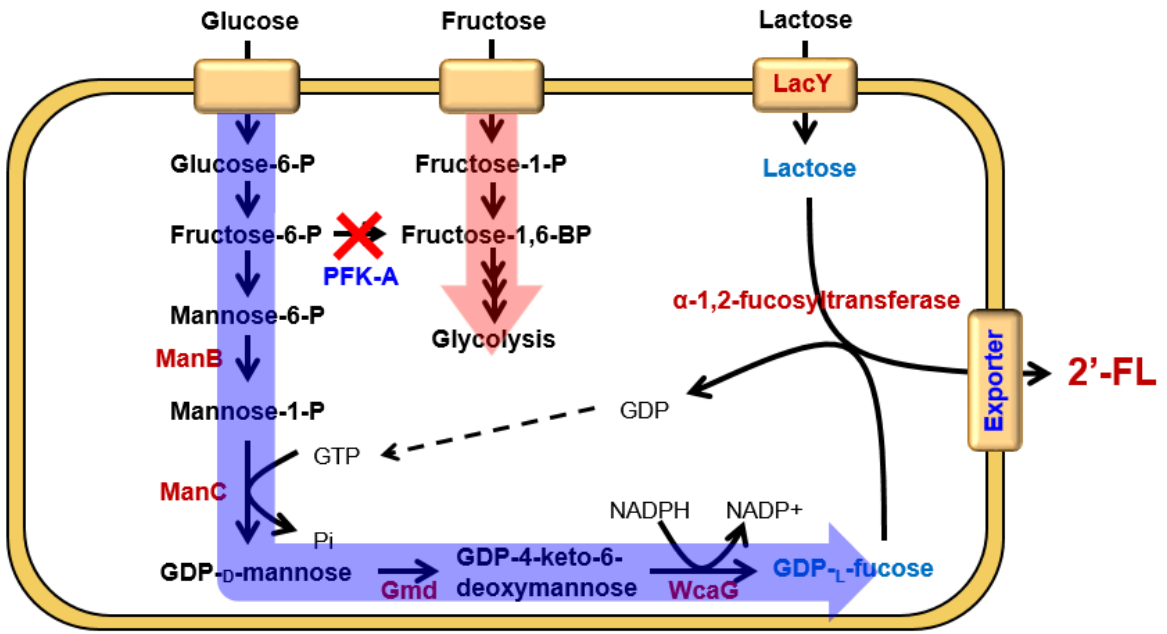
2. Disruption of phosphofructokinase A gene (*pfkA*)

2.1. New strategy for 2'-FL production

The current 2'-FL production system in engineered *C. glutamicum* produces 2'-FL by synthesizing GDP-$_{L}$-fucose from glucose (Fig. 6). In this case, the overall metabolic flux is thought to flow into glycolysis, which is related to cell growth rather than to synthesis of GDP-$_{L}$-fucose (Becker, Klopprogge et al. 2005). For the synthesis of GDP-$_{L}$-fucose, GDP-$_{D}$-mannose should be synthesized. Fructose 6-phosphate (F6P) which is a key intermediate of the first main step of glycolysis is converted to fructose 1,6-bisphosphate (F16BP) by the enzyme phosphofructokinase A (PFK-A). If the *pfkA* is disrupted to block the entry of F6P into glycolysis, the chance of going from F6P to GDP-$_{D}$-mannose would increase. However, in this case, since the pathway of glycolysis, which is important for cell growth, is blocked, there would be a problem with cell growth. In order to solve this problem, a strategy of using a mixture of glucose and fructose as a carbon source was established.

First, it is known that *C. glutamicum* can simultaneously consume glucose and fructose (Dominguez, Cocaign-Bousquet et al. 1997, Wendisch, de Graaf et al. 2000). There is no effect of carbon catabolite repression (CCR). Based on the above facts, a two-track system that utilizes fructose for cell growth through glycolysis and glucose for synthesis of GDP-$_{L}$-fucose, was developed (Figure 14)

Figure 14. New strategy for 2'-FL production.



ManB : Phosphomannomutase
ManC : GTP-mannose-1-phosphate guanylyltransferase
Gmd : GDP-D-mannose-4,6-dehydratase from *E. coli* K-12

WcaG : GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase from *E. coli* K-12
LacY : Lactose permease from *E. coli* K-12
PFK-A : Phosphofructokinase A

2.2. Construction of *pfkA* knock-out strain

To disrupt the *pfkA* (Ncgl1202), 311 bp from the start codon of *pfkA* (called F region) and 382 bp from the middle of *pfkA* to the stop codon (called R region) were respectively amplified with primers (Table 4). The F-R fragment was constructed by overlapping PCR using F1_*Bam*HI_*pfkA*_dis and R2_*Eco*RI_*pfk*A_dis (Table 4) and *pfkA* knock-out vector pk19-Δ*pfkA* was constructed. When making the F-R fragment to replace *pfkA* on chromosome, the length of the F-R fragment was set to a multiple of 3 so that the translation ends at the original stop codon of *pfkA*. The *pfkA* knock-out strain ΔP was constructed by a double crossover method (Schäfer, Tauch et al. 1994). Disruption of *pfkA* was confirmed by colony PCR using the primer pairs F1_*Bam*HI_*pfkA*_dis and R2_*Eco*RI_*pfk*A_dis (Table 4). The complete *pfkA* represents a length of 1041 bp, but the disrupted *pfkA* represents a length of 693 bp. The result of *pfkA* disruption is shown in Figure 15.

The *pfkA* gene and disrupted *pfkA* sequences are as follows.

Ncgl1202 *pfkA* (1041 bp) F region, 311 bp / R region 382 bp

The F and R region are marked with shading.

ATGGAAGACATGCGAATTGCTACTCTCACGTCAGGCGGCG
ACTGCCCCGGACTAAACGCCGTCATCCGAGGAATCGTCCGC
ACAGCCAGCAATGAATTTGGCTCCACCGTCGTTGGTTATCAA
GACGGTTGGGAAGGACTGTTAGGCGATCGTCGCGTACAGCT
GTATGACGATGAAGATATTGACCGAATCCTCCTTCGAGGCGG
CACCATTTTGGGCACTGGTCGCCTCCATCCGGACAAGTTTAA

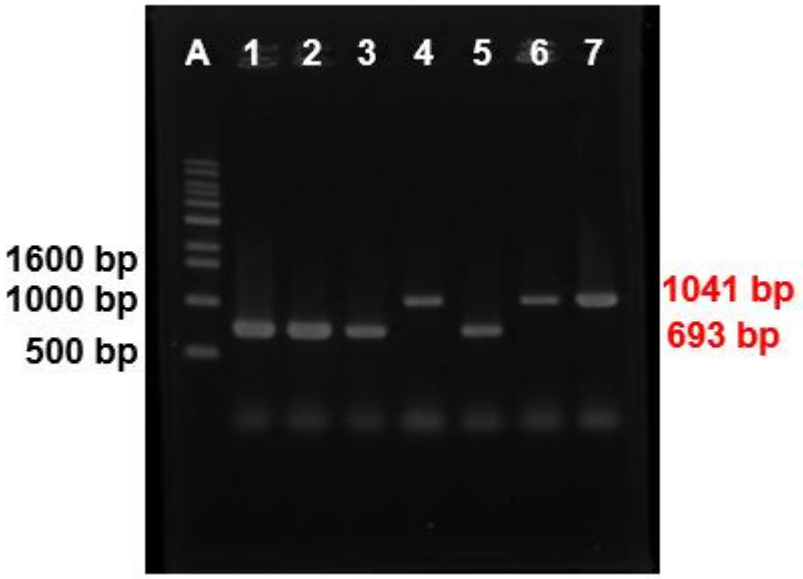
GGCCGGAATTGATCAGATTAAGGCCAACTTAGAAGACGCCG
GCATCGATGCCCTTATCCCAATCGGTGGCGAAGGAACCCTGA
AGGGTGCCAAGTGGCTGTCTGATAACGGTATCCCTGTTGTCG
GTGTCCCAAAGACCATTGACAATGACGTGAATGGCACTGAC
TTCACCTTCGGTTTCGATACTGCTGTGGCAGTGGCTACCGAC
GCTGTTGACCGCCTGCACACCACCGCTGAATCTCACAACCG
TGTGATGATCGTGGAGGTCATGGGCCGCCACGTGGGTTGGA
TTGCTCTGCACGCAGGTATGGCCGGCGGTGCTCACTACACC
GTTATTCCAGAAGTACCTTTCGATATTGCAGAGATCTGCAAG
GCGATGGAACGTCGCTTCCAGATGGGCGAGAAGTACGGCAT
TATCGTCGTTGCGGAAGGTGCGTTGCCACGCGAAGGCACCA
TGGAGCTTCGTGAAGGCCACATTGACCAGTTCGGTCACAAG
ACCTTCACGGGAATTGGACAGCAGATCGCTGATGAGATCCA
CGTGCGCCTCGGCCACGATGTTCGTACGACCGTTCTTGGCCA
CATTCAACGTGGTGGAACCCCAACTGCTTTCGACCGTGTTCT
GGCCACTCGTTATGGTGTTCGTGCAGCTCGTGCGTGCCATGA
GGGAAGCTTTGACAAGGTTGTTGCTTTGAAGGGTGAGAGCA
TTGAGATGATCACCTTTGAAGAAGCAGTCGGAACCTTGAAG
GAAGTTCCATTCGAACGCTGGGTTACTGCCCAGGCAATGTTT
GGATAG

Disrupted *pfkA* (F-R fragment, 693 bp: a multiple of 3)

ATGGAAGACATGCGAATTGCTACTCTCACGTCAGGCGGCG
ACTGCCCCGGACTAAACGCCGTCATCCGAGGAATCGTCCGC
ACAGCCAGCAATGAATTTGGCTCCACCGTCGTTGGTTATCAA
GACGGTTGGGAAGGACTGTTAGGCGATCGTCGCGTACAGCT

GTATGACGATGAAGATATTGACCGAATCCTCCTTCGAGGCGG
CACCATTTTGGGCACTGGTCGCCTCCATCCGGACAAGTTTAA
GGCCGGAATTGATCAGATTAAGGCCAACTTAGAAGACGCCG
GCATCGATGCCCTTATCCCAATCATTATCGTCGTTGCGGAAG
GTGCGTTGCCACGCGAAGGCACCATGGAGCTTCGTGAAGGC
CACATTGACCAGTTCGGTCACAAGACCTTCACGGGAATTGG
ACAGCAGATCGCTGATGAGATCCACGTGCGCCTCGGCCACG
ATGTTCGTACGACCGTTCTTGGCCACATTCAACGTGGTGGAA
CCCCAACTGCTTTCGACCGTGTTCTGGCCACTCGTTATGGTG
TTCGTGCAGCTCGTGCGTGCCATGAGGGAAGCTTTGACAAG
GTTGTTGCTTTGAAGGGTGAGAGCATTGAGATGATCACCTTT
GAAGAAGCAGTCGGAACCTTGAAGGAAGTTCCATTCGAAC
GCTGGGTTACTGCCCAGGCAATGTTTGGATAG

Figure 15. Confirmation of *pfkA* disruption by colony PCR with primer pairs, F1_*Bam*HI_*pfkA*_dis and R2_*Eco*RI_*pfkA*_dis (Table 4)



A: 1 kb ladder

1, 2, 3, 5: disrupted *pfkA*

4, 6, 7: wild-type *pfkA*

2.3. Optimization of carbon source condition

To determine a carbon source suitable for the ΔP, in which *pfkA* is disrupted, growth properties of ΔP were compared in various ratios of glucose and fructose mixture (Fig. 16). During 46 hours culture, cells hardly grew under only glucose 40 g/L and glucose 20 g/L-fructose 20 g/L (5:5) mixture condition. Under only fructose 40 g/L condition, the maximum cell mass was only 80% of the control level (maximum DCW was 12-13 g/L when the wild-type strain was cultured under glucose condition). Under the rest of the conditions, lag phase in the early time of fermentation was somewhat longer, but the maximum cell mass was similar to that of control level. Among them, it was determined to use glucose 12 g/L-fructose 28 g/L (3:7) mixture as a carbon source for 2'-FL production, because the concentration of glucose for GDP-L-fucose synthesis is the highest.

To construct the Δ*pfkA* strain to produce 2'-FL, ΔP BCGW TTLE(CO) harboring plasmids pVBCLE and pEGWTT(CO) were constructed.

Flask fermentation was performed in CGXII medium containing various ratios of glucose and fructose. The total amount of carbon source was set to 40 g/L because 4% glucose is used for CGXII medium. The fermentation results are shown in Figure 17 and Table 6. Under fructose 40 g/L condition, 0.88 g/L of 2'-FL was produced, but cell mass was only 70% of the control level. Under glucose 4 g/L-fructose 36 g/L (1:9) condition, 1.07 g/L of 2'-FL was produced, but cell mass was also only 70% of the control level. Under the rest of the conditions, cell mass was similar to that of the control level, and 2'-FL production was 1.22 g/L under glucose 12 g/L-fructose 28 g/L (3:7) and 1.18 g/L

under glucose 8 g/L-fructose 32 g/L (2:8) condition. As expected above, glucose 12 g/L-fructose 28 g/L (3:7) mixture was the best for producing 2'-FL in ΔP BCGW TTLE(CO).

Figure 16. Growth properties of ΔP in various ratios of glucose and fructose mixture. (A) OD_{600} during 46 hours culture (B) Maximum dry cell weight during 46 hours culture

Symbols: ● and **1**, Glc 40 g/L; ■ and **2**, Glc 20 g/L-Fru 20 g/L (5:5); ▲ and **3**, Glc 12 g/L-Fru 28 g/L (3:7); ▼ and **4**, Glc 8 g/L-Fru 32 g/L (2:8); ◆ and **5**, Glc 4 g/L-Fru 36 g/L (1:9); ★ and **6**, Fru 40 g/L

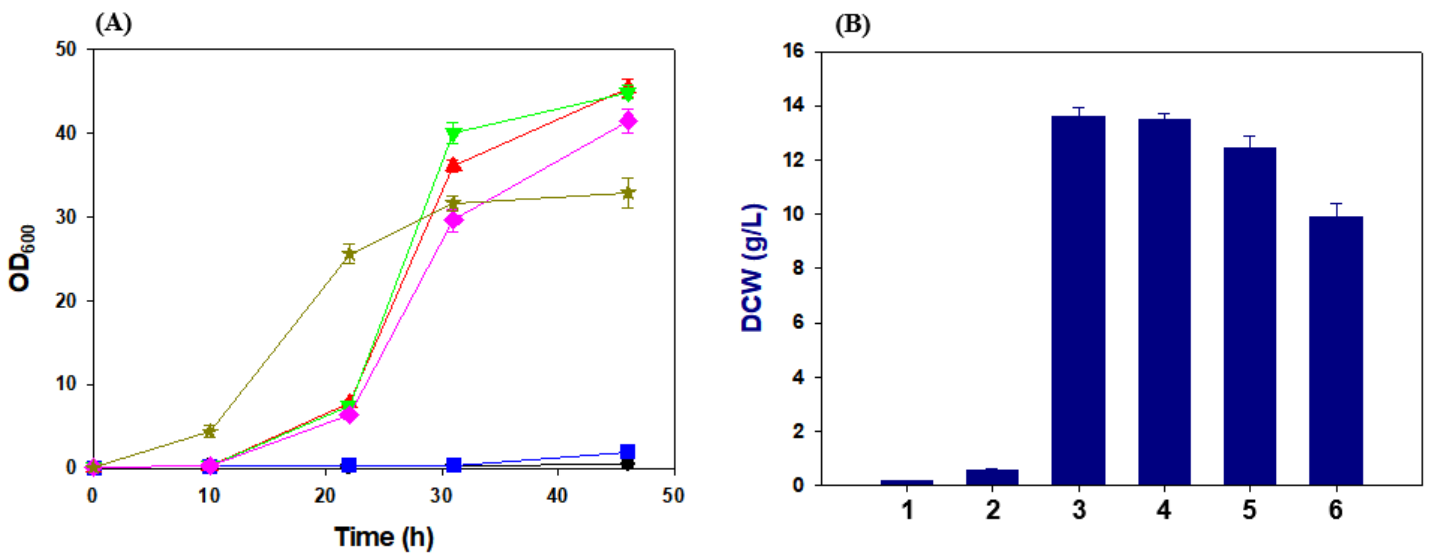
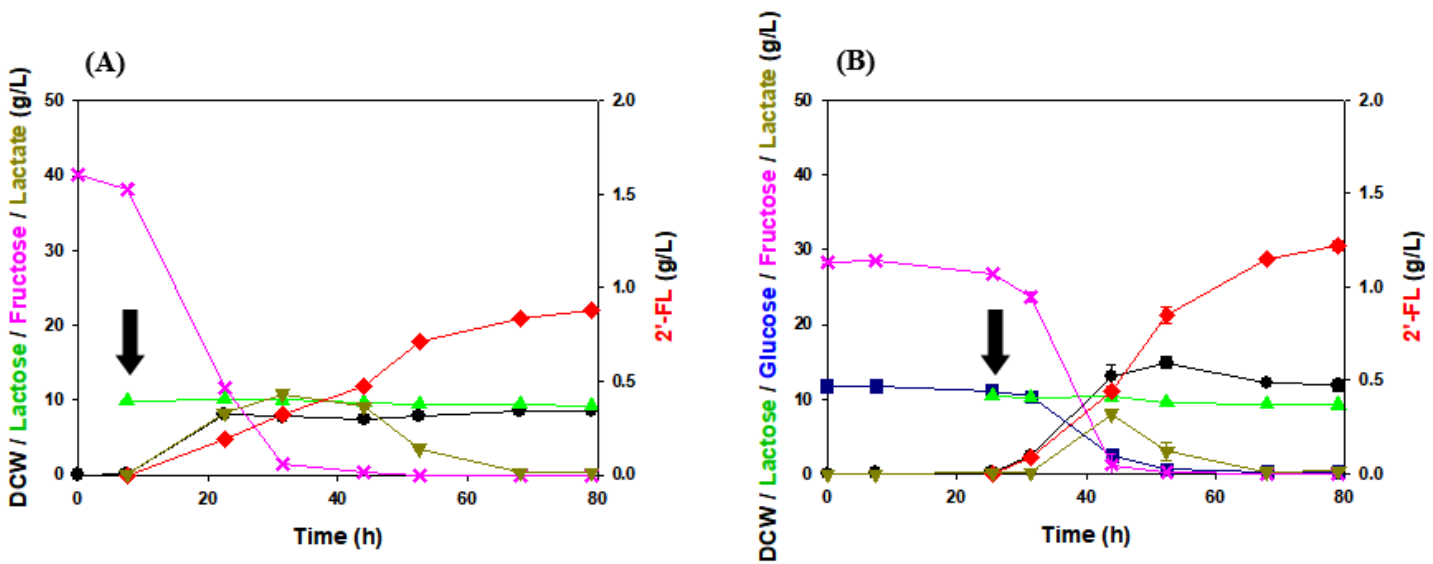
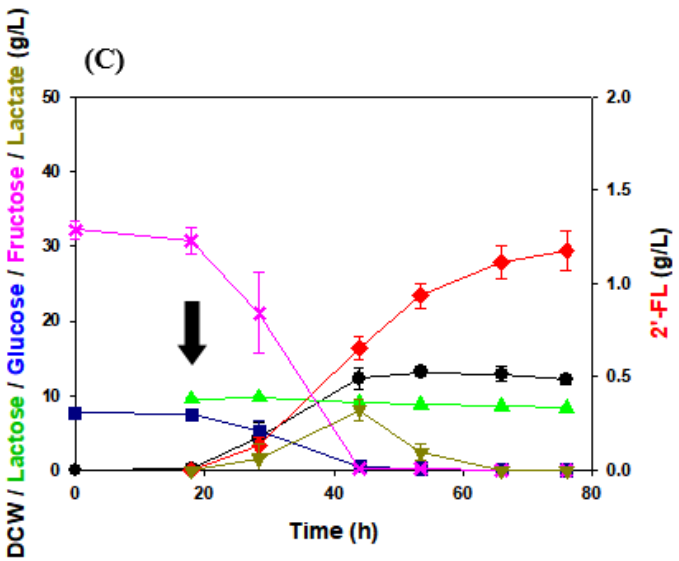


Figure 17. Flask fermentation of ΔP BCGW TTLE(CO). (A) Fru 40 g/L (B) Glc 12 g/L-Fru 28 g/L (3:7) (C) Glc 8 g/L-Fru 32 g/L (2:8) (D) Glc 4 g/L-Fru 36 g/L. As OD_{600} reached 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; **X**, Fructose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL




(C)
DCW / Lactose / Glucose / Fructose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
50
0.0
0.5
1.0
1.5
2.0
0
20
40
60
80

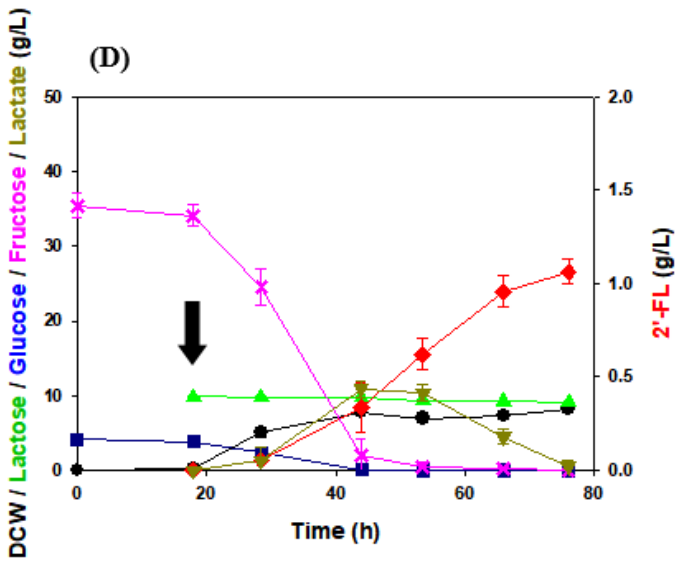

(D)
DCW / Lactose / Glucose / Fructose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
50
0.0
0.5
1.0
1.5
2.0
0
20
40
60
80

Table 6. Summary of flask fermentation of ΔP BCGW TTLE(CO) under the various mixed sugar conditions

Carbon source condition	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Yield (mole 2'-FL/ mole lactose)	*Productivity (mg/L/h)
Fru 40 g/L	8.58	0.88	0.87	11.1
Glc 12 g/L-Fru 28 g/L (3:7)	14.8	1.22	0.73	15.4
Glc 8 g/L-Fru 32 g/L (2:8)	13.1	1.18	0.65	15.4
Glc 4 g/L-Fru 36 g/L (1:9)	8.15	1.07	0.96	14

*2'-FL yield and productivity were calculated based on total fermentation time.

3. Fed-batch fermentation for production of 2’-FL

3.1. Fed-batch fermentation of ΔP BCGW TTLE(CO)

Fed-batch fermentation of ΔP BCGW TTLE(CO) was performed in a 2.5 L bioreactor with 1 L initial working volume of CGXII medium containing glucose 12 g/L-fructose 28 g/L (3:7) mixture as a carbon source. To prepare the cells for inoculation into the main culture, pre-culture was performed in a baffled flask with BHI medium. After complete consumption of carbon source added initially, glucose 240 g/L-fructose 560 g/L (3:7) mixture (total 800 g/L) was fed continuously. 2’-FL began to be produced after lactose addition and IPTG induction for expression of the genes associated with 2'-FL biosynthesis enzymes.

The fermentation results are shown in Figure 18 and Table 7. During 180 hours fermentation, ΔP BCGW TTLE(CO) produced 12.6 g/L 2’-FL. In particular, it was observed that about 50 hours of a lag phase appeared in the middle part of fermentation. This lag phase appeared at the start of feeding after IPTG induction and lasted about 50 hours. Also in the flask fermentation of ΔP BCGW TTLE(CO), a rather long lag phase was observed under the condition of high glucose concentration (Fig. 17). It is probably due to intracellular accumulation of sugar phosphates such as glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P). It is known that the accumulation of G6P or its analogues in bacteria like *E. coli* causes stress that appears in the form of growth inhibition (Richards, Patel et al. 2013). However, the reason why cell growth is inhibited by the accumulation of sugar phosphates is unknown. So far, it is expected that the accumulation of sugar phosphates itself may be toxic to the cell (for example, due to the

formation of toxic by-products such as methylglyoxal) (Hopper and Cooper 1971, Kadner, Murphy et al. 1992, Morita, El-Kazzaz et al. 2003, Vanderpool 2007, Richards and Vanderpool 2011, Richards, Patel et al. 2013). Also, depletion of glycolytic intermediates could be the cause of stress (Kimata, Tanaka et al. 2001, Morita, El-Kazzaz et al. 2003, Vanderpool 2007, Richards and Vanderpool 2011, Richards, Patel et al. 2013). It was reported that the high level of F6P caused by *pfkA* mutation seemed to induce stress as evidenced by *ptsG* mRNA degradation (Morita, El-Kazzaz et al. 2003, Richards, Patel et al. 2013). In the case of the ΔP strain, G6P and F6P accumulate when glucose is present, and therefore growth inhibition is expected to occur at that time.

Figure 18. Fed-batch fermentation of ΔP BCGW TTLE(CO). After depletion of carbon source (glucose-fructose 3:7 mixture, total 40 g/L), IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; **X**, Fructose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL

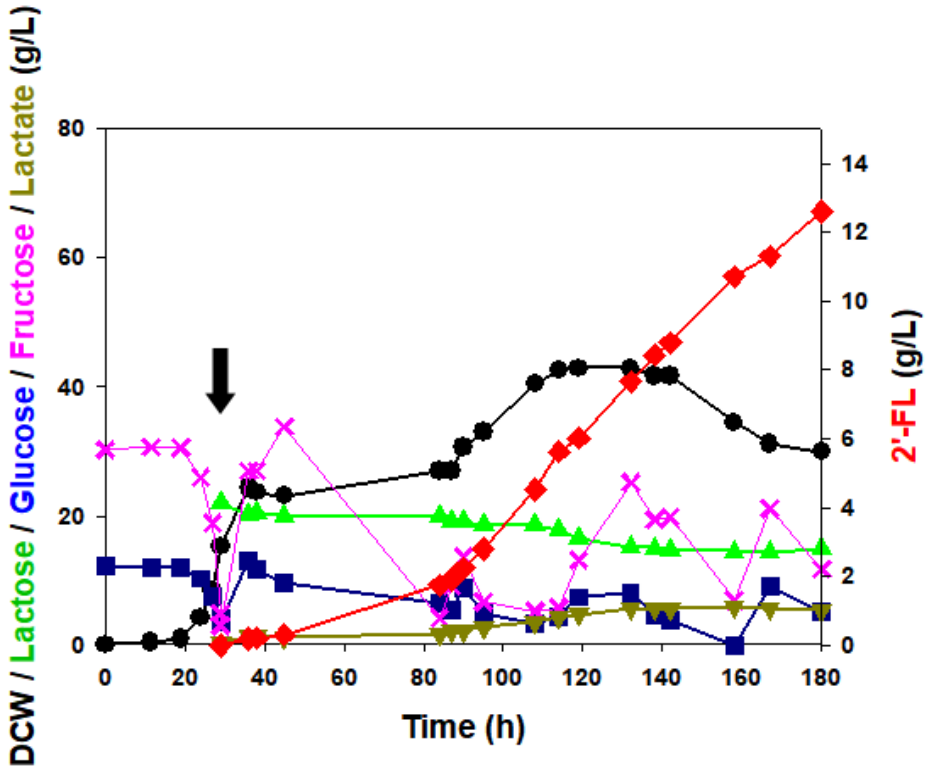


Table 7. Summary of fed-batch fermentation of ΔP BCGW TTLE(CO) under the glucose-fructose 3:7 mixture condition

Strains	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Yield (mole 2'-FL/mole lactose)	*Productivity (g/L/h)
ΔP BCGW TTLE(CO)	42.9	12.6	1.18	0.07

*2'-FL yield and productivity were calculated based on total fermentation time.

3.2. Optimization of fed-batch fermentation process

Fed-batch fermentation ΔP BCGW TTLE(CO) feeding glucose-fructose (3:7) mixture had a problem that a very long lag phase appears in the middle of fermentation. Additionally, since the consuming rate of the two sugars during fermentation was not constant, it is difficult to keep the concentrations of the two sugars appropriately (Fig. 18). As mentioned above, glucose concentration can affect cell growth in case of Δ*pfkA* strain. Therefore, a new fermentation strategy was sought to solve these problems.

First, it was devised that feeding solution was prepared with only fructose and glucose was intermittently added into the medium. Prior to this, in order to set the appropriate glucose concentration to maintain during fermentation, an experiment was conducted on a flask-scale in which glucose was added simultaneously with IPTG induction while culturing under only fructose condition. 2'-FL production and cell growth were compared at various glucose concentrations of 4, 10, 15, and 20 g/L. The results are shown in Figure 19 and Table 8. 2'-FL production was 1.19 g/L under the 4 g/L glucose condition, 2.05 g/L under the 10 g/L glucose condition, 1.41 g/L under the 15 g/L glucose condition and 1.61 g/L under the 20 g/L glucose condition. Especially, under the 10 g/L glucose condition, 2'-FL production was improved by 68% compared to the use of glucose-fructose (3:7) mixture. Under the 4 g/L glucose condition, the production of 2'-FL decreased compared to other cases and maximum cell mass was found to be poor. Thus, it was determined that it would be appropriate to maintain the glucose concentration around 10-20 g/L during the fermentation. Fed-batch

fermentation was performed based on the above experimental results. To prepare the cells for inoculation, pre-culture was performed in a baffled flask with BHI medium. After complete consumption of 40 g/L fructose added initially, fructose was fed continuously. Simultaneously, for production of 2'-FL, IPTG induction was performed and 20 g/L lactose and 20 g/L glucose was added. At the same time, however, cell growth was inhibited again (Fig. 20).

Second, in order to solve the problem of inhibition of cell growth, a method of cell adaptation at the pre-culture step was devised. So far, pre-culture as a preparation step of cells for inoculation into the main culture was performed with BHI medium. However, when cells enter the main culture, the conditions for the cells to adapt vary, such as minimal medium (CGXII medium), glucose-fructose mixture, lactose and IPTG induction. By establishing similar conditions from the pre-culture step, it was thought that the cells could adapt quickly to the environment of the main culture. Based on this idea, pre-culture was performed in the same manner as flask fermentation. It was performed in a baffled flask with CGXII medium containing 40 g/L fructose. As OD_{600} reached 0.8, 1mM IPTG, 10 g/L lactose and 20 g/L glucose were added. To prepare the adapted cells to the above conditions, cells were cultured when OD_{600} reached 20 (mid-log phase). After that, the pre-culture broth was inoculated into the main culture and IPTG, lactose and glucose were added to the medium. Feeding solution was prepared with only fructose (800 g/L) and fed by continuous feeding method when cells reached log phase. Glucose was supplied intermittently to maintain a level of 10-20 g/L. The fermentation results are shown in

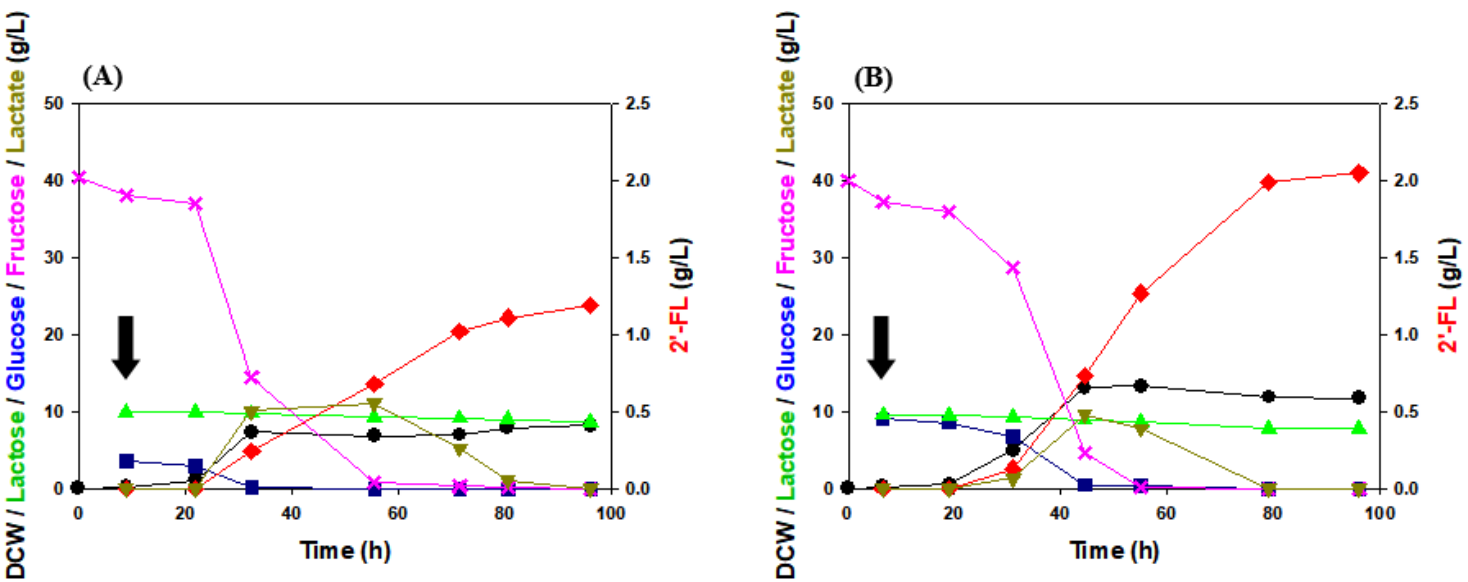
Figure 21 and Table 9.

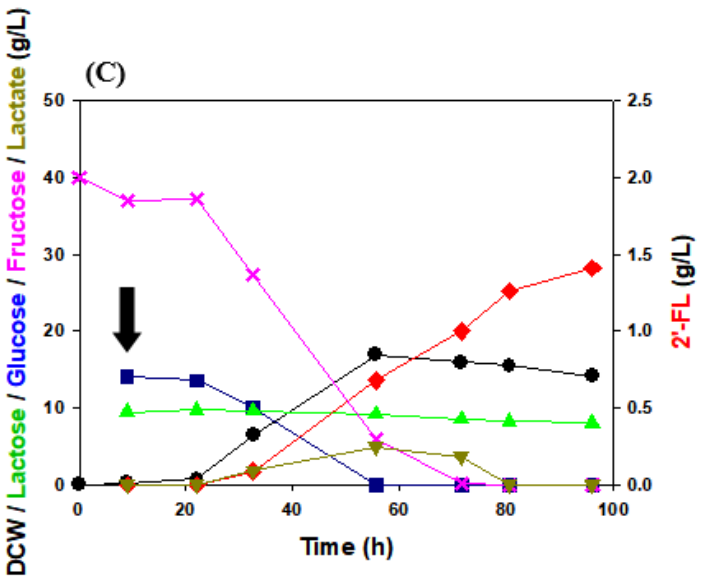
During 180 hours fermentation, 21.5 g/L of 2'-FL was produced. This result is 87% higher than that of BCGW TTL(CO) constructed in the previous research (Jo, Thesis. 2016) and 71% higher than that of ΔP BCGW TTLE(CO) feeding glucose-fructose (3:7) mixture as carbon source. The lag phase, which appeared in the middle of the fermentation, was not observed. However, the yield of 2'-FL from lactose was higher than the theoretical yield of 1, which seems to be due to two reasons. First, there is a substance that overlaps with the peak of lactose, which makes it difficult to analyze. The substance was found to be trehalose by quantification using GC/TOF MS by Prof. K. H. Kim at Korea University (data not shown). The retention time of trehalose and lactose is similar in the currently used Rezex ROA-organic acid H^+ Column (Phenomenex, USA), so peak separation did not work properly (Fig. 22). It is known that trehalose is produced in *C. glutamicum* when cells are subjected to osmotic stress (Wolf, Krämer et al. 2003, Tropis, Meniche et al. 2005). Trehalose is a glucose disaccharide and it is formed by the α-1,1-glucoside bond. Lactose is also a disaccharide composed of glucose and galactose. So, the molecular weights of the two are the same and peak separation appears to be difficult.

Second, as the fermentation time becomes longer, the medium evaporates in the latter half of fermentation, and the metabolites seem to be concentrated. Taken together, in the latter part of the fermentation, due to the accumulation of trehalose by osmotic stress and the evaporation of the medium, it is difficult to estimate the amount of consumed lactose.

Figure 19. Flask fermentation of ΔP BCGW TTLE(CO). (A) Glc 4 g/L (B) Glc 10 g/L (C) Glc 15 g/L (D) Glc 20 g/L (initial carbon source: 40 g/L fructose only). As OD_{600} reached 0.8, IPTG, lactose and glucose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; **X**, Fructose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL




(C)
DCW / Lactose / Glucose / Fructose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
50
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60
80
100

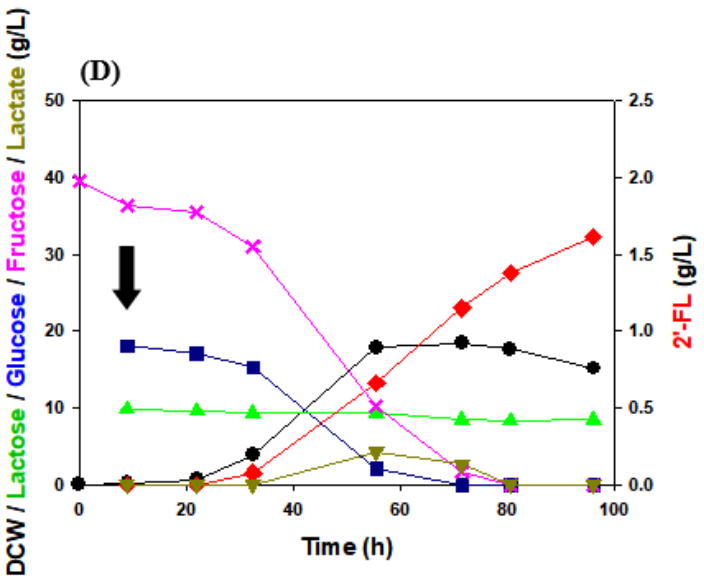

(D)
DCW / Lactose / Glucose / Fructose / Lactate (g/L)
2'-FL (g/L)
Time (h)
0
10
20
30
40
50
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60
80
100

Table 8. Summary of flask fermentation of ΔP BCGW TTLE(CO) under the various glucose addition conditions (initial carbon source: 40 g/L fructose only)

Glucose dumping condition	Maximum dry cell weight (g/L)	Maximum 2-FL concentration (g/L)	*Yield (mole 2-FL/mole lactose)	*Productivity (mg/L/h)
4 g/L	8.19	1.19	0.71	12.4
10 g/L	13.35	2.05	0.81	21.4
15 g/L	16.98	1.41	0.68	14.7
20 g/L	18.45	1.61	0.77	16.7

*2'-FL yield and productivity were calculated based on total fermentation time.

Figure 20. Fed-batch fermentation of ΔP BCGW TTLE(CO). After depletion of 40 g/L fructose, IPTG, lactose and glucose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; **X**, Fructose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL

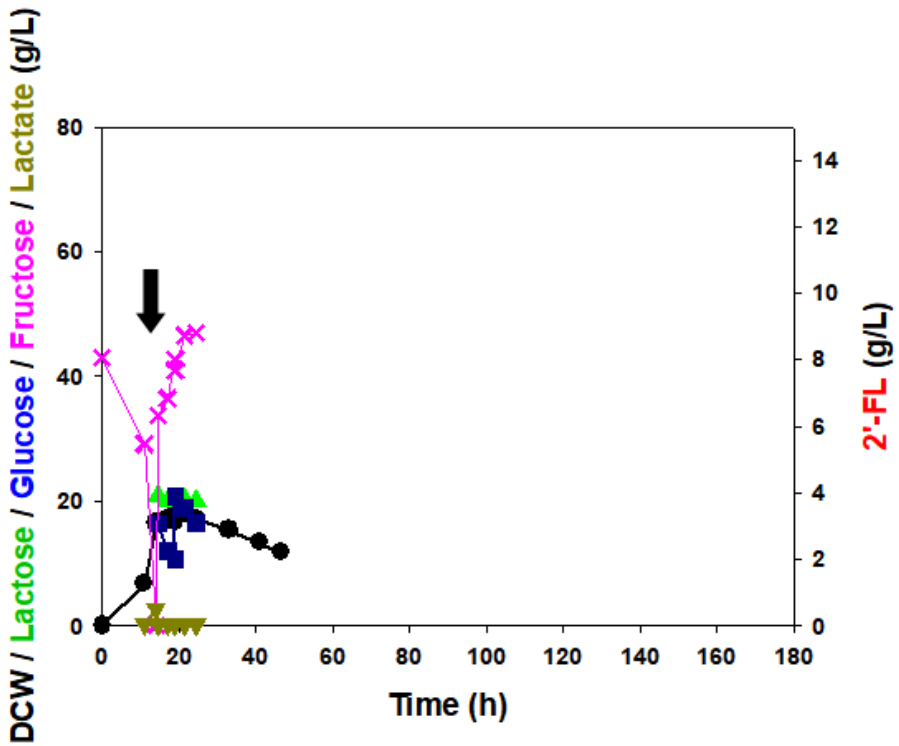


Figure 21. Fed-batch fermentation of ΔP BCGW TTLE(CO). After cell adaptation at the pre-culture step, inoculation was conducted. At the same time as the inoculation, IPTG, lactose and glucose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; **X**, Fructose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL

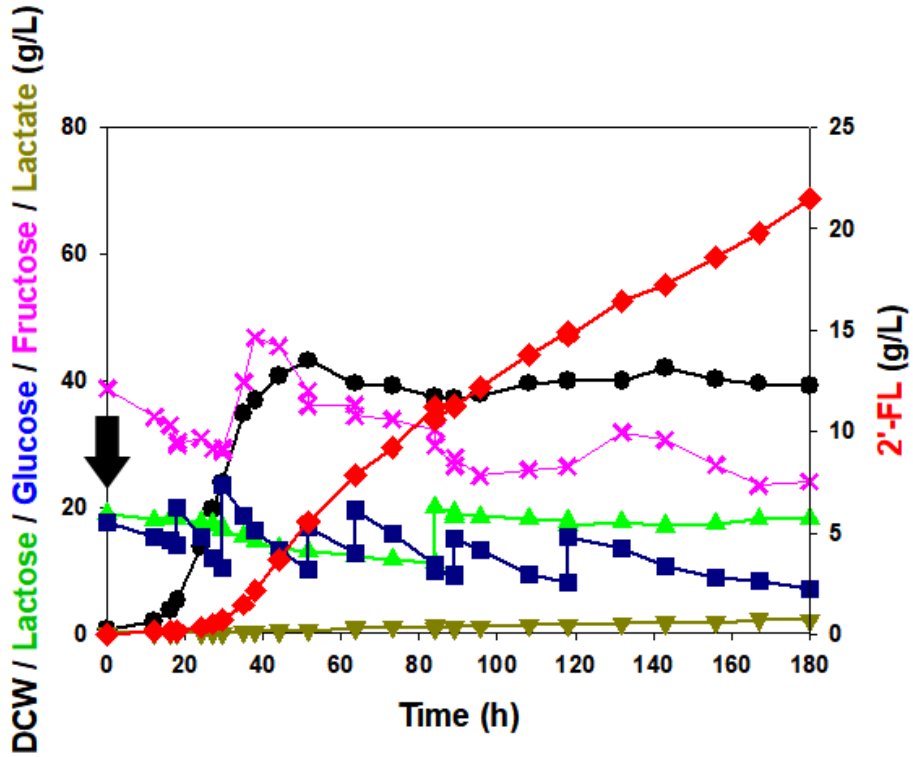
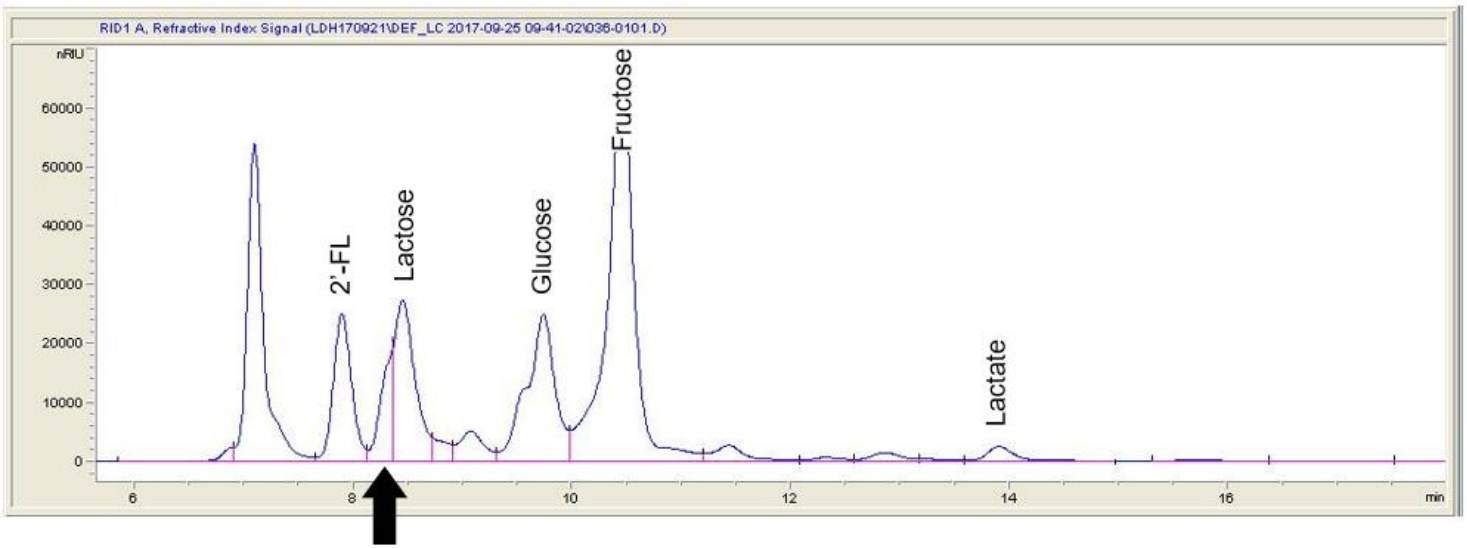


Table 9. Summary of fed-batch fermentation of ΔP BCGW TTLE(CO) by a strategy based on cell adaptation at the pre-culture step.

Strains	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Yield (mole 2'-FL/mole lactose)	*Productivity (g/L/h)
ΔP BCGW TTLE(CO)	43.2	21.5	1.41	0.119

*2'-FL yield and productivity were calculated based on total fermentation time.

Figure 22. LC profile of 84-hour fed-batch fermentation sample of ΔP BCGW TTLE(CO). Trehalose peak is indicated by the thick arrow.



4. Change of the culture temperature condition

Previously, the exporter was introduced to export intracellular 2'-FL, but a considerable amount of 2'-FL was present in the cells. In the case of fed-batch fermentation of ΔP BCGW TTLE(CO), there was about 6-7% intracellular g 2'-FL per g cell (Fig. 23). Thus, to more excellently export 2'-FL out of the cell, a strategy to increase the culture temperature was devised. It was thought that the higher the culture temperature, the greater the fluidity of the cell membrane and the more intracellular 2'-FL would go out of the cell. Similarly heat shock is also generally performed in bacteria when DNAs are transformed.

To confirm the effect of the change of culture temperature condition on 2'-FL production, flask fermentation was performed. At various time points, the culture temperature was changed from 30℃ to 37℃. The fermentation results are shown in Figure 24 and Table 10. When the fermentation was performed at 37℃ from the beginning, cells did not grow for 50 hours (data not shown). In particular, the enhanced 2'-FL production was confirmed when the temperature was changed from 30℃ to 37℃, when OD_{600} reached 40 (maximum cell growth) and after complete consumption of carbon source. In the former case, the amount of 2'-FL produced was 2.43 g/L and in the latter case, 2.62 g/L. The 2.62 g/L 2'-FL is 28% higher than that of 2'-FL produced in ΔP BCGW TTLE(CO) fermented at 30℃ and 379% higher than that of 2'-FL produced in BCGW TTL(CO) fermented at 30℃ (Jo, Thesis. 2016).

After fermentation, the amount of intracellular 2'-FL was also compared according to the method described above. The results are shown in Figure 25. When the culture temperature was changed at early

time during fermentation, the amount of intracellular 2'-FL tended to decrease. In particular, in the case of changing the culture temperature to 37℃ after depletion of the carbon source, the total amount of 2’-FL was similar to that of 2’-FL produced at 30℃. However, in the former, the amount of extracellular 2'-FL increased and the amount of intracellular 2'-FL decreased compared to the latter. Therefore, it is considered that the remaining 2'-FL in the cell went slightly out of the cell. The results suggested that the increased culture temperature while cells grows affected the cell growth, especially maximum cell mass, and reduced the total amount of 2’-FL rather than the control. However, the amount of extracellular 2’-FL was similar or higher compared to control because of reduced intracellular 2’-FL. It seemed that the fluidity of the cell membrane increased and the chance for the intracellular 2’-FL to go out of the cell increased. The increased amount of extracellular 2’-FL was higher than the decreased amount of intracellular 2’-FL. When the incubation temperature was changed, the cell mass was further reduced after fermentation. The improved extracellular 2’-FL production maybe also due to cell lysis.

If this fermentation strategy is also applied to fed-batch fermentation, the 2'-FL production is expected to be further improved.

Figure 23. Intracellular 2'-FL confirmed in fed-batch fermentation of ΔP BCGW TTLE(CO)

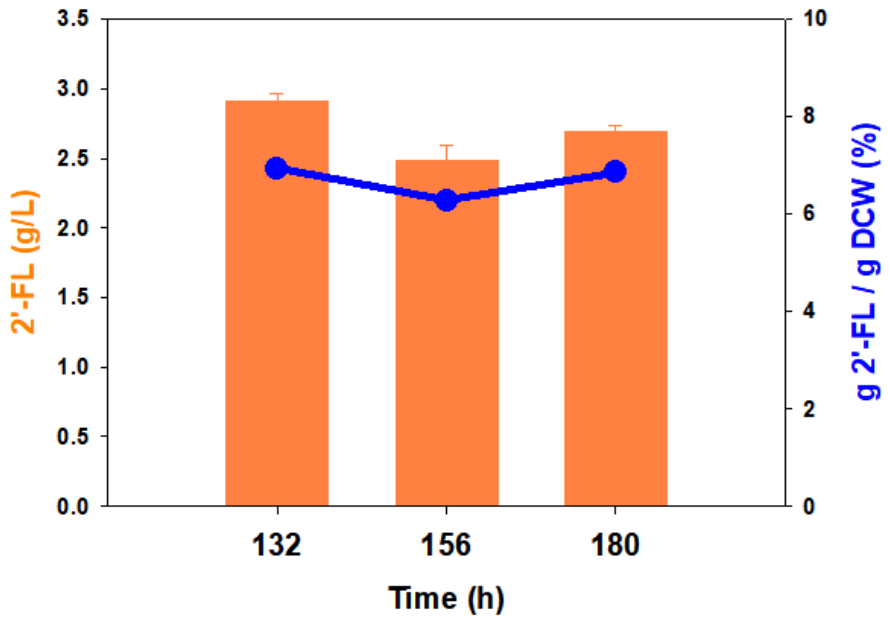
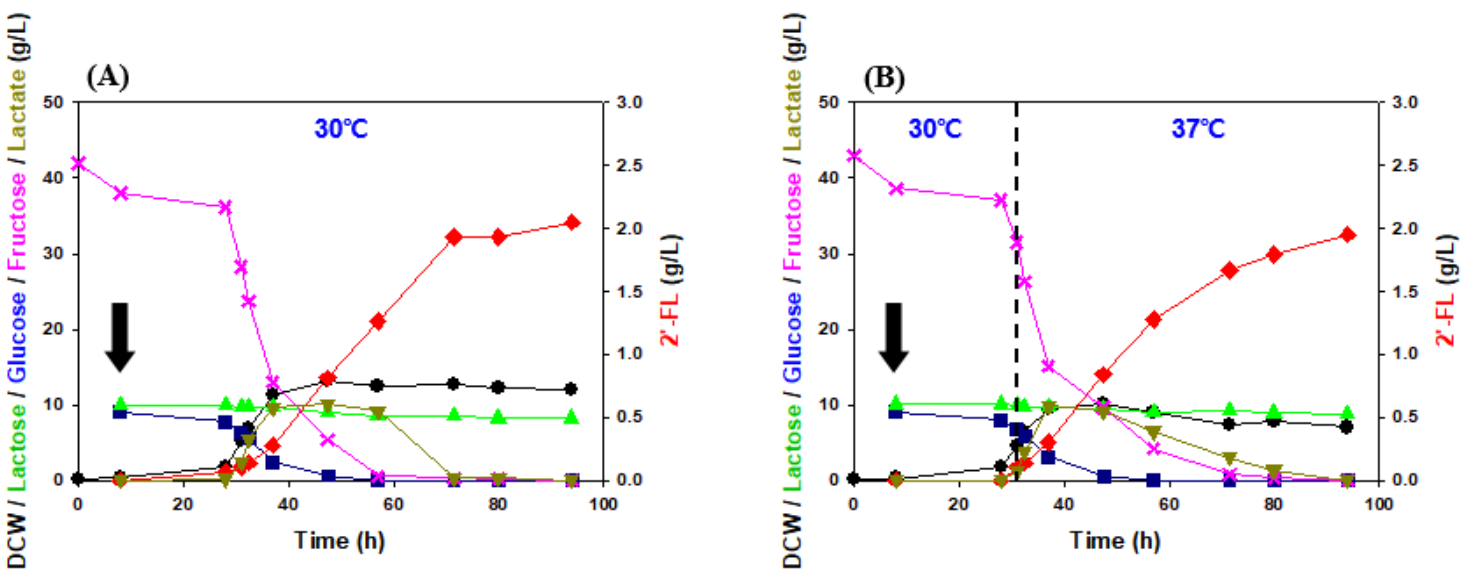
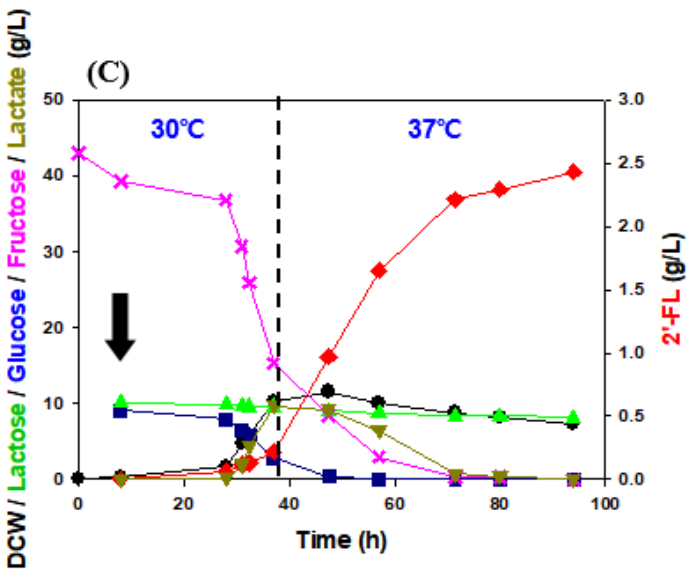


Figure 24. Flask fermentation of ΔP BCGW TTLE(CO). (A) 30℃. As OD_{600} reached 20 (B) and 40 (C) respectively, the culture temperature was changed from 30℃ to 37℃. (D) After complete consumption of carbon source, the culture temperature was changed from 30℃ to 37℃.

As OD_{600} reached 0.8, IPTG, lactose and glucose were added (thick arrow).

Symbols: ●, DCW; ■, Glucose; **X**, Fructose; ▲, Lactose; ▼, Lactate; ◆, 2'-FL




(C)
30°C
37°C
DCW / Lactose / Glucose / Fructose / Lactate (g/L)
2'-FL (g/L)
Time (h)

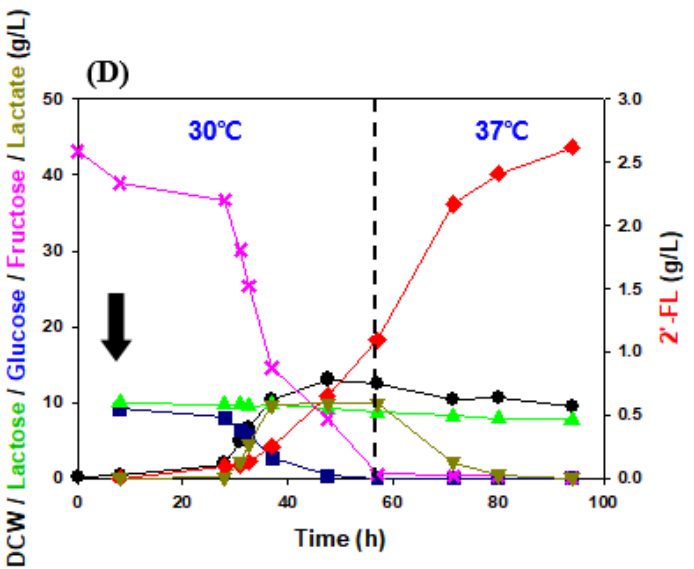

(D)
30°C
37°C
DCW / Lactose / Glucose / Fructose / Lactate (g/L)
2'-FL (g/L)
Time (h)

Table 10. Summary of flask fermentation of ΔP BCGW TTLE(CO) under the various fermentation conditions

†Culture temperature condition	Maximum dry cell weight (g/L)	Maximum 2'-FL concentration (g/L)	*Yield (mole 2'-FL/mole lactose)	*Productivity (mg/L/h)
A	13.14	2.05	0.82	21.8
B	10.11	1.95	1.04	20.7
C	11.55	2.43	0.86	25.9
D	13.02	2.62	0.83	27.9

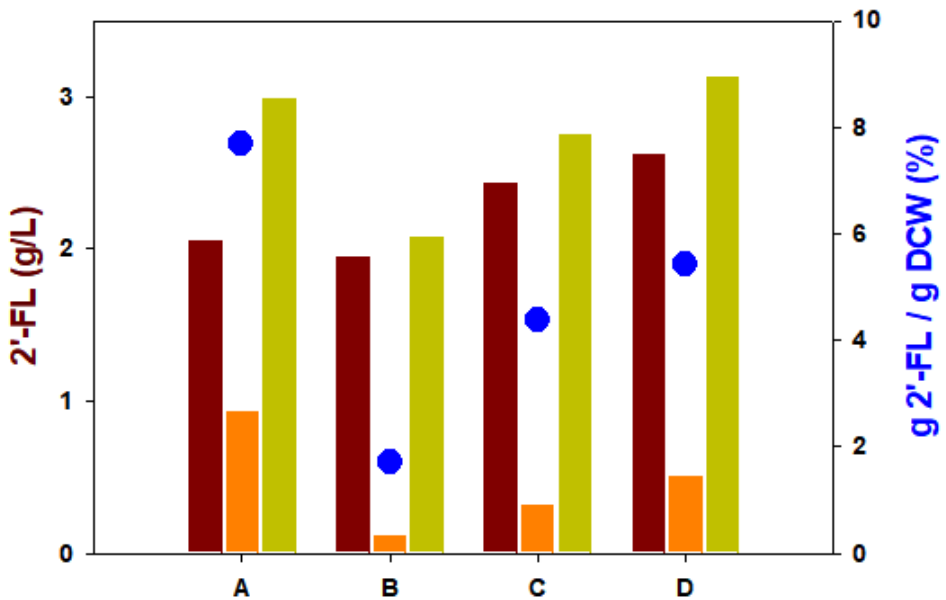
†Details are shown in Figure 24.

*2'-FL yield and productivity were calculated based on total fermentation time.

Figure 25. Intracellular 2'-FL of ΔP BCGW TTLE(CO) under the various fermentation conditions. Details of A, B, C and D are shown in Figure 24.

Colors: Brown (first bar), Extracellular 2'-FL; Orange (second bar), Intracellular 2'-FL;

Yellow green (third bar), Total 2'-FL, Blue dot, g intracellular 2'-FL / g DCW (%)



IV. CONCLUSIONS

This thesis can draw the following conclusions:

(1) By introducing the 2'-FL exporter from *B. infantis* into BCGW TTL(CO), 2'-FL production was improved. In flask fermentation, 0.83 g/L of 2'-FL was produced, which was improved by 52% compared to BCGW TTL(CO).

(2) To enhance the metabolic flux for GDP-$_L$-fucose, a two-track system that utilizes fructose for cell growth through glycolysis, and glucose for synthesis of GDP-$_L$-fucose, was developed by disruption of the *pfkA* gene. In flask fermentation using glucose-fructose 3:7 mixture as a carbon source, ΔP BCGW TTLE(CO) produced 1.22 g/L of 2'-FL.

(3) Fed-batch fermentation of ΔP BCGW TTLE(CO) using glucose-fructose 3:7 mixture as feeding solution resulted in 12.6 g/L of 2'-FL concentration. However, cell growth was inhibited in the middle of fermentation, so cell adaptation was performed at pre-culture step to solve this problem. As a result, fed-batch fermentation of ΔP BCGW TTLE(CO) resulted in 21.5 g/L of 2'-FL concentration.

2’-FL production is enhanced by 87% compared to BCGW TTL(CO).

(4) To export 2’-FL out of the cell excellently, a strategy to increase the culture temperature during the fermentation was devised. 2’-FL production was improved by increasing the culture temperature in the middle of fermentation. When the culture temperature was changed from 30℃ to 37℃ after the carbon source depletion in the flask fermentation of ΔP BCGW TTLE(CO), 2.62 g/L of 2'-FL was produced. This result is 379% higher than that of 2’-FL produced in BCGW TTL(CO) fermented at 30℃.

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국 문 초 록

모유에는 다른 포유류의 젖과는 특이적으로 올리고당이 약 15 g/L 가량 함유되어 있다. 이 모유 올리고당은 약 200~300 여 종류가 존재하는 것으로 알려져 있으며, 그 중 50~80% 가량이 푸코실화 되어있는 푸코실올리고당이다. 푸코실올리고당은 유산균의 먹이로써 활용되는 프리바이오틱 효과, 병원균으로부터의 감염 방지, 면역반응의 조절, 두뇌 발달 등의 기능성을 갖는 것으로 알려져 있으며, 신생아의 초기 성장단계에서 장내 미생물총의 형성에 기여하는 것으로 보고되고 있다. 이러한 푸코실올리고당 중 유당의 갈락토오스에 α-1,2 결합으로 푸코실화 되어있는 2'-푸코실락토오스 (2'-fucosyllactose, 2'-FL)의 함량이 가장 높으며, 이 2'-FL 은 프리바이오틱 효과와 더불어 *Campylobacter jejuni*, *Pseudomonas* 등의 병원균 및 *Escherichia coli* 의 enterotoxin 과 같은 독소가 장내에 부착되는 것을 막고 염증반응을 완화하는 등의 다양한 기능을 갖는 물질임이 밝혀져 이를 기능성 식품 소재로써 활용하기 위한 시도들이 이루어지고 있다.

선행연구에서는, GRAS (Generally Recognized As Safe)로 인증된 코리네박테리움 글루타미쿰을 이용하여 2'-FL 을 생산하는 기술이 개발되었다. 2'-FL 을 합성하기 위해서는 GDP-L-fucose 와 유당이 필요하다. 야생형 코리네박테리움

글루타미쿰은 GDP-L-fucose 생합성 경로가 존재하지 않아 선행연구를 통해 GDP-L-fucose 를 생합성하기 위한 유전자들이 도입되었다. 또한 야생형 코리네박테리움 글루타미쿰은 유당대사를 할 수 없어 유당을 세포 안으로 들여올 수 없으므로 대장균 유래의 β-galactosidase 가 파쇄된 *lacYA* 오페론을 도입하여 유당을 세포 내로 수송하도록 하였으며, 푸코오스 전이를 위하여 헬리코박터 파일로리 유래의 α-1,2 fucosyltransferase 유전자인 *fucT2* 를 코돈 최적화하여 도입하였다. 위와 같이 대사공학적으로 구축된 균주를 이용하여 회분식 발효를 실시한 결과 547 mg/L 의 2'-FL 이 생산되었으며, 유가식 발효 결과 11.5 g/L 의 2'-FL 이 생산되었다.

본 연구에서는 기존의 시스템을 대사공학적으로 개량함으로써 2'-FL 생산을 증대시키고자 하였다. 우선, 비피도박테리움 인팬티스 유래의 ABC transporter permease 를 도입하여 세포 내부에 존재하는 2'-FL 을 세포 밖으로 수송하고자 하였다. 그 결과 기존 균주보다 그람 균체 당 38% 가량 향상된 수송능력을 보였다. 배지 상에 존재하는 2'-FL 의 농도는 830 mg/L 로 기존 균주보다 약 52% 향상되었다.

다음으로 GDP-L-fucose 생합성 경로를 최적화하여 GDP-L-fucose 로의 metabolic flux 를 강화하는 연구를 수행하였다. 포도당을 이용하여 GDP-L-fucose 를 합성하는 시스템은 metabolic flux 가 세포생장과 관련 있는 해당과정으로 대부분

집중되는 문제점이 있었다. 이를 해결하기 위하여 pK19mobsacB 벡터를 이용한 double crossover 방법으로 phosphofructokinase A (*pfkA*) 유전자를 파쇄함으로써, 해당과정의 첫 번째 주요경로인 과당 6-인산 (Fructose 6-phosphate)에서 과당 1,6-이인산 (Fructose 1,6-bisphosphate)으로의 대사경로가 차단된 균주를 구축하였다. 그 후 포도당과 과당 혼합당을 탄소원으로 이용함으로써, 포도당은 GDP-L-fucose 의 합성에, 과당은 세포생장에 이용되도록 하는 이중경로 시스템을 구축하였다. 그 결과 회분식 발효에서 1.22 g/L 의 2'-FL 이 생산되었고, 유가식 발효에서는 12.6 g/L 의 2'-FL 이 생산되었다.

그러나 발효 중반부에 세포생장이 지연되고, 포도당과 과당의 소모속도가 지속적으로 변하여 발효 동안 일정수준의 농도를 유지하기 어렵다는 문제점이 나타났다. 이를 해결하기 위하여, 과당 단일당을 공급하면서 포도당은 간헐적으로 공급함으로써 일정농도를 유지하는 전략을 세웠다. 그 결과 회분식 발효에서 2.05 g/L 의 2'-FL 이 생산되었다. 하지만 유가식 발효에서는 포도당 공급 시 세포생장이 오랫동안 지연되는 현상이 또다시 발생하였다. 이를 해결하고자 주배양에 접종할 세포를 배양하는 단계인, 전배양 단계에 세포적응과정을 부여하였다. 주배양과 비슷한 환경을 조성한 뒤, 중간지수생장기까지 세포를 배양하여 주배양에 접종하였다. 그 결과 세포생장이 지연되는 현상을 해결할 수

있었고, 21.5 g/L 의 2’-FL 이 생산되었다. 이는 선행연구보다 87% 향상된 결과이다.

끝으로 발효 후반부에 배양온도를 30℃에서 37℃로 변경함으로써, 세포막의 유동성을 증가시켜 세포 내에 잔존하는 2’-FL 이 세포 밖으로 분출되도록 하였다. 그 결과 회분식 발효에서 선행연구보다 379% 향상된 2.61 g/L 의 2’-FL 이 생산되었다.

본 연구에서는 안전한 미생물인 코리네박테리움 글루타미쿰을 이용하여 모유 올리고당의 핵심소재인 2’-FL 을 고농도로 생산하는 생물공정을 개발하였다. 본 연구는 기능성 식품소재로 각광받고 있는 2’-FL 을 산업적으로 생산할 수 있는 기술적 근거를 제공할 것으로 기대된다.

주요어: 대사공학, 2’-푸코실락토오스, GDP-$_{L}$-fucose, 2’-푸코실락토오스 수송체, pK19mobsacB, phosphofructokinase A, 유가식 발효, 코리네박테리움 글루타미쿰

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