A Thesis for the Degree of Master of Science

Production of 3-fucosyllactose in engineered *Corynebacterium glutamicum*

대사공학적으로 설계된
코리네박테리움 글루타미쿰을 이용한
3-푸코실락토오스 생산에 관한 연구

By

Hyeong-Do Jeon

Department of Agricultural Biotechnology
Seoul National University
February 2019

A Thesis for the Degree of Master of Science

Production of 3-fucosyllactose in engineered *Corynebacterium glutamicum*

Advisor : Professor Jin-Ho Seo

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

By

Hyeong-Do Jeon

Department of Agricultural Biotechnology
Seoul National University
February 2019

農 學 碩 士 學 位 論 文

Production of 3-fucosyllactose in engineered *Corynebacterium glutamicum*

대사공학적으로 설계된 코리네박테리움 글루타미쿰을 이용한 3-푸코실락토오스 생산에 관한 연구

指導敎授 徐鎭浩

이論文을 農學碩士學位論文으로 提出함
2019年 2月

서울大學校大學院
農生命工學部 食品生命工學 專攻

全炯度

全炯度의 農學碩士學位論文을 認准함
2019年 2月

委員長	강 동 현 (인)
副委員長	서 진 호 (인)
委員	이 기 원 (인)

ABSTRACT

Human milk contains a substantial amount of oligosaccharides (15-25 g/L) in contrast with bovine milk. Human milk oligosaccharides (HMOs) have many biological functions involving prebiotic effects, prevention of pathogenic infection, modulation of immune systems, brain development of infants and anti-inflammatory effects. Particularly, 3-fucosyllactose (3-FL) which is one of the most abundant ones among 200 different oligosaccharides is magnified as a pharmaceutical or functional food material.

In this research, *Corynebacterium glutamicum* was used as a 3-FL producer since it is recognized as GRAS (Generally Recognized As Safe) and has been traditionally used for industrial production of nucleotides. In order to produce 3-FL in *C. glutamicum*, lactose and GDP-$_L$-fucose are essential. As the GDP-$_L$-fucose biosynthetic pathway does not exist in wild type *C. glutamicum*, the GDP-$_L$-fucose biosynthetic genes were introduced in the previous research. *C. glutamicum* does not have any lactose permease for transport of lactose into the cell. In order to transport lactose into the cell, lactose

permease derived from *Esherichia coli* was introduced as a form of the *lacYA* operon. Lastly, α-1,3-fucosyltransferase is needed for production of 3-FL by coupling lactose with GDP-L-fucose. α-1,3-Fucosyltransferases derived from various organisms were tested for confirmation of 3-FL production. The *azoT* gene from *Azospirillum brasilense* showed the highest production of 3-FL (390 mg/L) in a flask culture. In a fed-batch fermentation for high production of 3-FL, this strain produced 3-FL titer of 3.23 g/L.

In order to increase production of 3-FL further, the expression of the *azoT* gene was modulated at levels of translation and transcription. Firstly, the polycistronic expression of the *azoT* gene was converted into monocistronic expression for enhancing the expression of the *azoT* gene at a transcription level. In order to build the system of monocistronic expression of the *azoT* gene, the *tac* promoter was introduced in front of the *azoT* gene. Next, the *azoT* gene was codon-optimized for enhancing its expression at a translation level. The strain with CO*azoT* under monocistronic expression produced 3-FL titer of 590mg/L in a flask culture. In a fed-batch fermentation, this strain produced 3-FL titer of 4.00 g/L.

To increase the biosynthetic flux to GDP-L-fucose, the GDP-mannose

4,6-dehydratase gene from *A. brasilense (noeL)* was introduced. The strain with the *noeL* gene produced 3-FL titer of 1.33 g/L in a flask culture and 10.0 g/L in a fed-batch fermentation.

Lastly, to optimize the fermentation process, the medium used for pre-culture was replaced with the medium used for the main culture. After establishing an environment similar to that of the main culture, the cells were cultured up to the mid-log phase and inoculated in to the main culture. As a result, the delay of cell growth was solved and 17.1 g/L of 3-FL was produced in a fed-batch fermentation.

The microbial system developed in this study would be advantageous for industrial 3-FL production, as *C. glutamicum* used as host is recognized as GRAS.

Keywords: Metabolic engineering, 3-fucosyllactose, α-1,3-fucosyltransferase, engineered *Corinebacterium glutamicum*, fed-batch fermentation

Student Number: 2017-28426

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 effect, 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 is similar to that of bovine milk (Table 1). However there are significant differences in both contents and composition of oligosaccharides. Oligosaccharides content of human milk is much higher than that of bovine milk and about 80% of oligosaccharides are fucosylated. The high concentration of oligosaccharides is the most distinctive feature of human milk. Perhaps, the oligosaccharides contained in human milk seem to be 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)

2.1. Structures of HMOs

The oligosaccharides contained in human milk is 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 one 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 a Gal residue followed by further addition of Gal in a 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).

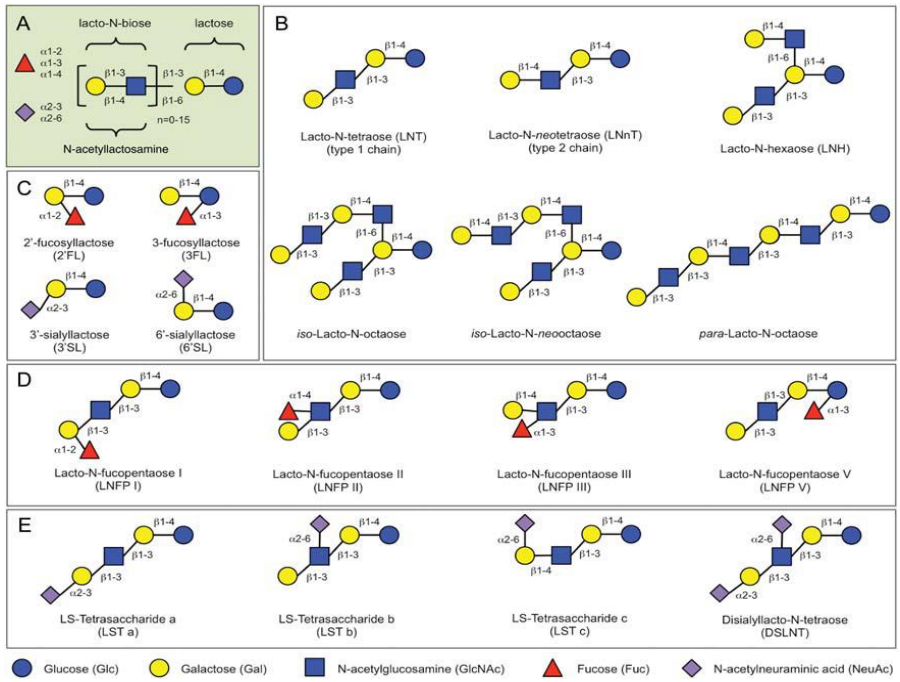


Figure 1. Typical HMO structures. (A) HMOs follow a basic structural blueprint. (Monosaccharide key is shown at the bottom of the figure.) (B) Lactose can be fucosylated or sialylated in different linkages to generate trisaccharides. (C) Lactose can be elongated by addition of either lacto-*N*-biose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

2.2. Functionalities of HMOs

The functionality of HMOs 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 and 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 strains and types 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 (LoCascio, Ninonuevo et al. 2007, Marcobal, Barboza et al. 2010, Asakuma, Hatakeyama et al. 2011).

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, viruses to epithelial surfaces owing to HMOs are soluble receptor analogues of epithelial cell-surface carbohydrates (Bode 2006, Kunz and Rudloff 2006, Boehm and Stahl 2007). For example, the 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 (Ruiz-Palacios, Cervantes et al. 2003, Morrow, Ruiz-Palacios et al. 2004). The antiadhesive effects is also applied to certain protozoan parasites like

Entamoeba histolytica, which causes amoebic dysentery or amoebic liver abscess (Pritt and Clark 2008, Jantscher-Krenn, Lauwaet et al. 2012). Fucosylated HMOs also interact with the guanylyl cyclase receptor for the stable toxin of *E. coli*, thereby inhibiting toxin binding (Crane, Azar 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 et al. 2000, Kunz and Rudloff 2006, Schumacher, Bakowsky 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 (Bode 2012).

3. 3-Fucosyllactose (3-FL)

3.1. What is 3-FL?

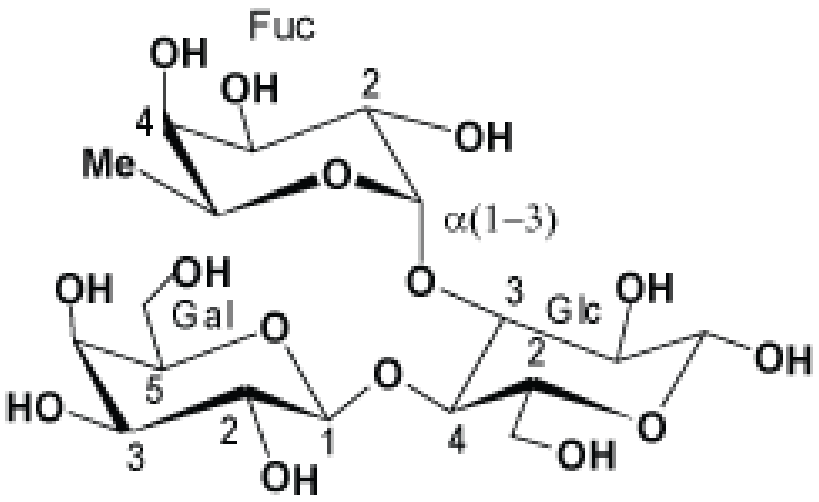
As mentioned above, about 200 kinds of HMOs have been found in human milk. Most HMOs are fucosylated, Fucosyloligosaccharides. 50~80% of the HMOs are fucosylated and 10~20% are sialylated (Kunz, Rudloff et al. 2000, Ninonuevo, Park et al. 2006, Bode 2012). They contain fucose and 3-8 sugars in size, in some case 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, 3-fucosyllactose (3-FL) is the second abundant fucosyloligosaccharide in human milk next to 2'-fucosyllactose (2-FL). (Table 2) (Chaturvedi, Warren et al. 2001, Castanys- Muñoz, Martin et al. 2013, Smilowitz, O'Sullivan et al. 2013).

3-FL is a trisaccharide composed of lactose and fucose (Fig. 2). Fucose binds to the glucose of lactose through α1-3 linkage different from 2-

FL which has α1-2 linkage of fucose and glactose. Although 3-FL and 2-FL have different structures, but it is reported that 3-FL inhibits adhesion of *Campylobacter jejuni*, enteropathogenic *E. coli*, *Salmonella enterica* serovar *fyris* and *Pseudomonas aeruginosa* to the intestinal human cell line Caco-2 as could be shown for 2-FL (Weichert 2013). Therefore, it is considered that 3-FL has all the good characteristic of 2-FL. So, 3-FL is emerging as an additive for functional food and therapeutic material.

L-Fucose



D-Galactose D-Glucose

Figure 2. Structure of 3-fucosyllactose (3-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 production of 3-FL

To produce 3-FL, activated sugar nucleotide GDP-$_L$-fucose is a key compound as a fucose donor (Fig. 3). In biological and microbial methods, two different metabolic pathways could be utilized for GDP-$_L$-fucose biosynthesis; the salvage pathway and *de novo* pathway.

For the salvage pathway, L-fucose kinase (EC 2.7.1.52) phosphorylates L-fucose at the expense of ATP. Then, L-fucose-1-phosphate guanylyltransferase (EC 2.7.7.30) combines L-fucose-2-phosphate with GTP to produce GDP-L-fucose (Becker and Lowe 2003).

In the *de novo* pathway, fructose-6-phosphate, produced in the glycolysis, is converted into mannose-1-phosphate by mannose-6-phosphate isomerase (ManA, E.C. 5.3.1.8) and phosphomannomutase (ManB, E.C. 5.4.2.8). Mannose-1-phosphate is combined with GTP by mannose-1-phosphate guanyltransferase (ManC, E.C. 2.7.7.22), resulting in the formation of GDP-D-mannose. GDP-D-mannose 4,6-dehydratase (Gmd, E.C. 4.2.1.27) then removed a water molecule from GDP-D-mannose. GDP-L-fucose synthase (WcaG, EC 1.1.1.271) catalyzes the reduction of the keto group at the C_4 position of GDP-4-keto-6-deoxymannose to synthesize GDP-$_L$-fucose, where reduced

NADPH is supplied as a reducing power (Lee et al. 2011).

Although GDP-L-fucose is synthesized through a short process in the salvage pathway, the cost of fucose, a starting material for GDP-L-fucose, is so expensive that the salvage pathway is not economically viable in production of 3-FL. On the other hand, the *de novo* pathway consists of multiple steps and the starting material is economical. In the previous research, the *de novo* pathway was constructed in *C. glutamicum* (Fig. 4) (Chin, Park et al. 2013). In this research, a 3-FL producing strain was constructed using the *de novo* pathway based on the previous research.

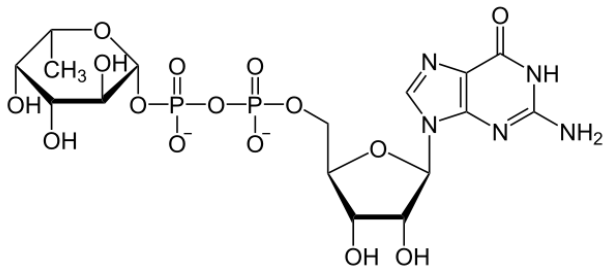


Figure 3. Structure of guanosine 5'- diphospho-β-L-fucose (GDP-L-fucose)

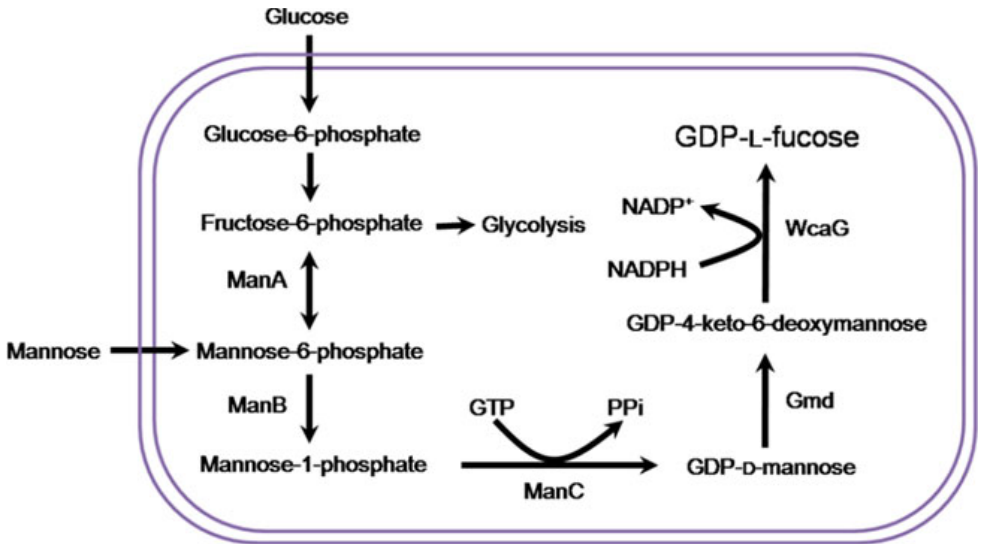


Figure 4. *De novo* biosynthetic pathway of GDP-L-fucose.

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).

3.3. Fucosyltransferse

3-FL is synthesized by fucosylation of lactose by α-1,3-fucosyltransferase. This enzyme catalyzes the transfer of fucose from GDP-$_L$-fucose to glucose 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, 3-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-1,3-Fucosyltransferases is a key enzyme in 3-FL production. This enzyme could catalyze the last step of 3-FL synthesis that is transferring fucose from GDP-$_{L}$-fucose to lactose, an acceptor in formation of 3-FL, in α-1,3 glycosidic linkage between fucose and the glucose residue of lactose. However, there are not many reports on α-1,3-fucosyltransferases so far. Results for production of 3-FL through a fed-batch fermentation were not published and only the result of producing 3-FL of 0.58 g/L through a flask fermentation was published (Yu et al. 2018).

4. *Corynebacterium glutamicum*

4.1. Characterization of *Corynebacterium glutamicum*

In the middle of 1950s, a bacterium was isolated which excretes L-glutamic acid extracellularly. Originally, this bacterium was named *Micrococcus glutamicus* (KINOSHITA, UDAKA et al. 1957). In 2000, the name changed to *Corynebacterium glutamicum* according to the taxonomy (Kumagai 2000). Since decades when *C. glutamicum* was discovered, *C. glutamicum* has played an important role in producing amino acids and nucleotides, such as amino acids L-valine, L-histidine, L-phenylalanine, L-tryptophan, L-glutamate and L-lysine (Ikeda 2003) and nucleotides 5'-inosinic acid (IMP), 5'- guanylic acid (GMP), 5'-xanthylic acid (XMP), in the industrial scale.

C. glutamicum is an aerobic or facultative anaerobic, Gram-positive, non-spore forming bacterium. It has a rod-shape, somewhat irregular ("coryneform") morphology (Fig .5) (Eggeling and Bott 2005). In the early stages, tailoring superior strains depended mostly on multiple rounds of random mutation and screening. Although this method has led to high productivities and yields, it is impossible to understand the reason for such an improvement. Recently, many genetic engineering

tools have been developed. 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, respectively.

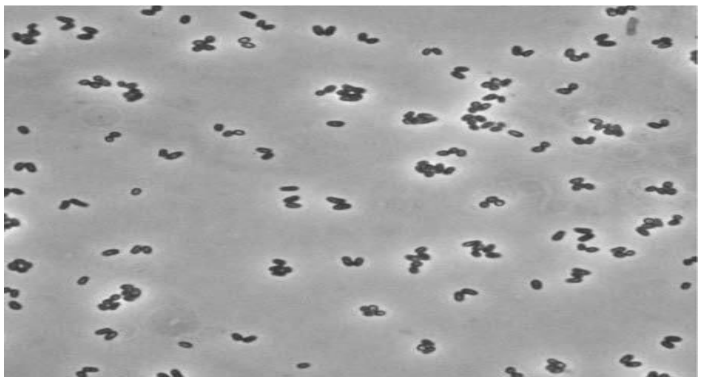
4.2. *C. glutamicum* as a 3-FL producer

Production of 2-FL through microorganisms have been performed both in *E. coli* and *C. glutamicum*. Highest titer of 2-FL produced in *E. coli* is 23.1 g/L (Chin et al. 2016) and in *C. glutamicum* is 25.5 g/L (Park, Thesis. 2018). However, there are no studies have been conducted on the production of 3-FL in *C. glutamicum*. In this study,

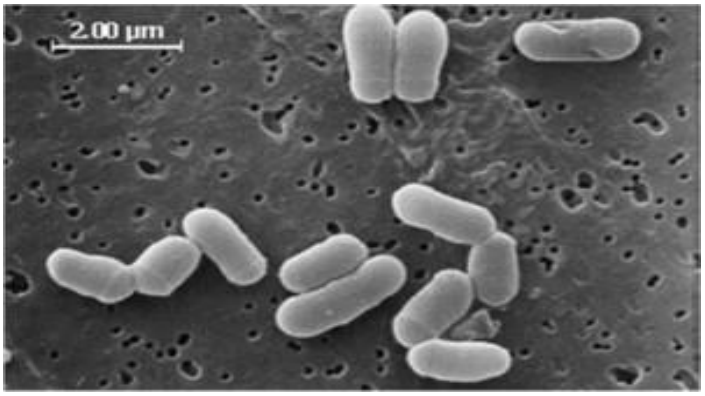
the production of 3-FL in *C. glutamicum* was conducted.

C. glutamicum has a high capacity for NADPH regeneration. 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 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 in 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). In addition, *C. glutamicum* is classified as a 'Generally Recognized As Safe' (GRAS) microorganism. Therefore, it is believed that *C. glutamicum* has 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 3-FL.



(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).

5. Research objectives

This research was focused on the development of an engineered *C. glutamicum* system for production of 3-FL. The specific objectives of this research were described as follows.

(1) To search for candidates for α-1,3-fucosyltransferase gene and construct an expression system of α-1,3-fucosyltransferase for production of 3-FL

(2) To improve expression levels of α-1,3-fucosyltransferase gene at transcription and translations steps

(3) To optimize the biosynthetic pathway of GDP-$_L$-fucose by introducing GDP-mannose 4,6-dehydratase from *Azospirillum brasilense*

(4) To enhance 3-FL production in engineered *C. glutamicum* by optimization of fed-batch fermentation

II. MATERIALS AND METHODS

1. Reagents and Enzymes

Experiments were performed using chemicals of reagent grade. Lactose, ethidium bromide, isoniazid, protocatechuic acid, biotin, cupric sulfate, sulfuric acid and antifoam 204 were got 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 got from Duksan (Ansan, Korea). Kanamycin monosulfate, IPTG and MOPS were purchased from Duchefa (Haarlem, The Netherlands). Calcium chloride, zinc sulfate, manganese(II) sulfate and Nickel(II) chloride were got from Junsei Chemical (Tokyo, Japan). Brain heart infusion, bacto-tryptone, yeast extract and bacto-agar were got from Difco (Detroit, MI., USA).

Restriction enzymes and calf intestinal alkaline phosphatase (CIP) were got from New England Biolabs (Beverly, MA, USA). T4 ligation mix and In-Fusion® HD cloning kit were got 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, Su-won, Korea) was used as host strain for 3-FL production.

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 by Prof. J. B. Park at Ewha Womans University. They were used as the backbone plasmids for the expression of heterologous genes or overexpression of innate genes.

Plasmid pVBCLE was previously constructed. It harbors the *lacYA* operon from *E. coli* and *manB, manC* genes from *C. glutamicum* and *blon_2204-2203* cluster from *Bifidobacterium infantis* under the *tac* promoter. Plasmid pEGWTA(CO) harbors the codon optimized α-1,3-fucosyltransferase gene(*azoT)* derived from *A. brasilense* and the *gmd-*

wcaG gene cluster derived from *E. coli* under the two *tac* promoter.

Plasmid pENGWTA(CO) harbors GDP-mannose 4,6-dehydratase gene(*noeL*) derived from *A. brasilense* in pEGWTA(CO).

Plasmids pVmBC and pEGW were previously constructed for overexpression of the genes for GDP-$_{L}$-fucose biosynthesis enzymes (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 AL	*C. glutamicum* ATCC 13032 harboring pVBCL and pEGWA	This study
BCGW TALE(CO)	*C. glutamicum* ATCC 13032 harboring pVBCLE and pEGWTA(CO)	This study
BCNGW TALE(CO)	*C. glutamicum* ATCC 13032 harboring pVBCLE and pENGWTA(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)
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*	(Lee, Thesis, 2018)
pEGWA	pEGW + *azoT*	This study
pEGWTA(CO)	pEGW + tac promoter + CO*azoT*	This study
pENGWTA(CO)	pEGWTA(CO) + *noeL*	This study

Table 4. List of primers used in this study

Name	Sequence
F_inf_sacI_RBS_a*zoT* (pEGWA)	GCTTTCGGGGGTAA**GAGCTC** *AAGGAGATATACA* ATGCTCGATCAGCGGACAAGC
R_inf_sacI_a*zoT* (pEGWA, pEGWTA)	CGGCCAGTGAATTC**GAGCTC** TTACAGCCGGCTCTCGATCC
F_BamH1_RBS_*azoT*(CO) (pVA(CO) for tac_RBS_*azoT*(CO))	CGC**GGATCC** *AAGGAGATATACA* ATGCTCGATCAACGTACGAGC
R_Kpn1_*azoT*(CO) (pVA(CO) for tac_RBS_*azoT*(CO))	CGG**GGTACC** TTATAAGCGGGATTCGATCCAGTC
F_inf_Sac1_Tac_RBS_*azoT* (pEGWTA, pEGWTA(CO))	GCTTTCGGGGGTAA**GAGCTC** TCAGGCAGCCATCGGAAG
R_inf_sacI_CO*azoT* (pEGWTA(CO))	CGGCCAGTGAATTC**GAGCTC** TTATAAGCGGGATTCGATCCAGTC
F_inf_KpnI_*noeL* (pENGWTA(CO))	CTAGAGGATCCCCG**GGTACC** TTGGCGCGGATCGCATTGATCTTCG
R_inf_KpnI_*noeL* (pENGWTA(CO))	TTGTATATCTCCTT**GGTACC** TCAAGGTTGCGGCAGGGC

The italic sequences present the RBS (ribosome binding site) and spacer.

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

Figure 6. Genetic maps of plasmids pVBCLE and pEGWTA(CO)

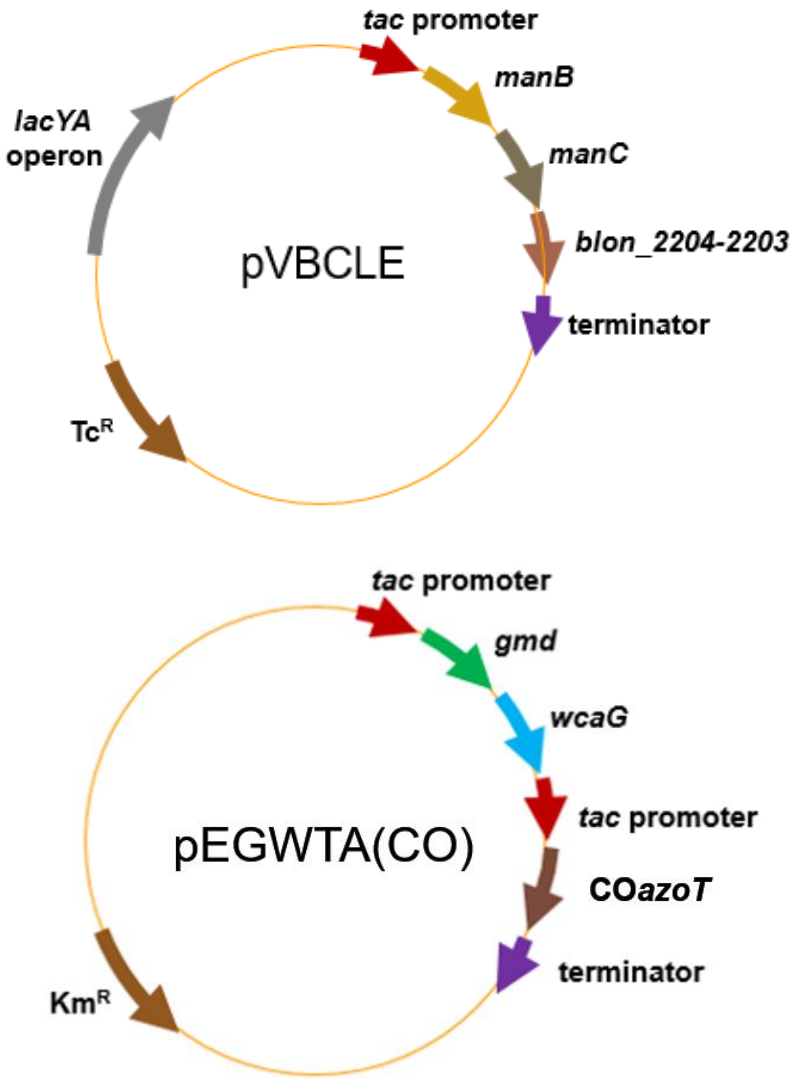
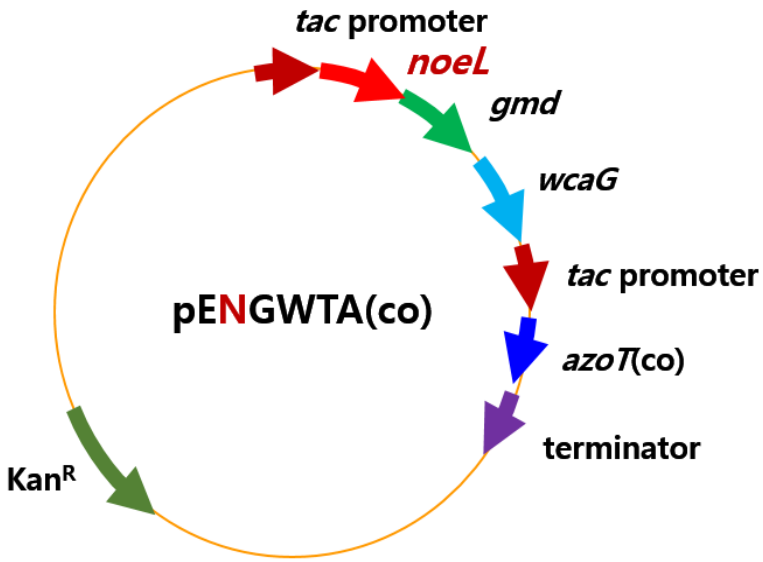


Figure 7. Genetic maps of plasmid pENGWTA(CO)



3. DNA Manipulation and Transformation

3.1. Preparation of DNA

Mini-scale preparation of DNA was performed by using DNA-spin™ Plasmid DNA Purification Kit from iNtRON (Sung-nam, Korea). Preparation of *C. glutamicum* and *A. brasilense* chromosomal DNAs for PCR template was performed by using DNeasy Blood & Tissue Kit from QIAGEN (Düsseldorf, Germany). 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 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 gene cloning 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 for 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 *Sac*I, *BamH*I and *Kpn*I and calf intestinal alkaline phosphatase (CIP) were got from New England Biolabs (Beverly, USA). The Ligation Mix and In-Fusion® HD cloning kit from Takara (Otsu, Japan) were used for ligation of PCR products and plasmids.

3.4. Transformation of *E. coli*

Transformation of *E. coli* was performed 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 new 50 mL LB medium and cultured for OD_{600} = 0.5. Cells were harvested by centrifugation at 6,000 rpm for 5 min at 4℃ and 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 transformed to *E. coli* competent cell through heat-shock at 42℃ for 45 sec, and 1 mL of LB medium was added to the test tubes and incubated at 37℃ for 1 hour to allow the *E. coli* to express the antibiotic resistance. Transformed *E. coli* 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). Incubated at 30℃, overnight cultures of the *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℃, 250 rpm cultured for OD_{600} = 1.75. Cells were 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. 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 heat-shock process. Then, the transformant was incubated for 1 hour at 30°C, 250 rpm 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℃ for 2 days.

4. Media and Culture conditions

4.1. 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 culture of *E. coli*. Brain heart infusion (BHI) (Difco, USA) containing with appropriate antibiotics (25 μg/mL kanamycin, 5 μg/mL tetracycline) was used for culture 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-morpholinopropanesulfonic 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).

4.2. 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℃ and 250 rpm in a shaking incubator (Vision, Korea). Recombinant *C. glutamicum* containing single vector (pVWEx2 derived plasmid) was added 5 μg/mL tetracycline and the case of dual vector system (pEKEx2 and pVWEx2 derived plasmids) was added 25 μg/mL kanamycin and 5 μg/mL tetracycline.

For the flask fermentation, 1 mL of BHI culture grown overnight was inoculated in a 500 mL baffled flask (NALGENE, USA) with 100 mL CGXII (4% glucose) media and grown at 30℃ and 250 rpm. The appropriate antibiotics were added. As an optical density reached at OD_{600} = 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 3-FL production.

A fed-batch fermentation was performed in a bioreactor of 2.5 L jar (Kobiotech, Korea) with 1 L initial volume of CGXII medium containing 4% glucose and antibiotics of the same concentration as flask culture. The 100 ml pre-culture was performed with in a 500 mL baffled flask with 100 ml BHI with the same method with the flask fermentation. And then, the culture solution was inoculated into the bioreactor for an initial OD_{600} = 1. 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 6.98 ~ 7.02 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. When 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 a substrate for α-1,3-fucosyltransferase.

5. Fermentation analysis

5.1. Dry cell weight

Cell growth was measured by monitoring the optical density of culture broth. It was measured absorbance at 600nm 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 (DCW) by using the following conversion equation:

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

5.2. Quantification of metabolites concentration

The concentrations of glucose, lactose, lactate, acetate and 3-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℃. 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.

III. RESULTS AND DISCUSSIONS

1. Introduction of α-1,3-fucosyltransferase gene from *Azospirillum brasilense* (*azoT*)

1.1. Construction of the strain with α-1,3-fucosyltransferase gene

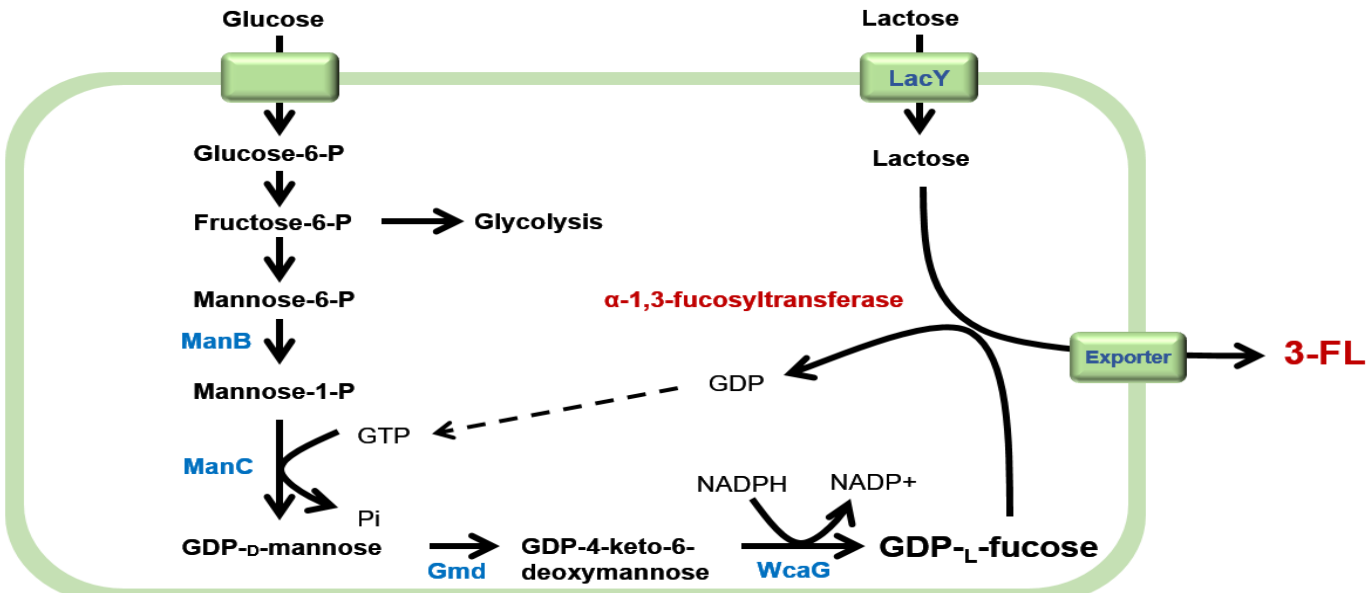
To produce 3-FL in engineered *C. glutamicum*, GDP-$_L$-fucose and lactose are required for substrates. And also, activity of α-1,3-fucosyltransferase are required. The strain capable of biosynthesizing GDP-$_L$-fucose was previously constructed (Chin, Park et al. 2013). Plasmid pVBCL was previously constructed for expression of lactose permease gene from *E. coli* to improve the ability to import lactose into the cell (Jo, Thesis. 2016) and pVBCLE was previously constructed for expression of the exporter gene from *Bifidobacterium infantis* to improve the ability to export 3-FL into the media (Lee, Thesis. 2018). In this study, the α-1,3-fucosyltransferase gene was searched and introduced to *C. glutamicum* to produce 3-FL through the pathway as shown in the (Fig. 7).

In order to identify the best α-1,3-fucosyltransferase gene for production of 3-FL in *C. glutamicum*, it was tried to search for α-1,3-

fucosyltransferase gene from various organisms could produce 3-FL. The candidates for α-1,3-fucosyltransferase gene were selected on the basis of amino acid similarity in the carbohydrate-active enzyme database (CAZy). 7 candidates are as follows (Table 5).

To introduce α-1,3-fucosyltransferase to *C. glutamicum*, each of the amplified α-1,3-fucosyltransferase gene from various organisms was digested with *SacI* and ligated into plasmid pEGW. The constructed plasmids were identified by the restriction enzyme (*SacI*) and DNA sequencing (SolGent, Daejeon, Korea). These plasmids were transformed in *C. glutamicum* by electroporation with plasmid pVBCL to provide an ability to produce 3-FL.

Figure 8. Biosynthetic pathway of 3-FL from glucose and lactose in engineered *C. glutamicum*.



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

Table 5. Candidates for α-1,3-fucosyltransferase

Organisms	Protein name	Gene bank
Helicobacter pylori ATCC 26695	pylT	CP010436.1
Helicobacter pylori ATCC 26695	pylT(E) (protein engineered)	-
Helicobacter pylori NCTC 11637	fucT	AF008596.1
Helicobacter pylori NCTC 11637	fucT(E) (protein engineered)	-
Bacteroides fragilis ATCC 25285	fra1	CR626927.1
Azospirillum brasilense ATCC 29145	azoT	CP007794.1
Coraliomargarita akajimensis KCTC 12865	cakT	CP001998.1

1.2. Flask fermentation of the strain expressing α-1,3-fucosyltransferase genes

To confirm 3-FL production in engineered *C. glutamicum*, flask fermentation with the strains harboring the plasmid pVBCL and pEGW with α-1,3-fucosyltransferase genes from various organisms was carried out (Table 6). Flask fermentations were performed in CGXII medium containing 40 g/L glucose. When optical density reached OD_{600} of 0.8, 0.1 mM IPTG was added for gene expression and 10 g/L lactose was added for 3-FL production.

Among those strains, the BCGW AL strain with the *azoT* gene from *A. brasilense* ATCC 29145 produced the highest 3-FL titer in a flask fermentation (Table 6). This strain produced 3-FL titer of 390 mg/L, which is 2.3 fold higher than the second best strain, yield of 0.32 mole 3-FL/mole lactose and productivity of 5.5mg/L/h (Fig.8, Table 7). This strain was used for subsequent experiments.

Table 6. Summary of flask fermentation of strains with α-1,3-fucosyltransferase genes from various organisms

Organisms	Protein name	3-FL titer (mg/L)
Helicobacter pylori ATCC 26695	pylT	0.15
Helicobacter pylori ATCC 26695	pylT(E) (protein engineered)	0.13
Helicobacter pylori NCTC 11637	fucT	0.17
Helicobacter pylori NCTC 11637	fucT(E) (protein engineered)	0.16
Bacteroides fragilis ATCC 25285	fra1	N.D.
Azospirillum brasilense ATCC 29145	azoT	0.39
Coraliomargarita akajimensis KCTC 12865	cakT	N.D.

Figure 9. Flask fermentation of BCGW AL. When OD_{600} reached at 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 3-FL

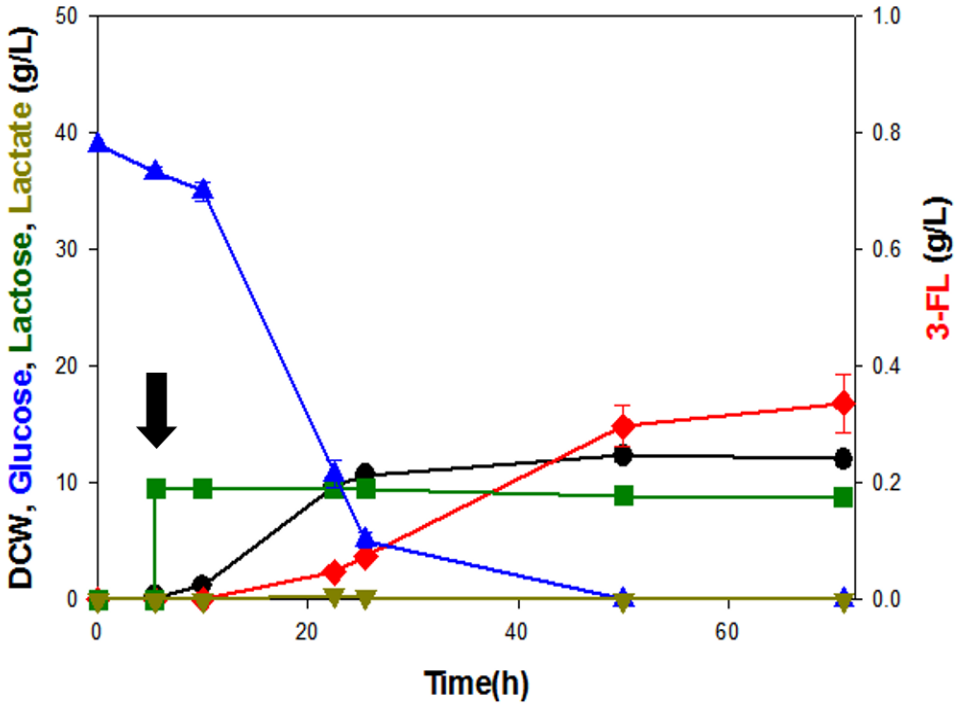


Table 7. Summary of flask fermentation of BCGW AL.

Strain	Maximum dry cell weight (g/L)	Maximum 3-FL Titer (g/L)	Yield (mole 3-FL / mole lactose)	Productivity (g/L/h)
BCGW AL	13.1	390	0.32	5.5
*BCGW FL	13.9	170	0.2	2.4

* The second best strain expressing *fucT* gene

1.3. Fed-batch fermentation of the strain expressing *azoT*

For the high cell density culture and high production of 3-FL, a fed-batch fermentation of the BCGW AL strain was performed in a 2.5 L bioreactor with 1 L CGXII medium containing 40 g/L glucose. To prepare the cells for inoculation to the main culture, a pre-culture was performed in a baffled flask with BHI medium. After complete consumption of initial 40 g/L glucose, 800 g/L glucose solution was fed continuously. 3-FL began to be produced after lactose addition and IPTG induction for expressing the genes associated with 3-FL biosynthesis.

During 144 hour of culture, this strain produced 3-FL titer of 3.23 g/L, yield of 0.12 mole 3-FL/mole lactose and productivity of 0.03 g/L/h. After IPTG induction, production of 3-FL was increased steadily while cell growth rate was decreased. This showed production of 3-FL was not associated with cell growth. Lactate, recognized as a by-product, did not accumulate (Fig 9, Table 8).

Figure 10. Fed-batch fermentation of BCGW AL. After depletion of initial carbon source (40 g/L glucose), IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 3-FL

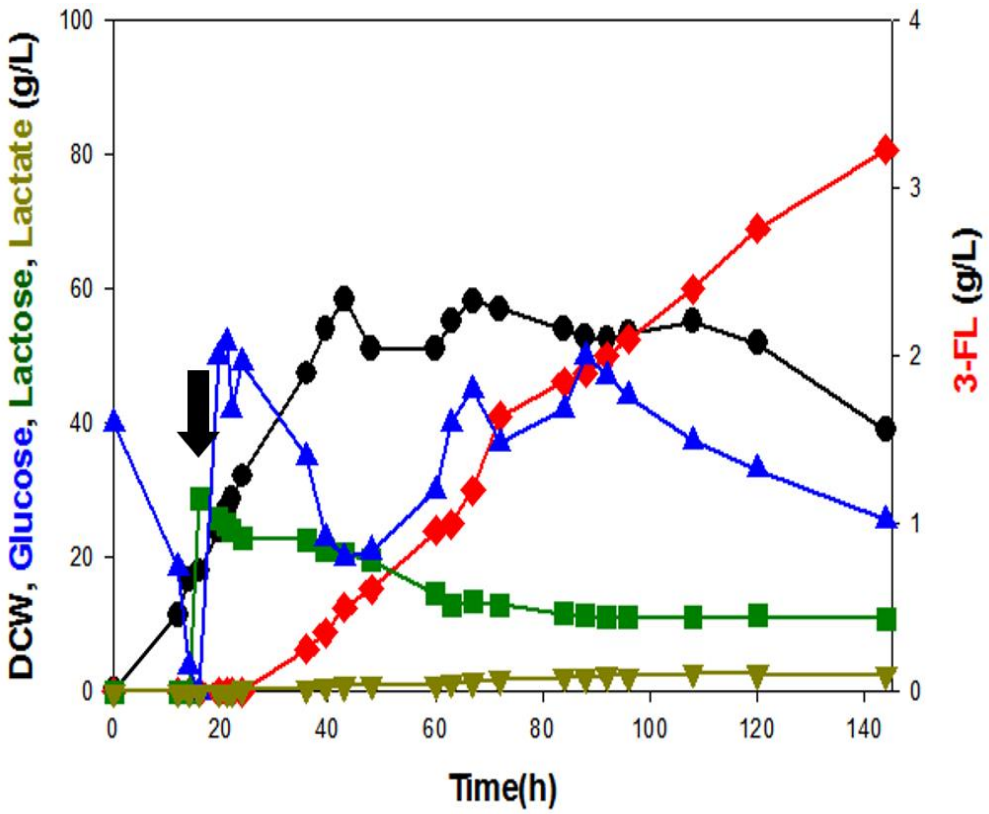


Table 8. Summary of fed-batch fermentation of BCGW AL.

Strain	Maximum dry cell weight (g/L)	Maximum 3-FL Titer (g/L)	Yield (mole 3-FL / mole lactose)	Productivity (g/L/h)
BCGW AL	58.2	3.23	0.12	0.03

2. Enhanced expression of *azoT*

2.1. Conversion of polycistronic into monocistronic expression of *azoT*

The expression system of the *azoT* gene in the strain BCGW AL is the polycistronic expression system by using episomal plasmid pEWGA, which transcripts the *gmd*, *wcaG* and *azoT* genes all together under tac promoter (Fig. 10-A).

To enhance the expression of the *azoT* gene at a transcription level, the polycistronic expression system of the *azoT* gene was converted into a monocistronic expression system through addition of the tac promoter in front of the *azoT* gene (Fig. 10-B)

2.2 Codon optimization of *azoT*

Heterologous protein production in *C. glutamicum* may be decreased due to codon bias. Codon usage bias is differences in the frequency of occurrence of synonymous codons in coding DNA (Ermolaeva 2001). Enhanced heterologous expression of genes may be obtained by replacing natural codons to more commonly used codons.

To enhance the expression of the *azoT* gene at a translation level, the *azoT* gene from *A. brasilense* was codon optimized. The codon optimization of the *azoT* gene was conducted by BIONEER (Daejeon, Korea). As a result, there were 16% differences between sequences of the *azoT* and codon optimized *azoT* (COazoT) genes (Fig. 11).

The *azoT* gene sequences are as follows.

ATGCTCGATCAGCGGACAAGCGCGTTTCTTGAGGAATTCC
TGGCGAAGCCGGGCGGCGATCCCGAGCGGCTCGACCGCTTC
CTGCTGCACGGCCCGTACCGCGGCCGGCGCGGCGGCAAACC
GCGGCTGAAGCTGGCCTTCCACGACTTCTGGCCGGAGTTCG
ACAAGGGCACGAACTTCTTCATCGAGATCCTGTCCAGCCGC
TTCGACCTGTCGGTGGTCGAGGACGACAGCGACCTCGCCAT
CGTGTCGGTCTTCGGCGGGCGGCACCGCGAGGCGCGCAGCC
GCCGCACCCTGTTCTTCACCGGGGAGAACGTGCGCCCGCCG
TTGGACGGCTTCGACATGGCGGTGTCCTTCGACCGCGTCGA
CGACCCGCGCCATTACCGCCTGCCGCTCTACGTCATGCACG
CCTACGAGCACATGCGGGAGGGGGCGGTGCCGCATTTCTGT
TCGCCGGTCCTGCCGCCGGTGCCGCCGACGCGGGCGGCCTT

CGCGGAGCGCGGCTTCTGCGCCTTCCTCTACAAGAACCCGA

ACGGGGAGCGCCGCAACCGCTTCTTCCCGGTGCTGGACGGG

CGGCGGCGCGTCGATTCGGTGGGCTGGCACCTGAACAACAC

CGGCAGCGTCGTCAAGATGGGCTGGCTGTCGAAGATCCGCG

TCTTCGAACGCTACCGTTTCGCCTTCGCCTTCGAGAACGCCA

GCCATCCCGGCTATCTGACGGAAAAGATCCTGGACGTCTTC

CAGGCCGGGGCGGTGCCGCTCTATTGGGGTGATCCCGACCT

GGAGCGCGAGGTGGCGGTCGGCAGCTTCATCGACGTGTCGC

GCTTCGCCACGGACGAGGAGGCGGTGGACCACATCCTTGCG

GTGGACGACGATTACGACGCCTATTGCGCCCACCGCGCCGT

GGCGCCCTTCCTGGGGACGGAGGAGTTTTATTTCGACGCCT

ACCGCCTCGCCGACTGGATCGAGAGCCGGCTGTAA

The CO*azoT* gene sequences are as follows.

ATGCTCGATCAACGTACGAGCGCATTCTTGGAAGAATTTC

TGGCGAAACCGGGAGGTGATCCCGAGCGACTCGACCGCTTC

TTACTTCACGGCCCGTACCGTGGCCGGCGGGGCGGCAAGCC

GCGATTGAAACTTGCGTTCCACGATTTCTGGCCAGAGTTTGA

CAAGGGAACCAATTTTTTCATCGAAATTCTGTCCTCCCGCTT

CGATCTGTCGGTAGTGGAAGACGATTCTGACCTCGCCATTG

TGTCTGTCTTCGGCGGGCGGCACCGCGAGGCTCGCTCACGC

CGCACCCTCTTCTTCACCGGAGAGAACGTTCGCCCACCGCTT

GACGGTTTCGATATGGCTGTGTCCTTCGACCGCGTTGACGAT

CCACGCCATTACAGGCTGCCACTCTACGTCATGCACGCCTA

CGAGCACATGCGAGAGGGCGCGGTGCCACATTTTTGTTCAC

CTGTCCTGCCACCAGTGCCTCCGACAAGAGCTGCTTTTGCAG

AACGTGGATTTTGCGCCTTTTTGTACAAGAATCCTAACGGTG

AAAGGCGTAACCGCTTTTTCCCGGTGCTGGACGGTCGTCGA

CGTGTTGATTCTGTGGGCTGGCATCTAAACAATACCGGTTCC

GTCGTGAAAATGGGATGGTTGTCGAAGATCCGTGTCTTCGA

ACGCTACCGTTTCGCCTTCGCATTCGAGAACGCTAGCCATCC

CGGTTATCTTACTGAAAAGATCCTGGACGTCTTCCAGGCCG

GCGCGGTGCCTTTGTATTGGGGTGATCCCGATCTAGAACGC

GAAGTTGCAGTCGGCAGCTTTATCGACGTGAGTCGCTTCGC

AACTGATGAGGAAGCTGTGGACCACATTCTTGCAGTGGATG

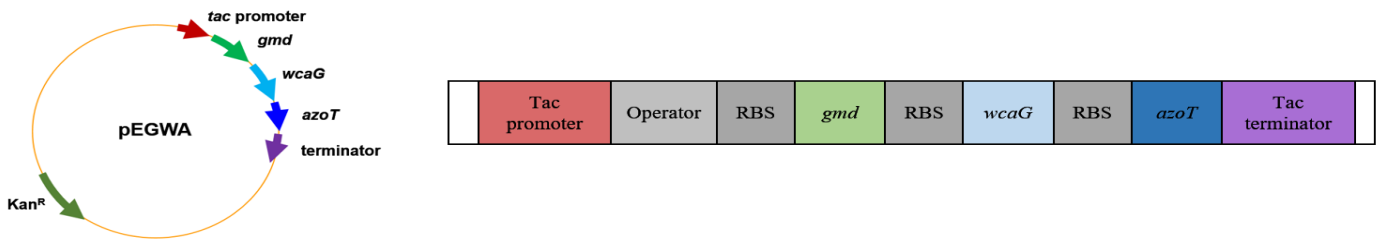
ATGATTACGACGCCTACTGCGCACACCGCGCAGTTGCGCCT

TTTCTGGGGACCGAGGAATTTTATTTCGATGCCTACCGCCTC

GCTGACTGGATCGAATCCCGCTTATAA

Figure 11. Conversion of polycistronic into monocistronic expression of *azoT* gene (A) Polycistronic expression of genes. (B) Monocistronic expression of the *azoT* gene.

(A) Polycistronic expression



(B) Monocistronic expression of *azoT* gene

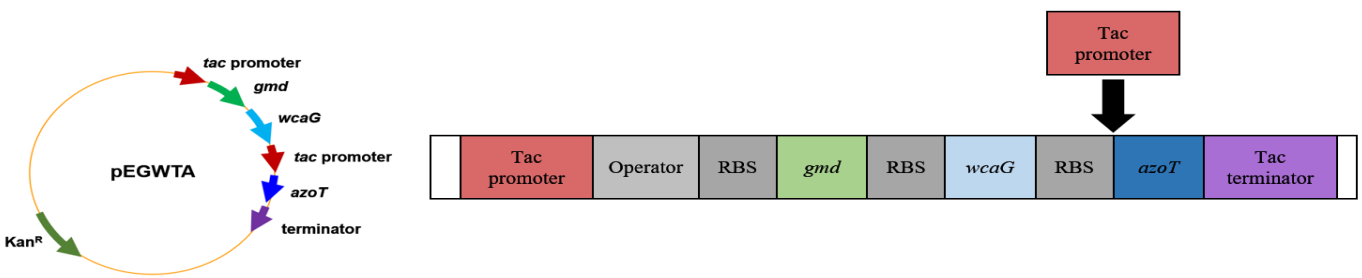
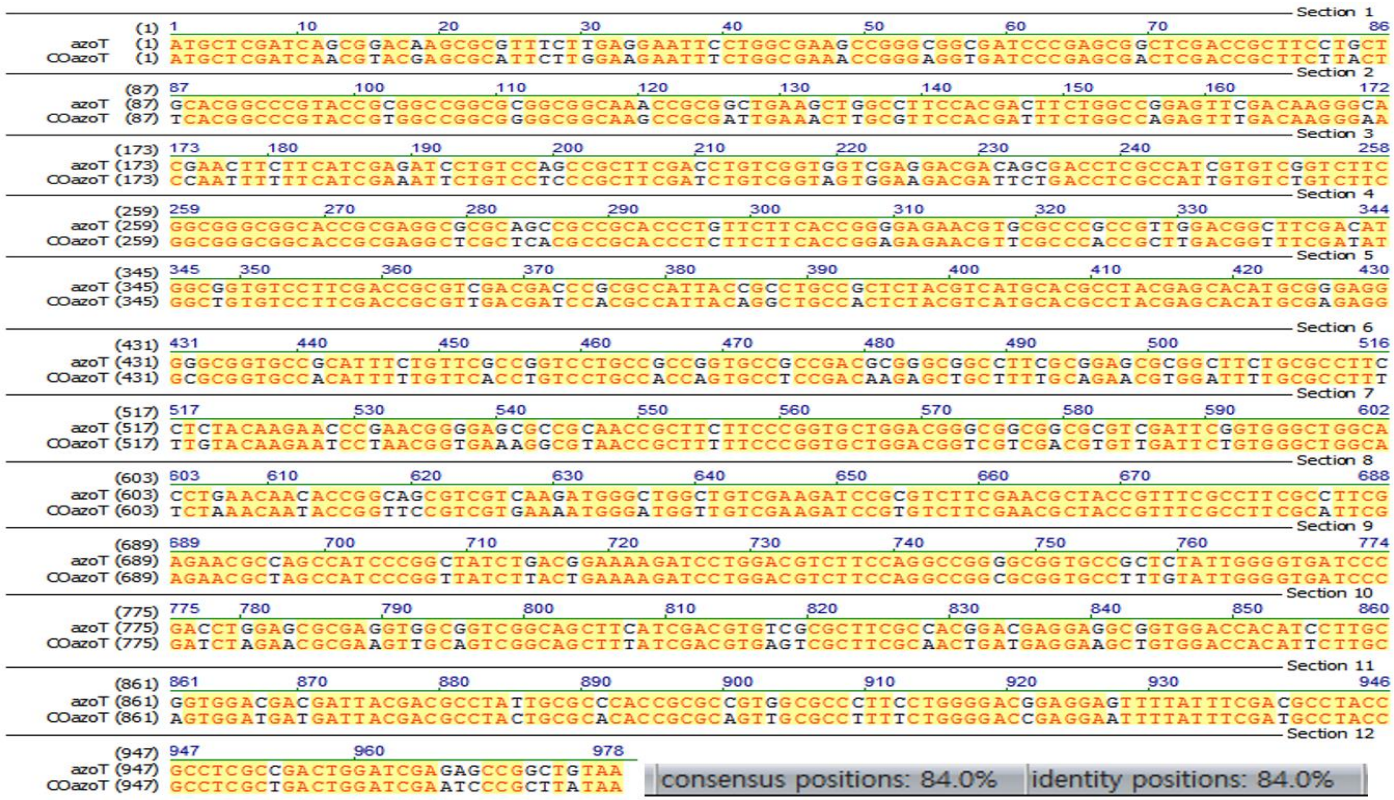


Figure 12. Differences between sequences of *azoT* and CO*azoT* gene.



2.3 Flask and fed-batch fermentation of the strain with CO*azoT* under monocistronic expression

To construct the strain with CO*azoT* gene under monocistronic expression, plasmid pEGWTA(CO) was constructed. First, amplified CO*azoT* gene from pBHA(CO*azoT*) was digested with *BamH*I and *Kpn*I and ligated into the plasmid pVWEx2 to construct plasmid pVA(CO). Next, amplified CO*azoT* gene with the *tac* promoter from the plasmid pVA(CO) was digested with *Sac*I and ligated into the plasmid pEGW to construct plasmid pEGWTA(CO). The constructed plasmid were proved by the restriction enzyme digestion and DNA sequencing. And then, the plasmids pEGWTA(CO) and pVBCLE were transformed in *C. glutamicum* ATCC 13032 by electroporation to produce 3-FL.

To confirm the effect of the monocistronic expression of the CO*azoT* gene for the 3-FL production, flask fermentation of the BCGW TALE(CO) strain with the *tac* promoter in front of the CO*azoT* gene was carried out. Flask fermentation was performed in CGXII medium containing 40 g/L glucose. When optical density reached OD_{600} =0.8, 0.1 mM IPTG was added for gene expression and 10 g/L lactose was added for 3-FL production. During 77 hours of fermentation, this strain

produced 3-FL titer of 590 mg/L, yield of 0.29 mole 3-FL/mole lactose and productivity of 7.7mg/L/h (Fig.12, Table 9).

Figure 13. Flask fermentation of BCGW TALE(CO). When OD_{600} reached at 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 3-FL

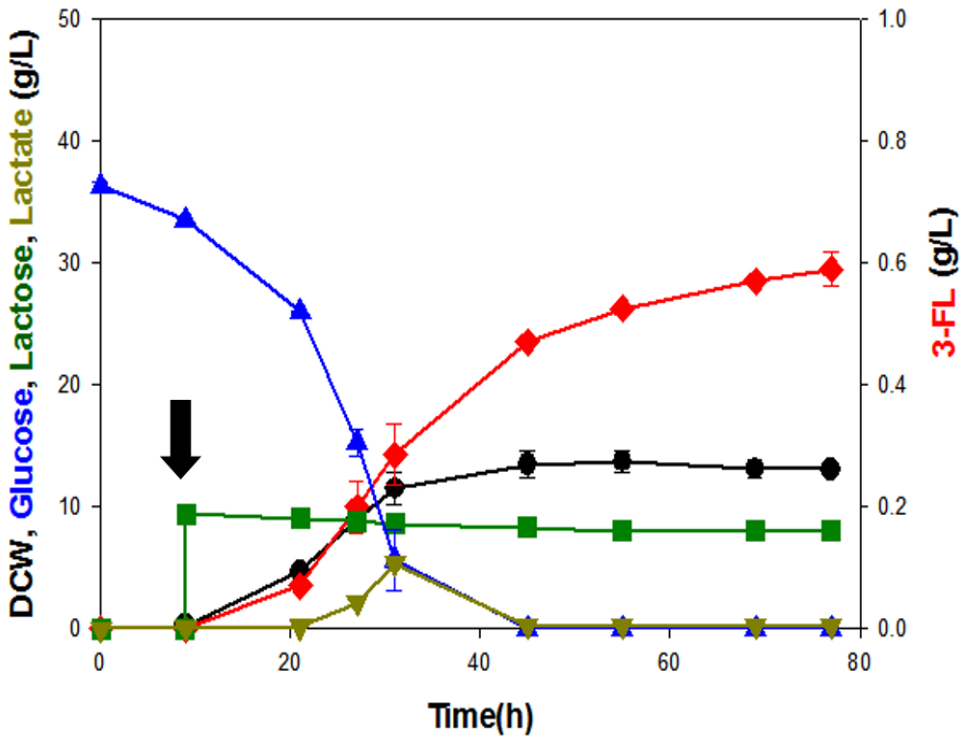


Table 9. Summary of flask fermentation of BCGW TALE(CO)

Strain	Maximum dry cell weight (g/L)	Maximum 3-FL Titer (mg/L)	Yield (mole 3-FL / mole lactose)	Productivity (mg/L/h)
BCGW TALE(CO)	13.7	590	0.29	7.7

For high cell density culture and mass production of 3-FL, a fed-batch fermentation of the BCGW TALE(CO) strain was performed in a 2.5 L bioreactor with 1 L CGXII medium containing 40 g/L glucose. The fermentation methods are same as those of the previous fed-batch fermentation of BCGW AL strain.

During 155 hours of culture, this strain produced 3-FL titer of 4.00 g/L, yield of 0.20 mole 3-FL/mole lactose and productivity of 0.03 g/L/h. The 3-FL titer of BCGW TALE(CO) is 25% higher than the previous BCGW AL strain. (Fig 13, Table 10)

Figure 14. Fed-batch fermentation of the BCGW TALE(CO). After depletion of initial carbon source (40 g/L glucose), IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 3-FL

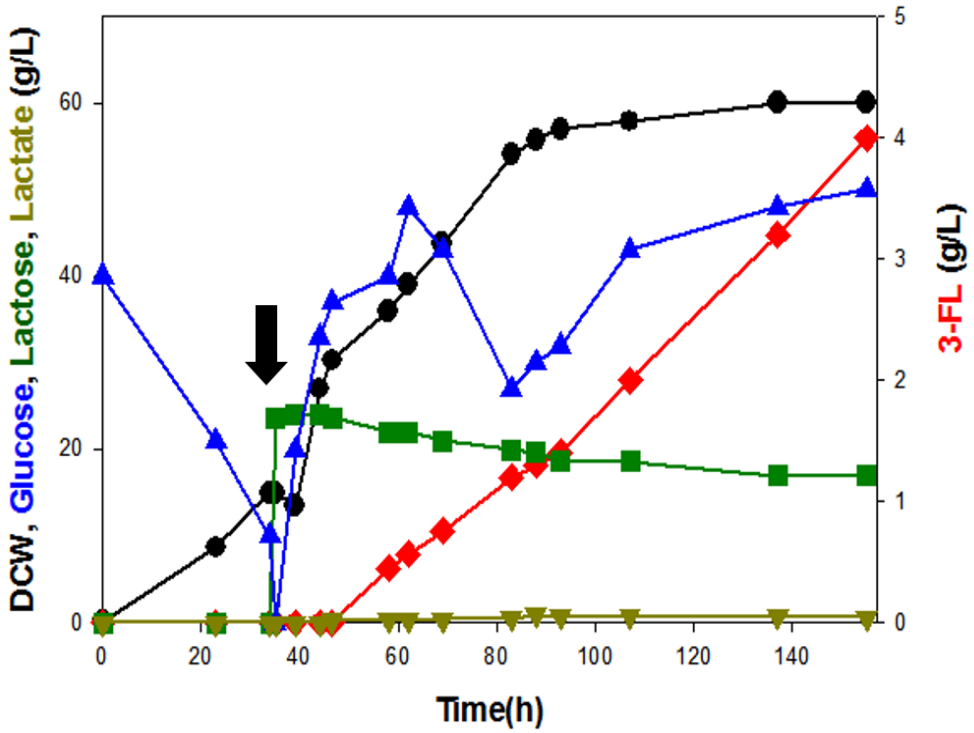


Table 10. Summary of fed-batch fermentation of BCGW TALE(CO).

Strain	Maximum dry cell weight (g/L)	Maximum 3-FL Titer (g/L)	Yield (mole 3-FL / mole lactose)	Productivity (g/L/h)
BCGW TALE(CO)	60	4.00	0.20	0.03

3. Introduction of GDP-mannose 4,6-dehydratase gene from *A. brasilense* (*noeL*)

3.1. Construction of strain expressing *noeL*

A. brasilense is a well studied nitrogen-fixing bacterium. It has the same optimal growth conditions as *C. glutamicum* such as pH 7.0, 30 and aerobic atmosphere condition. So, it has an advantage in expressing the genes from *A. brasilense* in *C. glutamicum*.

To produce 3-FL in engineered *C. glutamicum*, the GDP-$_{L}$-fucose biosynthetic pathway is essential and this pathway is constructed previously (Chin, Park et al. 2013). But in this pathway, the genes for the biosynthesis of GDP-$_{L}$-fucose are from *E. coli*, which have different optimal growth conditions. So, in this study, the GDP-mannose 4,6-dehydratase gene from *A. brasilense* was introduced additionally to *C. glutamicum* to improve a GDP-$_{L}$-fucose biosynthetic ability for 3-FL production.

To introduce the GDP-mannose 4,6-dehydratase gene (*noeL*) to *C. glutamicum*, plasmid pENGWTA(CO) was constructed. The amplified *noeL* gene from *A. brasilense* colony was digested with *Kpn*I and ligated into the plasmid pEGWTA(CO). The constructed plasmid were

proved by the restriction enzyme digestion and DNA sequencing. And then, the plasmids pENGWTA(CO) and pVBCLE were transformed in *C. glutamicum ATCC* 13032 by electroporation to produce 3-FL.

3.2. Flask and fed-batch fermentation of the strain expressing *noeL*

To confirm the effect of the *noeL* gene expression on the 3-FL production, flask fermentation of the BCNGW TALE(CO) strain was carried out. Flask fermentation was performed in CGXII medium containing 40 g/L glucose. When optical density reached OD_{600} of 0.8, 0.1 mM IPTG was added for gene expression and 10 g/L lactose was added for 3-FL production. During 72 hours of fermentation, this strain produced 3-FL titer of 1.33 g/L, yield of 0.35 mole 3-FL/mole lactose and productivity of 0.02 g/L/h (Fig.14, Table 11)

For the high cell density culture and high production of 3-FL, a fed-batch fermentation of the BCNGW TALE(CO) strain was performed in a 2.5 L bioreactor with 1 L CGXII medium containing 40 g/L glucose. The fermentation methods are the same as those of the previous fed-batch fermentation of the BCGW AL strain.

During 156 hours of culture, this strain produced 3-FL titer of 10.0 g/L, yield of 0.53 mole 3-FL/mole lactose and productivity of 0.06 g/L/h. The 3-FL titer of BCNGW TALE(CO) is a 3.1 fold higher than the previous BCGW AL strain. (Fig 15, Table 12).

Figure 15. Flask fermentation of BCNGW TALE(CO). When OD_{600} reached at 0.8, IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 3-FL

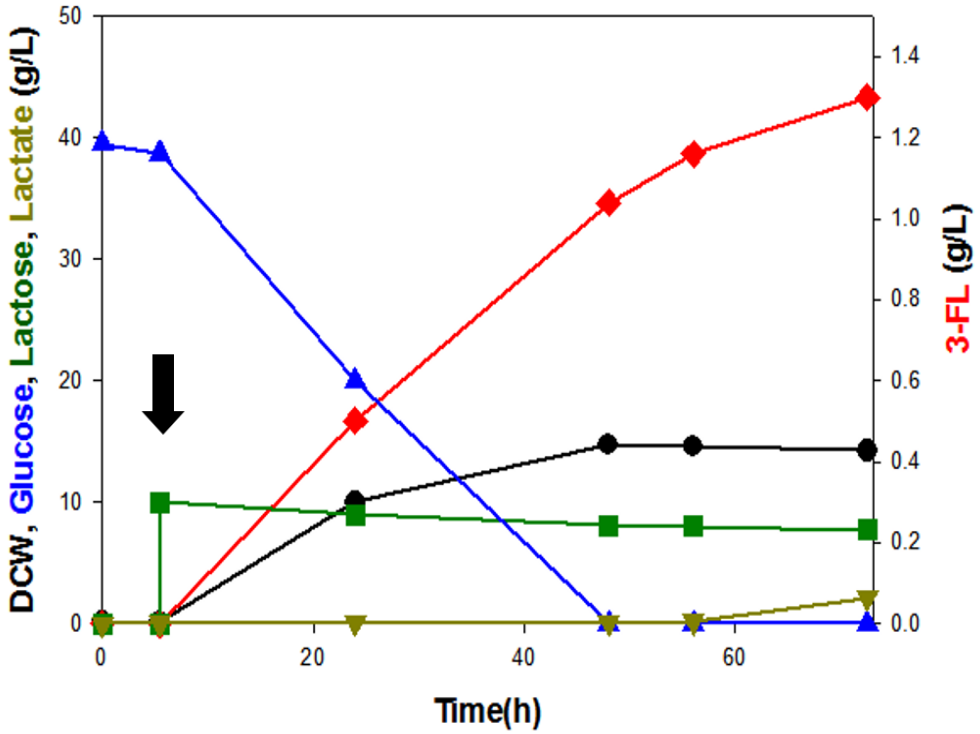


Table 11. Summary of flask fermentation of BCNGW TALE(CO).

Strain	Maximum dry cell weight (g/L)	Maximum 3-FL Titer (g/L)	Yield (mole 3-FL / mole lactose)	Productivity (g/L/h)
BCNGW TALE(CO)	14.3	1.33	0.35	0.02

Figure 16. Fed-batch fermentation of BCNGW TALE(CO). After depletion of initial carbon source (40 g/L glucose), IPTG and lactose were added (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 3-FL

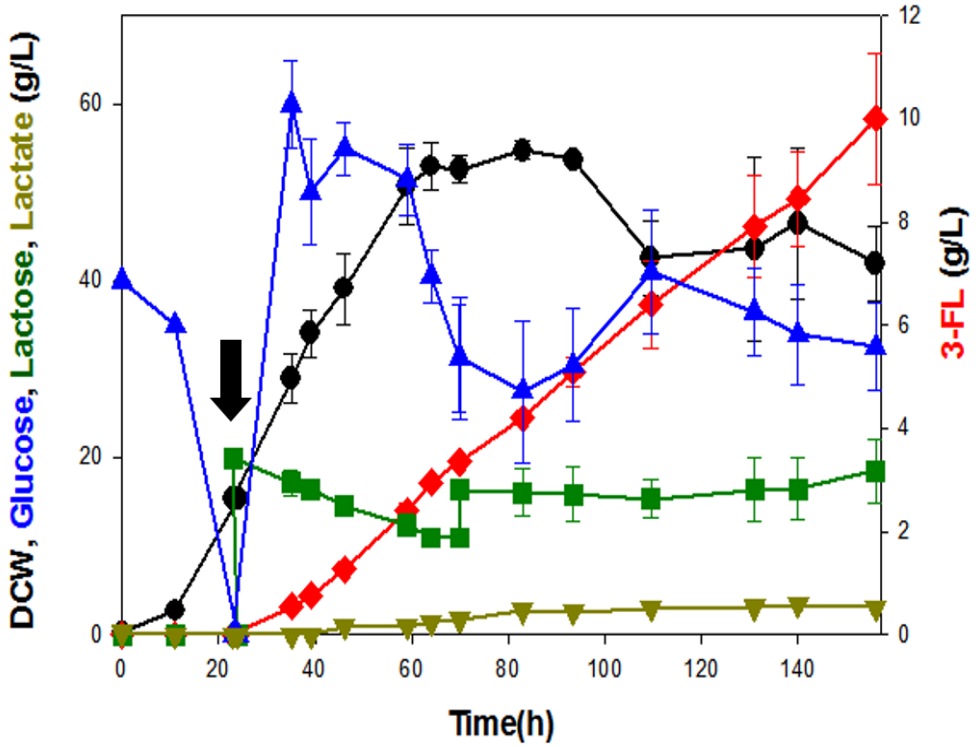


Table 12. Summary of fed-batch fermentation of BCNGW TALE(CO).

Strain	Maximum dry cell weight (g/L)	Maximum 3-FL Titer (g/L)	Yield (mole 3-FL / mole lactose)	Productivity (g/L/h)
BCNGW TALE(CO)	55.5	10.0	0.53	0.06

4. Optimization of fed-batch fermentation process

To reduce the lag phase at the beginning of the fed-batch fermentation and to increase the production of 3-FL, the optimization of fed-batch fermentation process was conducted.

First, the medium at the pre culture was replaced. Previously, BHI medium was used in the pre culture for cell growth. In this study, the medium for the pre culture was replaced to CGXII medium, which is used for a main culture. Through the replacement of medium, the same environment as main culture was realized in the pre culture to adapt cells to the main culture. The cells were cultured up in CGXII medium and inoculated in to the main culture for $OD_{600} = 4$.

Next, the timing of the IPTG induction was changed. By adding IPTG in the pre culture, cells were grown to be as suitable for production as 3-FL before inoculation to main culture.

Through these changes, 140 hours of fermentation of the BCNGW TALE(CO) strain produced 3-FL titer of 17.1 g/L, yield of 0.65 mole 3-FL/mole lactose and productivity of 0.11 g/L/h. The 3-FL titer of fermentation through optimization process is 70% higher than the previous fermentation process with same strain and it is 5.3 fold higher than the previous BCGW AL strain. (Fig 16, Table 13).

Figure 17. Optimized fed-batch fermentation of BCNGW TALE(CO). Lactose was added at the same time for inoculation (thick arrow).

Symbols: ●, DCW; ▲, Glucose; ■, Lactose; ▼, Lactate; ◆, 3-FL

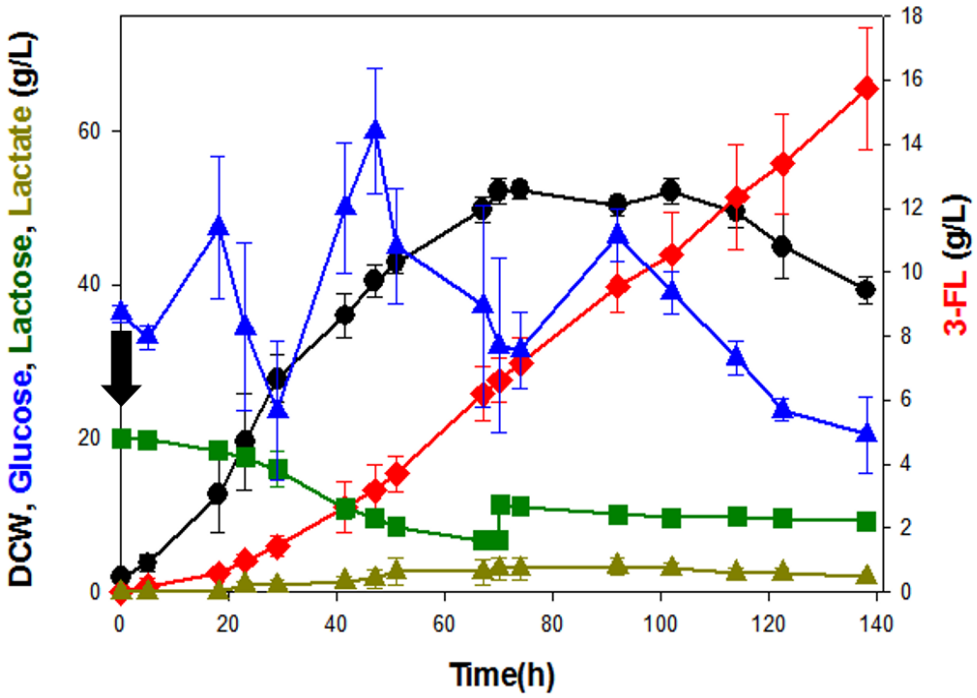


Table 13. Summary of optimized fed-batch fermentation of BCNGW TALE(CO).

Strain	Maximum dry cell weight (g/L)	Maximum 3-FL Titer (g/L)	Yield (mole 3-FL / mole lactose)	Productivity (g/L/h)
BCNGW TALE(CO) (fermentation optimized)	51.6	17.1	0.65	0.11

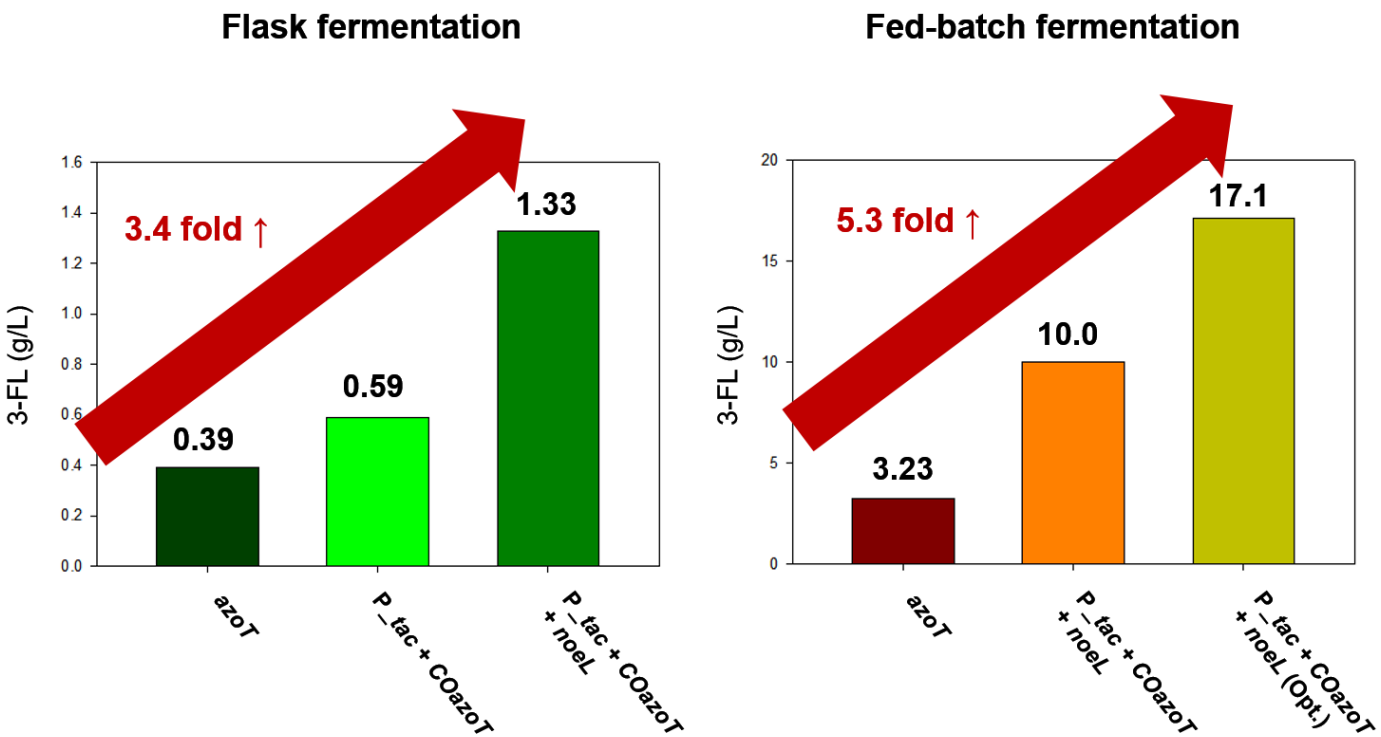
5. Summary

In this study, 7 candidates for the α-1,3-fucosyltransferase gene were introduced into *C. glutamicum* for the production of 3-FL. Among them, the BCGW AL strain constructed by introducing the *azoT* gene from *A. brasilense* produced the highest 3-FL titer of 390 mg/L in a flask fermentation. This strain produced 3.23 g/L of 3-FL in a fed-batch fermentation.

In order to enhance the expression of the *azoT* gene at a translational and transcriptional level, the *azoT* gene was codon optimized and the *tac* promoter was added in front of the *azoT* gene. In addition, the novel GDP-mannose 4,6-dehydratase gene(*noeL*) was introduced additionally to improve a GDP-$_{L}$-fucose biosynthetic ability for 3-FL production. With these strategies, the BCNGW TALE(CO) strain was constructed. This strain produced 1.33 g/L of 3-FL in a flask fermentation and 10.0 g/L of 3-FL in a fed-batch fermentation.

Finally, optimization of a fed-batch fermentation process was performed by replacing pre culture medium and by changing the time of IPTG induction. With these fermentation methods, the BCNGW TALE(CO) strain produced 17.1 g/L of 3-FL in a fed-batch fermentation, which was enhanced by a 5.3 fold compared to the BCGW AL strain (Fig. 18).

Figure 18. Summary of 3-FL titer in flask fermentation and fed-batch fermentation



IV. CONCLUSIONS

This thesis can draw the following conclusions:

(1) By introducing the α-1,3-fucosyltransferase gene (*azoT*) from *A. brasilense*, the BCGW AL strain produced 0.39 g/L of 3-FL in a flask fermentation and 3.23 g/L of 3-FL in a fed-batch fermentation

(2) By modulating α-1,3-fucosyltransferase gene expression and by introducing the GDP-mannose 4,6-dehydratase gene (*noeL*) from *A. brasilense*, the BCNGW TALE(CO) strain produced 1.33 g/L of 3-FL in a flask fermentation and 10.0 g/L of 3-FL in a fed-batch fermentation.

(3) By optimizing a fed-batch fermentation process, the BCNGW TALE(CO) strain produced 17.1 g/L of 3-FL in a fed-batch fermentation, which was enhanced by a 5.3 fold compared to the control strain BCGW AL.

V. REFERENCES

ABE, S., K.-I. TAKAYAMA and S. KINOSHITA (1967). "Taxonomical studies on glutamic acid-producing bacteria." The Journal of General and Applied Microbiology **13**(3): 279-301.

Aharonowitz, Y. and A. L. Demain (1978). "Carbon catabolite regulation of cephalosporin production in *Streptomyces clavuligerus*." Antimicrobial Agents and Chemotherapy **14**(2): 159-164.

Albermann, C., J. Distler and W. Piepersberg (2000). "Preparative synthesis of GDP-β-l-fucose by recombinant enzymes from enterobacterial sources." Glycobiology **10**(9): 875-881.

Albermann, C., W. Piepersberg and U. F. Wehmeier (2001). "Synthesis of the milk oligosaccharide 2′-fucosyllactose using recombinant bacterial enzymes." Carbohydrate Research **334**(2): 97-103.

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

Becker, D. J. and J. B. Lowe (2003). "Fucose: biosynthesis and biological function in mammals." Glycobiology **13**(7): 41R-53R.

Becker, J., C. Klopprogge, O. Zelder, E. Heinzle and C. Wittmann (2005). "Amplified expression of fructose 1,6-bisphosphatase in *Corynebacterium glutamicum* increases in vivo flux through the pentose phosphate pathway and lysine production on different carbon sources." Appl Environ Microbiol **71**(12): 8587-8596.

Bode, L. (2012). "Human milk oligosaccharides: every baby needs a sugar mama." Glycobiology **22**(9): 1147-1162.

Boehm, G. and B. Stahl (2007). "Oligosaccharides from milk." Journal of Nutrition **137**(3): 847S.

Breton, C., R. Oriol and A. Imberty (1998). "Conserved structural features in eukaryotic and prokaryotic fucosyltransferases." Glycobiology **8**(1): 87-94.

Castanys-Muñoz, E., M. J. Martin and P. A. Prieto (2013). "2′-fucosyllactose: an abundant, genetically determined soluble glycan present in human milk." Nutrition Reviews **71**(12): 773-789.

Chaturvedi, P., C. Warren, M. Altaye, A. Morrow, G. Ruiz-Palacios, L. Pickering and D. Newburg (2001). "Fucosylated human milk oligosaccharides vary between individuals and over the course of lactation." Glycobiology **11**(5): 365.

Chin, Y.-W., J.-B. Park, Y.-C. Park, K. H. Kim and J.-H. Seo (2013). "Metabolic engineering of *Corynebacterium glutamicum* to produce GDP-l-fucose from glucose and mannose." Bioprocess and Biosystems Engineering **36**(6): 749-756.

Chin, Y. W., N. Seo, J. H. Kim and J. H. Seo (2016). "Metabolic engineering of *Escherichia coli* to produce 2′-fucosyllactose via salvage pathway of guanosine 5′-diphosphate (GDP)-l-fucose." Biotechnology and Bioengineering **113**(11): 2443-2452.

Coppa, G., P. Pierani, L. Zampini, I. Carloni, A. Carlucci and O. Gabrielli (1999). "Oligosaccharides in human milk during different phases of lactation." Acta Paediatrica **88**(s430): 89-94.

Dominguez, H., M. Cocaign-Bousquet and N. Lindley (1997). "Simultaneous consumption of glucose and fructose from sugar mixtures during batch growth of *Corynebacterium glutamicum*." Applied Microbiology and Biotechnology **47**(5): 600-603.

Drouillard, S., H. Driguez and E. Samain (2006). "Large-Scale Synthesis of H-Antigen Oligosaccharides by Expressing *Helicobacter pylori* α1,2-Fucosyltransferase in Metabolically Engineered *Escherichia coli* Cells." Angewandte Chemie **118**(11): 1810-1812.

Eggeling, L. and M. Bott (2005). Handbook of Corynebacterium glutamicum, CRC press.

Eikmanns, B. J., E. Kleinertz, W. Liebl and H. Sahm (1991). "A family of *Corynebacterium glutamicum*/*Escherichia coli* shuttle vectors for cloning, controlled gene expression, and promoter probing." Gene **102**(1): 93-98.

Gokhale, U. B., O. Hindsgaul and M. M. Palcic (1990). "Chemical synthesis of GDP-fucose analogs and their utilization by the Lewis* A (1→ 4) fucosyltransferase." Canadian Journal of Chemistry **68**(7): 1063-1071.

Hahn-Zoric, M., F. Fulconis, I. Minoli, G. Moro, B. Carlsson, M. Bottiger, N. RAHA and L. HANSON (2008). "Antibody responses to parenteral and oral vaccines are impaired by conventional and low protein formulas as compared to breast-feeding." Acta Paediatrica **79**(12): 1137-1142.

Han, N. S., T. J. Kim, Y. C. Park, J. Kim and J. H. Seo (2012). "Biotechnological production of human milk oligosaccharides." Biotechnology Advances **30**(6): 1268-1278.

Haynes, J. A. and M. L. Britz (1989). "Electrotransformation of *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*: growth in Tween 80 increases transformation frequencies." FEMS Microbiology Letters **61**(3): 329-333.

Hopper, D. and R. Cooper (1971). 'The regulation of *Escherichia coli* methylglyoxal synthase; a new control site in glycolysis?" FEBS letters **13**(4): 213-216.

Ikeda, M. (2003). Amino acid production processes. Microbial production of l-amino acids, Springer**:** 1-35.

Ikeda, M. and R. Katsumata (1998). "A novel system with positive selection for the chromosomal integration of replicative plasmid DNA in *Corynebacterium glutamicum*." Microbiology **144**(7): 1863-1868.

Jang, M. H., W. H. Lee, S. Y. Shin, N. S. Han, J. H. Seo and M. D. Kim (2010). "Molecular Cloning of the Genes for GDP-mannose 4, 6-dehydratase and GDP-L-fucose Synthetase from *Bacteroides thetaiotaomicron*." Food Science and Biotechnology **19**(3): 849-855.

Jantscher-Krenn, E. and L. Bode (2012). "Human milk oligosaccharides and their potential benefits for the breast-fed neonate." Minerva Pediatr **64**: 83-99.

Jenness, R. (1979). The composition of human milk.

Kadner, R. J., G. P. Murphy and C. M. Stephens (1992). "Two mechanisms for growth inhibition by elevated transport of sugar phosphates in *Escherichia coli*." Microbiology **138**(10): 2007-2014.

Kameyama, A., H. Ishida, M. Kiso and A. Hasegawa (1991). "Total synthesis of sialyl Lewis X." Carbohydrate Research **209**: C1-C4.

Katsumata, R., A. Ozaki, T. Oka and A. Furuya (1984). "Protoplast transformation of glutamate-producing bacteria with plasmid DNA." Journal of Bacteriology **159**(1): 306-311.

Kimata, K., Y. Tanaka, T. Inada and H. Aiba (2001). "Expression of the glucose transporter gene, *ptsG*, is regulated at the mRNA degradation step in response to glycolytic flux in *Escherichia coli*." The EMBO Journal **20**(13): 3587-3595.

KINOSHITA, S., S. UDAKA and M. SHIMONO (1957). "Studies on the amino acid fermentation." The Journal of General and Applied Microbiology **3**(3): 193-205.

Kiyoshi, M., M. Kazuhiko, T. Mahito, I. Koichi, I. Masaaki, T. Hiroshi, N. Shigeru and S. Konosuke (1985). "Construction of novel shuttle vectors and a cosmid vector for the glutamic acid-producing bacteria *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*." Gene **39**(2): 281-286.

Komata, Y. (1976). "Utilization in foods." Microbial Production of Nucleic Acid-related Substances. Kodansha/John Wiley and Sons. Tokyo, Japan. New York, NY: 299-319.

Kretzschmar, G. and W. Stahl (1998). "Large scale synthesis of linker-modified sialyl Lewis^X, Lewis^X and N-acetyllactosamine." Tetrahedron **54**(23): 6341-6358.

Kumagai, H. (2000). Microbial production of amino acids in Japan. History of Modern Biotechnology I, Springer**:** 71-85.

Kunz, C., S. Rudloff, W. Baier, N. Klein and S. Strobel (2000). "O LIGOSACCHARIDES IN HUMAN MILK: Structural, Functional, and Metabolic Aspects." Annual Review of Nutrition **20**(1): 699-722.

Lanting, C., M. Huisman, E. Boersma, B. Touwen and V. Fidler (1994). "Neurological differences between 9-year-old children fed breast-milk or formula-milk as babies." The Lancet **344**(8933): 1319-1322.

Lee, W. H., Y. W. Chin, N. S. Han, M. D. Kim and J. H. Seo (2011). "Enhanced production of GDP-L-fucose by overexpression of NADPH regeneration in recombinant *Escherichia coli*.", Applied Microbiology and Biotechnology **91**: 967-976

Lee, W. H., P. Pathanibul, J. Quarterman, J. H. Jo, N. S. Han, M. J. Miller, Y. S. Jin and J. H. Seo (2012). "Whole cell biosynthesis of a functional oligosaccharide, 2'-fucosyllactose, using engineered *Escherichia coli*." Microbial Cell Factories **11**: 9.

Ma, B., J. L. Simala-Grant and D. E. Taylor (2006). "Fucosylation in prokaryotes and eukaryotes." Glycobiology **16**(12): 158R-184R.

Marx, A., A. A. de Graaf, W. Wiechert, L. Eggeling and H. Sahm (1996). "Determination of the fluxes in the central metabolism of *Corynebacterium glutamicum* by nuclear magnetic resonance spectroscopy combined with metabolite balancing." Biotechnology and Bioengineering **49**(2): 111-129.

Matsuki, T., K. Yahagi, H. Mori, H. Matsumoto, T. Hara, S. Tajima, E. Ogawa, H. Kodama, K. Yamamoto, T. Yamada, S. Matsumoto and K. Kurokawa (2016). "A key genetic factor for fucosyllactose utilization affects infant gut microbiota development." Nat Commun **7**: 11939.

McVeagh, P. and J. Miller (2008). "Human milk oligosaccharides: only the breast." Journal of Paediatrics and Child Health **33**(4): 281-286.

Miller, J. and P. McVeagh (2007). "Human milk oligosaccharides: 130 reasons to breast-feed." British Journal of Nutrition **82**(05): 333-335.

Miyoshi, E. (2008). Fucosylation and Cancer. Experimental Glycoscience, Springer**:** 235-237.

Morita, T., W. El-Kazzaz, Y. Tanaka, T. Inada and H. Aiba (2003). "Accumulation of glucose 6-phosphate or fructose 6-phosphate is

responsible for destabilization of glucose transporter mRNA in *Escherichia coli*." J Biol Chem **278**(18): 15608-15614.

Morrow, A. L., G. M. Ruiz-Palacios, M. Altaye, X. Jiang, M. Lourdes Guerrero, J. K. Meinzen-Derr, T. Farkas, P. Chaturvedi, L. K. Pickering and D. S. Newburg (2004). "Human milk oligosaccharides are associated with protection against diarrhea in breast-fed infants." The Journal of pediatrics **145**(3): 297-303.

Nathoo, T. and A. Ostry (2009). The one best way?: breastfeeding history, politics, and policy in Canada, Wilfrid Laurier Univ. Press.

Newburg, D. (1997). "Do the binding properties of oligosaccharides in milk protect human infants from gastrointestinal bacteria?" Journal of Nutrition **127**(5): 980S.

Newburg, D. S., G. M. Ruiz-Palacios and A. L. Morrow (2005). "Human milk glycans protect infants against enteric pathogens." Annu Rev Nutr **25**: 37-58.

Ninonuevo, M. R., Y. Park, H. Yin, J. Zhang, R. E. Ward, B. H. Clowers, J. B. German, S. L. Freeman, K. Killeen and R. Grimm (2006). "A strategy for annotating the human milk glycome." Journal of Agricultural and Food Chemistry **54**(20): 7471-7480.

Richards, G. R., M. V. Patel, C. R. Lloyd and C. K. Vanderpool

(2013). "Depletion of glycolytic intermediates plays a key role in glucose-phosphate stress in *Escherichia coli*." J Bacteriol **195**(21): 4816-4825.

Richards, G. R. and C. K. Vanderpool (2011). "Molecular call and response: the physiology of bacterial small RNAs." Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms **1809**(10): 525-531.

Rivero-Urgell, M. and A. Santamaria-Orleans (2001). "Oligosaccharides: application in infant food." Early Human Development **65**: S43-S52.

SANTAMARíA, R., J. A. GIL, J. M. MESAS and J. F. MARTÍN (1984). "Characterization of an endogenous plasmid and development of cloning vectors and a transformation system in *Brevibacterium lactofermentum*." Microbiology **130**(9): 2237-2246.

Schäfer, A., J. Kalinowski, R. Simon, A. Seep-Feldhaus and A. Pühler (1990). "High-frequency conjugal plasmid transfer from gram-negative *Escherichia coli* to various gram-positive coryneform bacteria." Journal of Bacteriology **172**(3): 1663-1666.

Schäfer, A., A. Tauch, W. Jäger, J. Kalinowski, G. Thierbach and A. Pühler (1994). "Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection

of defined deletions in the chromosome of *Corynebacterium glutamicum*." Gene **145**(1): 69-73.

Schwarzer, A. and A. Pühler (1991). "Manipuiation of *Corynebacterium glutamicum* by Gene Disruption and Replacement." Nature Biotechnology **9**(1): 84-87.

Severin, S. and X. Wenshui (2005). "Milk biologically active components as nutraceuticals: review." Critical Reviews in Food Science and Nutrition **45**(7): 645-656.

Smilowitz, J. T., A. O'Sullivan, D. Barile, J. B. German, B. Lönnerdal and C. M. Slupsky (2013). "The Human Milk Metabolome Reveals Diverse Oligosaccharide Profiles." The Journal of Nutrition **143**(11): 1709-1718.

Tropis, M., X. Meniche, A. Wolf, H. Gebhardt, S. Strelkov, M. Chami, D. Schomburg, R. Kramer, S. Morbach and M. Daffe (2005). "The crucial role of trehalose and structurally related oligosaccharides in the biosynthesis and transfer of mycolic acids in *Corynebacterineae*." J Biol Chem **280**(28): 26573-26585.

Van der Rest, M., C. Lange and D. Molenaar (1999). "A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA." Applied Microbiology and Biotechnology **52**(4): 541-545.

Vanderpool, C. K. (2007). "Physiological consequences of small RNA-mediated regulation of glucose-phosphate stress." Current Opinion in Microbiology **10**(2): 146-151.

Weichert, S, S. Gennewein, E. Hufner, C. Weiss, J. Borkowski, J. Putze and H. Schroten (2013). "Bioengineered 2'-fucosyllactose and 3-fucosyllactose inhibit the adhesion of *Pseudomonas aeruginosa* and enteric pathogens to human intestinal and respiratory cell lines.", Nutrition Research **33**: 831-838.

Wendisch, V. and F. Jülich (1997). Physiologische und NMR-spektroskopische Untersuchungen zur in vivo-Aktivität zentraler Stoffwechselwege im Wildstamm und in rekombinanten Stämmen von Corynebacterium glutamicum, Forschungszentrum, Zentralbibliothek.

Wendisch, V. F., A. A. de Graaf, H. Sahm and B. J. Eikmanns (2000). "Quantitative determination of metabolic fluxes during coutilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose." Journal of Bacteriology **182**(11): 3088-3096.

Wolf, A., R. Krämer and S. Morbach (2003). "Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC13032 and their significance in response to osmotic stress." Molecular Microbiology **49**(4): 1119-1134.

Yoshihama, M., K. Higashiro, E. Rao, M. Akedo, W. Shanabruch, M. Follettie, G. Walker and A. Sinskey (1985). "Cloning vector system for *Corynebacterium glutamicum*." Journal of Bacteriology **162**(2): 591-597.

Yu, J. W., J. Shin, M. Park, Seydametova. E, S. M. Jung, J. H. Seo and D. H. Kweon (2018). "Engineering of α-1,3-fucosyltransferases for production of 3-fucosyllactose in *Escherichia Coli*." Metabolic Engineering **48**: 269-278

Zhang, L., K. Lau, J. Cheng, H. Yu, Y. Li, G. Sugiarto, S. Huang, L. Ding, V. Thon and P. G. Wang (2010). "*Helicobacter hepaticus* Hh0072 gene encodes a novel α1-3-fucosyltransferase belonging to CAZy GT11 family." Glycobiology **20**(9): 1077-1088

국 문 초 록

다른 포유류의 젖과는 달리 모유에는 올리고당이 특이적으로 많이 함유되어 있는 것으로 알려져 있다. 모유올리고당은 약 300 여 종이 존재하는데 그 중에서 약 80% 가량이 푸코실화 되어있는 푸코실올리고당이다. 푸코실올리고당 중 3-fucosyllactose (3-FL)는 두 번째로 많은 함량을 차지한다. 3-FL은 장내 유익균의 생육을 촉진하는 프리바이오틱효과, 병원성균의 감염 방지, 면역반응의 조절, 두뇌 발달 등의 우수한 기능성을 지녀 유아용 분유, 건강식품, 의약품 및 화장품의 소재로 각광받고 있다.

선행연구에서는 코리네박테리움 글루타미쿰을 이용하여 GDP-L-fucose를 생합성하는 경로를 구축하였고, lactose를 세포내로 수송하기 위하여 *lacYA* 오페론을 도입하였다. 본 연구에서는 이를 활용하여 코리네박테리움 글루타미쿰을 이용하여 3-FL을 생산하고, 그 생산량을 증대시키는 연구를 수행하였다. 3-FL은 GDP-L-fucose와 lactose가 효소에 의해 α-1,3 결합으로 푸코실화 됨으로써 생성된다. 따라서 α-1,3 결합을 형성하는

α-1,3-fucosyltransferase를 도입하는 전략을 활용하여 코리네박테리움 글루타미쿰에서 3-FL을 생산하고자 하였다. 이를 위하여 헬리코박터 파일로리, 박테로이데스 프라질리스 및 아조스피릴럼 브라실렌스 유래의 7가지의 α-1,3 fucosyltransferase 유전자를 도입하여 회분식 발효를 진행해본 결과 아조스피릴럼 브라실렌스 유래의 α-1,3-fucosyltransferase인 *azoT*를 도입한 균주에서 가장 많은 390 mg/L의 농도로 3-FL이 생산되었다. 고농도의 3-FL을 생산하기 위해 유가식 발효를 진행한 결과 3.23 g/L의 3-FL이 생산되었다.

다음으로 *azoT*의 발현을 조절하는 연구를 수행하였다. 먼저 *azoT*의 transcription level을 증가시키기 위해 *azoT*의 벡터 내에서의 polycistronic expression system을 monocistronic expression system으로 전환하고자 하였다. 이를 위해 *azoT* 앞에 *tac* promoter를 추가하였다. 다음으로 *azoT*의 translational level을 증가시키기 위해 아조스피릴럼 브라실렌스 유래의 *azoT*를 모균주인 코리네박테리움 글루타미쿰에 맞게 코돈 최적화하였다.이렇게 구축한 균주의 회분식 발효를 진행해본 결과 590

mg/L 의 3-FL이 생산되었고, 고농도의 3-FL을 생산하기 위해 유가식 발효를 진행한 결과 4.00 g/L의 3-FL이 생산되었다..

다음으로 GDP-L-fucose의 생합성경로를 최적화하는 연구를 수행하였다. 기존 GDP-L-fucose 생합성 경로에 이용되는 유전자들은 대장균 유래로서 활성의 최적 조건이 모균주인 코리네박테리움 글루타미쿰과는 맞지 않았다. 이를 해결하고자 코리네박테리움 글루타미쿰과 생육 최적 조건이 맞는 아조스피릴럼 브라실렌스 유래의 GDP-L-fucose 생합성에 관여하는 유전자인 *noeL*을 추가적으로 도입한 균주를 구축하였고 회분식 발효를 통해 1.33 g/L, 유가식 발효를 통해 10 g/L의 3-FL이 생산되었다.

마지막으로 유가식 발효의 공정을 최적화하는 연구를 수행하였다. 전 배양 단계에 사용하는 배지를 주 배양 단계에 사용하는 배지와 같도록 교체하여 전 배양에서 주 배양과 같은 환경을 조성한 뒤 중간지수생장기까지 세포를 배양하여 주 배양에 접종하는 전략을 사용하였다. 이를 통해 주 배양 초반에 나타나는 세포의 생장이 지연되는 현상을 해결할 수 있었

고, 최종적으로 17.1 g/L의 3-FL이 생산되었다.

본 연구에서 개발한 3-FL 생산 미생물 시스템은 GRAS (Generally Recognized As Safe)로 인증된 균주인 코리네박테리움 글루타미쿰을 이용했기 때문에 3-FL의 생산의 산업화에 이점을 가질 것으로 판단된다.

주요어: 대사공학, α-1,3-fucosyltransferase, 3-푸코실락토오스, 코리네박테리움 글루타미쿰, 유가식 발효

학번: 2017-28426