PRODUCTION OF A FUNCTIONAL HUMAN MILK OLIGOSACCHARIDE,
2'-FUCOSYLLACTOSE, USING MICROBIAL CELL FACTORIES

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Food Science and Human Nutrition
with a concentration in Food Science
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2015

Urbana, Illinois

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ABSTRACT

Human breast milk is the gold standard for infant nutrition. In human milk, the

oligosaccharides may protect babies by acting as decoy receptors for pathogens. Also, human

milk oligosaccharides (HMOs) enhance the proliferation of probiotics to strengthen the host

immune system. Among HMOs, 2'-fucosyllactose (2-FL), composed of L-fucose and D-lactose,

is known to possess an anti-infection capability against many harmful organisms such as

Campylobacter jejuni, enteropathogenic *Escherichia coli*, and even Norovirus. Consequently,

great quantities of 2-FL are being demanded for food applications and thorough investigation of

its biological properties. The current synthetic methods of 2-FL including chemical production

and enzymatic catalysis are complicated, expensive, and thus impractical for a large-scale

synthesis. In my research study, an alternative route to produce 2-FL using a microbial cell

factory was devised. First, *E. coli,* as a host strain to produce 2-FL, was engineered to

overexpress genes in the metabolic pathway of GDP-L-fucose, a donor of L-fucose, and harbor

the fucosyltransferase enzyme (FucT2) which catalyzes the transfer of L-fucose onto a lactose

molecule enabling 2-FL production. Second, the production conditions of 2-FL in the engineered

E. coli were optimized in order to pinpoint the appropriate conditions promoting the efficient and

consistent synthesis of 2-FL. Third, the key enzyme in 2-FL production, FucT2, from different

sources was compared in search for the FucT2 leading to an enhanced synthesis of 2-FL. Fourth,

the possibility to produce 2-FL was demonstrated in a different host, *Saccharomyces cerevisiae,*

which is a generally recognized as safe (GRAS) organism. Through metabolic engineering, the

minimum three components required to construct a 2-FL-producing *S. cerevisiae* consisting of

GDP-L-fucose production, lactose internalization, and functional expression of FucT2 were confirmed. Additionally, the gene target perturbation, deletion of *GDA1*, which resulted in an improved production of GDP-L-fucose was identified. GDP-L-fucose is a beneficial sugar nucleotide which serves as a precursor to innumerable compounds aside from 2-FL. From this study, the feasibility and scalability of employing the microbial cell factory approach for 2-FL and GDP-L-fucose production were manifested.

ACKNOWLEDGEMENTS

First, I would like to express my deepest gratitude to my thesis advisors, Drs. Yong-Su Jin and Michael Miller, for their continuous support, guidance, and patience throughout my graduate research. Also, I would like to extend my appreciation to the other members of my thesis committees for their valuable suggestions: Drs. Sharon Donovan and Timothy Garrow.

I would not have been able to complete my Ph.D. research without the help from friends in Dr. Jin's research group. I would like to specially thank Dr. Won-Heong Lee for his assistance when I first started doing experiments. I am grateful to Dr. Guochang Zhang and Suryang Kwak who are like my mentors and consultants in the lab. I am thankful to other colleagues, Drs. Soo Rin Kim, Suk-Jin Ha, Hyo-Jin Kim, Na Wei, Ching-Sung Tsai, Jingjing Liu, Jun-Seob Kim, Liujing Wei, and Lahiru Jayakody for their advice on my research. I am thankful to all other lab members, Eun Joong Oh, Heejin Kim, In Iok Kong, Hong Nan, Nurik Kuanyshev, Tim Turner, Seung-Oh Seo, Haiqing Xu, Pengfei Xia, Jaewon Lee, and Stephan Lane for our friendship and any kind of help you have given me. I would like to also thank Dr. Miller's lab friends for providing insights and assistance on my study whenever I needed. This study would not have been possible without the funding from the Royal Thai Government scholarship and the Food Science and Human Nutrition department, University of Illinois.

Last but not least, I would like to thank my parents, my sister, and my close friends in the U.S. and Thailand for their love and encouragement throughout my study.

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FACTORIES

1.1   Human milk oligosaccharides structure and health benefits

Human breast milk is the best source of nutrition for newborns. In addition to nutrients, it provides immunological components important for infant growth and development. Breastfeeding provides numerous health benefits to babies including but not limited to a lower incidence of infectious diarrhea caused by pathogens, a decreased risk of common autoimmune diseases, such as celiac disease, protection against excessive inflammation, faster brain development, a reduced risk of asthma, and decreased dental problems (Walker, 2010).

Human milk is composed mainly of lactose, lipids, proteins, and oligosaccharides other than lactose (**Fig. 1.1**). Human milk oligosaccharide (HMO), the third most abundant element, has received much attention in the past decade owing to its prebiotic property stimulating the growth of select beneficial gut microorganisms and inhibitory effect against pathogen adhesion to intestinal cells (Kunz et al., 2000; Morrow et al., 2004; Newburg et al., 2004). The structure of HMOs is based on a lactose backbone which can be further elongated by N-acetylglucosamine, L-fucose, or sialic acid to form a more complex oligosaccharide (Han et al., 2012). The most copious type of HMOs is the fucosylated (L-fucose containing) type constituting over 70% of total HMOs (Ninonuevo et al., 2006).

2'-fucosyllactose (2-FL) is the most prevalent fucosylated HMO accounting for about 30% of total HMOs in 2-FL-secreting mothers (Chaturvedi et al., 2001; Morrow et al., 2005). It plays a crucial role in guarding the host from biological harm such as microbial cells and toxins. The enteric pathogen *Campylobacter jejuni* binds to soluble 2-FL (acting as a receptor analog for pathogens) before binding to the epithelial cells and gets swept away with 2-FL without infecting the host (Chaturvedi et al., 2001; Morrow et al., 2004). In a recent finding, 2-FL synthesized by

whole-cell biocatalysis inhibited the adhesion of not only *C. jejuni* but also enteropathogenic *E. coli*, *Salmonella enterica* serovar Fyris, and *Pseudomonas aeruginosa* to the intestinal human cell line Caco-2 (reduction of 26%, 18%, 12%, and 17%, respectively) (Weichert et al., 2013). Protecting against viral pathogenesis, 2-FL was reported to block the attachment of Norovirus to host receptor glycans (Shang et al., 2013). Moreover, 2-FL exhibits a bifidogenic effect specifically boosting the growth of *Bifidobacterium bifidum* and *B. longum* subsp. *infantis* leading to a superior gut health (Asakuma et al., 2011).

1.2 Current approaches to produce 2'-fucosyllactose

Due to its various benefits, improved methods for production of 2-FL is needed in order to further evaluate its biological functions and to enable food applications such as incorporation in infant formula. Current access to 2-FL is limited because of the high cost of 2-FL in the market (roughly $100 per mg) which hinders the extension of 2-FL research. To obtain 2-FL, human milk is extensively purified, or methods of chemical or enzymatic synthesis are available. While there are many approaches for producing 2-FL, the current processes pose several disadvantages (Han et al., 2012). Isolation of 2-FL from human milk requires several steps of purification which results in the overall lengthy production time and makes this technique impractical for a large-scale production. Chemical synthesis of 2-FL can be accomplished through chemical reactions, however, the use of toxic reagents is involved during the process, the yield of the end product is low, and the complete operation can be rather expensive. To produce 2-FL via the enzymatic route, the present shortcomings are high costs of substrate and enzyme purification.

1.3 Production of 2'-fucosyllactose using a microbial cell factory through metabolic engineering

Metabolic engineering is the term used for targeted alteration of metabolic pathways in an organism in order to utilize cellular metabolism for production of a desired compound (Lessard, 1996). Modification of cellular processes involves the use of recombinant DNA techniques to express new genes in host cells, amplify endogenous enzymes, or knock out genes or modulate enzyme activities (Nielsen, 1998). The chemical reaction of 2-FL synthesis requires an activity of the enzyme α-1,2-fucosyltransferase (FucT2) catalyzing the transfer of L-fucose from GDP-L-fucose to the acceptor, D-lactose (Albermann et al., 2001). To enable the cells to produce 2-FL, substrates (GDP-L-fucose and lactose) must be intracellularly available for converting to the product. GDP-L-fucose may be obtained through an *in vivo* metabolic conversion from GDP-L-mannose (*de novo* pathway) (Ginsburg, 1960, 1961a,b) or L-fucose supplied from an external source or emancipated from catabolism of fucosylated glycans in the lysosome followed by an *in vivo* enzymatic conversion (salvage pathway) (Coffey et al., 1964; Kaufman and Ginsburg, 1968) (**Fig. 1.2**). The *de novo* pathway involves two enzymatic reactions of GDP-D-mannose-4, 6-dehydratase (GMD) and GDP-4-keto-6-deoxymannose-3, 5-epimerase-4-reductase (WcaG or FX) (Byun et al., 2007; Tonetti et al., 1996). In the salvage pathway, L-fucose provided extracellularly is internalized by L-fucose permease (FucP) and/or L-fucose released from the lysosome is transported to the cytosol. Then, an activity of the bifunctional enzyme, L-fucose kinase/L-fucose-1-phosphate guanylyltransferase (FKP), is required for the conversion of L-fucose to GDP-L-fucose (Coyne et al., 2005). The second substrate, lactose, can be fed into the cells to allow the product conversion. Another indispensable element, the enzyme

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FucT2, should be well expressed in the cytosol in order to facilitate the fucosylation of lactose yielding 2-FL synthesis. There are several putative FucT2 from different organisms. FucT2 from *Helicobacter pylori* has been the most extensively studied and shown to be functionally expressed in *E. coli* (Ma et al., 2006). Via the metabolic engineering approach, development of a cost-effective method of producing 2-FL directly from an inexpensive sugar, lactose, is attainable using the microbial cell as a manufacturing factory. This approach is also scalable by increasing the size of operation to a larger fermenter.

1.4   Metabolic engineering of *Escherichia coli* for 2'-fucosyllactose production

E. coli is an attractive choice as a host to produce a target metabolite due to the wide access of its genetic tools and ease of culture. To produce 2-FL in *E. coli*, we first need GDP-L-fucose. *E. coli* naturally possesses the *de novo* and salvage pathways for synthesizing GDP-L-fucose, because GDP-L-fucose is a precursor for the formation of colanic acid, an exopolysaccharide component found in *Enterobacteriaceae* (Stevenson et al., 1996). However, metabolic engineering techniques may be applied to increase the intracellular concentration of GDP-L-fucose. In regard to lactose, lactose can be made available inside the cells by lactose uptake from the medium. *E. coli* normally ferments lactose regulated by the *lac* operon consisting of a lactose permease (LacY) and a β-galactosidase (LacZ), which hydrolyzes lactose into glucose and galactose. Yet, intact lactose is needed for 2-FL production. Metabolic engineering strategies again may come into play to reserve more lactose for 2-FL synthesis, or sourcing for a strain which is inefficient in lactose utilization can also benefit 2-FL formation. In terms of FucT2, most laboratory *E. coli* strains do not express FucT2, though some other strains

in nature like *E. coli* O126 (Engels and Elling, 2014) and O128 (Shao et al., 2003) do. Accordingly, introduction of the functionally-expressed FucT2 in laboratory *E. coli* strains is necessary.

1.5   Previous endeavors on 2'-fucosyllactose production in the engineered *E. coli*

Drouillard et al. (2006) attempted to eliminate the competing pathway for 2-FL production by preventing colanic acid production from GDP-L-fucose. Hence, the recombinant *E. coli* was engineered to disrupt the enzyme colanic acid biosynthesis UDP-glucose lipid carrier transferase (WcaJ) which utilizes GDP-L-fucose for colanic acid formation. To increase the flux from the central metabolism toward the GDP-L-fucose pathway, the positive regulator protein of the colanic acid operon, RcsA, was overexpressed. In addition, the β-galactosidase (LacZ) enzyme which breaks down lactose into glucose and galactose was deactivated to preserve intact lactose for 2-FL formation. By supplementing 15 g/L of lactose to the growth medium, the recombinant *E. coli* was able to produce 11 g/L of 2-FL extracellularly.

More recently, Baumgartner et al. (2013) aimed to enhance the intracellular availability of GDP-L-fucose by chromosomally integrating additional copies of the enzymes associated with the *de novo* and salvage routes for GDP-L-fucose formation. Moreover, two copies of the FucT2 gene were integrated into the genome to gain a strong fucosylating activity. The resulting recombinant *E. coli* produced 20.28 g/L of 2-FL in the glycerol-limited fed-batch fermentation.

1.6   Metabolic engineering of *Saccharomyces cerevisiae* for GDP-L-fucose and 2'-fucosyllactose production

S. cerevisiae has a long history in alcoholic fermentations and baking. Through those practices, knowledge of this yeast about genetics, physiology, and biochemistry as well as molecular biology tools and techniques has compiled over time (Nevoigt, 2008). The U.S. Food and Drug Administration (FDA) classified *S. cerevisiae* as GRAS (generally recognized as safe) in 1986, which draws attention of this yeast to more food applications.

In regard to GDP-L-fucose and 2-FL production, *S. cerevisiae* could be a great host organism for this purpose for a few reasons. It has a large intracellular pool of GDP-D-mannose, a precursor for GDP-L-fucose synthesis, because it performs significant mannosylation such as adding mannose residues to proteins (Mattila et al., 2000). The fact that it does not contain a pathway for GDP-L-fucose biosynthesis may also be advantageous. GDP-L-fucose becomes a dead-end molecule that can then be totally concentrated on 2-FL formation without deviating to other metabolic pathways. Being considered as a safe organism, yeast secreting 2-FL can have a bright future in various perspectives such as food applications.

GDP-L-fucose production in *S. cerevisiae* can be accomplished via either *de novo* or salvage routes. The *de novo* approach necessitates expressions of heterologous GMD and WcaG to transform inherent GDP-D-mannose into GDP-L-fucose (Mattila et al., 2000; Nakayama et al., 2003). For the salvage mode, the sole expression of exogenous FKP complements the conversion of L-fucose to GDP-L-fucose when L-fucose is extracellularly supplemented (Liu et al., 2011).

To further enable *S. cerevisiae* to produce 2-FL, at least two more steps have to be executed aside from the intracellular synthesis of GDP-L-fucose. First, the cytosolic activity of

FucT2 is needed to permit the fucosylation of lactose for 2-FL formation. *S. cerevisiae* does not natively express FucT2. Therefore, the functional FucT2 from foreign organisms is required to be incorporated in the yeast. Second, the availability of lactose inside the cells is mandatory. Nevertheless, wild-type *S. cerevisiae* cannot assimilate lactose which makes it impossible to supply lactose in the cytosol. To correct this defect, the recombinant *S. cerevisiae* expressing a lactose transporter and the enzyme β-galactosidase from naturally lactose-consuming organisms like *Kluyveromyces* species, can be constructed (Sreekrishna and Dickson, 1985). The elements discussed above are prerequisites in generating a yeast strain platform for producing 2-FL. However, additional metabolic engineering actions may need to be implemented to acquire the actual 2-FL-producing yeast.

1.7   Previous endeavors on GDP-L-fucose and 2'-fucosyllactose production in the engineered *S. cerevisiae*

Mattila et al. (2000) constructed the *S. cerevisiae* strain harboring the *E. coli de novo* GMD and WcaG enzymes. The resulting strain produced 0.2 mg/L of GDP-L-fucose from innate GDP-D-mannose. By externally supplementing GDP-D-mannose, the amount of synthesized GDP-L-fucose was raised 3-fold as compared with the one without the supplementation. The author also affirmed the successful formation of GDP-L-fucose by performing the α-1,3-fucosyltransferase assay. The crude extract of the yeast expressing GMD and WcaG was used as the source of GDP-L-fucose in the assay reaction. The product, sialyl-Lewis X, was detected in the reaction after incubation with the acceptor molecule, sialyllactosamine, for one hour.

The same research group, Jarvinen et al. (2001), reported another GDP-L-fucose synthesis in *S. cerevisiae* from the expression of enzymes originated from *H. pylori*, GMD and GMER (WcaG in *E. coli*). The *S. cerevisiae* engineered with the *H. pylori* enzymes was able to synthesize 5 μmol/mL of GDP-L-fucose. Moreover, the fucosyltransferase assay similar to the previous work was conducted to verify the production of GDP-L-fucose. The α-1,3-fucosylated glycan product was observed at the end of the assay indicating the presence of L-fucose donor, GDP-L-fucose.

Later in 2003, Nakayama et al. expressed the GDP-L-fucose synthesizing genes from *Arabidopsis thaliana*, MUR1 and GER1, which are equivalent to the *E. coli* GMD and WcaG, respectively, in *S. cerevisiae*. The functional expression of the two genes was achieved which capacitated the yeast to accumulate 0.002 mg/$2x10^8$ cells of GDP-L-fucose *in vivo*.

Through a distinct route, Liu et al. (2011) successfully demonstrated the heterologous expression of FKP, the salvage pathway enzyme, from *Bacteroides fragilis* in *S. cerevisiae*. The recombinant *S. cerevisiae* was able to synthesize 0.13 mg of GDP-L-fucose in a 25 ml culture when 15 mM L-fucose was provided in the medium.

Although GDP-L-fucose formation in *S. cerevisiae* is certain, no article regarding 2-FL production in yeast has been published to date.

1.8   Figures
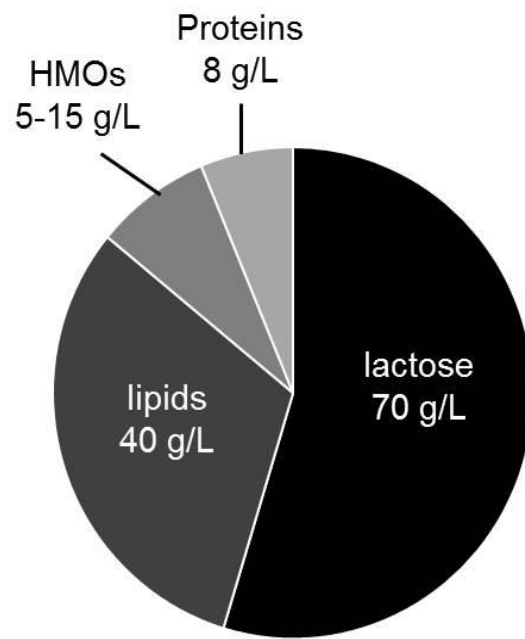
Fig. 1.1 Human milk composition (Zivkovic et al., 2011).
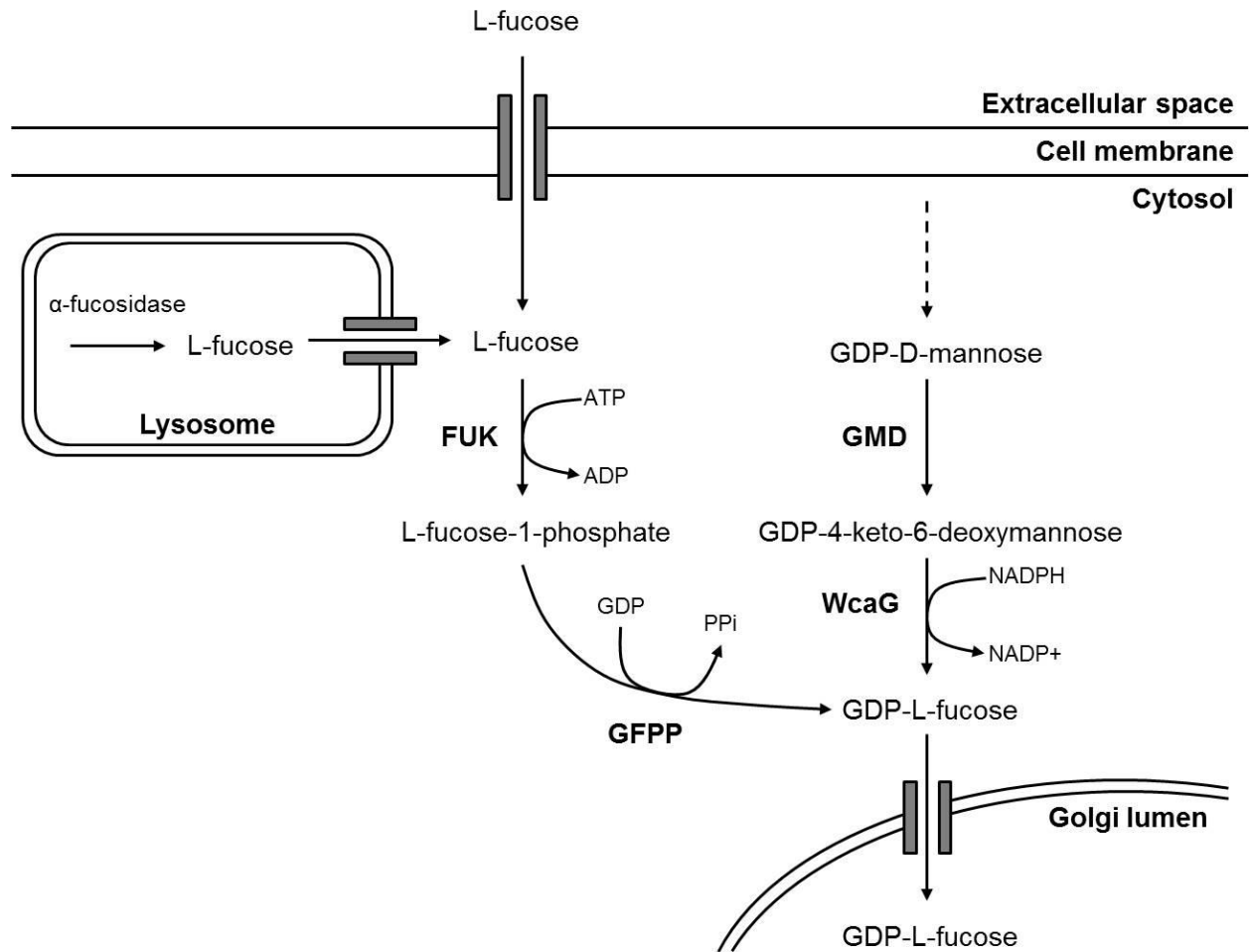
Fig. 1.2 GDP-L-fucose biosynthesis. The multi-step reactions on the left are the salvage pathway and the reactions on the right are the *de novo* pathway. FUK encodes L-fucose kinase. GFPP encodes GDP-L-fucose pyrophosphorylase. GMD encodes GDP-D-mannose-4, 6-dehydratase. WcaG encodes GDP-4-keto-6-deoxymannose-3, 5-epimerase-4-reductase (modified from Becker and Lowe, 2003).

CHAPTER II   WHOLE CELL BIOSYNTHESIS OF A FUNCTIONAL

OLIGOSACCHARIDE, 2'-FUCOSYLLACTOSE, USING

ENGINEERED *ESCHERICHIA COLI*

The content of this chapter has been accepted for publication by *Microbial Cell Factories*. I am the second author of the paper. Dr. Won-Heong Lee is the first author and designed the experiments. Josh Quarterman, Jung-Hyun Jo, Nam Soo Han, Michael J. Miller, Yong-Su Jin, and Jin-Ho Seo were co-authors. I performed the fermentation experiments of *E. coli* with the help from Dr. Won-Heong Lee. Dr. Yong-Su Jin was the director of the research.

2.1   Introduction

Human milk oligosaccharides (HMOs) are known to be the most relevant factor for the development of intestinal microbiota in breast-fed infants (Kunz and Rudloff, 2006). Also, HMOs have been reported to play important roles in preventing adhesion of pathogens and toxins to epithelial surfaces (Bode, 2006). Fucosyloligosaccharides, such as 2'-fucosyllactose, lacto-*N*-fucopentaose and lacto-*N*-difucohexaose, are common HMOs. Fucosylated oligosaccharides act as growth stimulating factors for select Bifidobacteria and soluble analogs of receptors for pathogenic bacteria, thereby protecting infants against infection from enteric pathogens and binding of toxins (Morrow et al., 2004; Newburg et al., 2004). Specifically, α-1,2-linked fucosylated oligosaccharides are reported to exhibit protective activity against several pathogens including *Campylobacter jejuni* (Chaturvedi et al., 2001; Morrow et al., 2004), *Salmonella* enteric serotype *Typhimurium* (Chessa et al., 2009), Enterotoxigenic *E. coli* (Newburg et al., 1990), *Helicobacter pylori* (Maqalhães and Reis, 2010), and noroviruses (Newburg et al., 2005). Among them, 2'-fucosyllactose (2-FL) is the most abundant fucosyloligosaccharide in human milk and accounts for more than 30% of total HMOs (Chaturvedi et al., 2001; Morrow et al., 2004). Low levels of 2-FL in the milk of sore mothers have been reported to be associated with a higher rate of diarrhea in breast-fed infants (Morrow et al., 2004). Hence, 2-FL is a promising oligosaccharide for nutraceutical and pharmaceutical purposes.

2-FL can be synthesized through the enzymatic fucosylation of lactose by α-1,2-fucosyltransferase (FucT2), which requires guanosine 5'-diphosphate (GDP)-L-fucose as a donor of L-fucose (Albermann et al., 2001). *Escherichia coli* is known to be able to synthesize GDP-L-

fucose since GDP-L-fucose is used for biosynthesis of colanic acid, one of the main components of the cell wall (Stevenson et al., 1996). Therefore, 2-FL can be produced via engineering of the GDP-L-fucose biosynthetic pathway and overexpression of the fucosyltransferase gene in metabolically engineered *E. coli*. **Fig. 2.1** shows the metabolic pathway for biosynthesis of GDP-L-fucose and 2-FL in recombinant *E. coli*.

Previously, biosynthesis of fucosyloligosaccharides using a recombinant microorganism and fucosyltransferase has been reported. The enzymatic synthesis of 2-FL was examined by using purified FucT2, GDP-L-fucose and lactose (Albermann et al., 2001), however, the high cost of GDP-L-fucose and FucT2 purification may be a limiting factor for large-scale production of fucosyloligosaccharides. Production of several fucose-containing lacto-oligosaccharides in recombinant *E. coli* was also reported through simultaneous overexpression of fucosyltransferase and the regulatory protein for colanic acid biosynthesis (Drouillard et al., 2006; Dumon et al., 2001), which suggested that whole cell synthesis of fucosyloligosaccharides through direct amplification of the GDP-L-fucose biosynthesis might be feasible.

To construct an efficient 2-FL production system by metabolic engineering, an understanding and detailed analysis of a cellular metabolic network involved in the 2-FL biosynthesis is important. Elementary flux mode (EFM) analysis has emerged as a powerful tool for metabolic pathway analysis. EFM analysis is a useful mathematical tool for defining and describing all metabolic routes that are both stoichiometrically and thermodynamically feasible for a group of enzymes. The EFM analysis can decompose a complex metabolic network of many highly interconnected reactions into uniquely organized pathways that support steady state of metabolism. EFM analysis can provide identification of all genetically independent pathways,

determination of the most efficient physiological state of a cell, and analysis of metabolic network properties such as robustness and regulation (Pfeiffer et al., 1999; Schuster et al., 2000; Trinh et al., 2009). Hence, it can be a useful tool for understanding dynamics of cellular metabolism and rational design of the host strain's metabolism for 2-FL production.

We have previously developed a recombinant *E. coli* system for efficient production of GDP-L-fucose by metabolic engineering. An enhancement of GDP-L-fucose production was achieved by modulation of several factors for biosynthesis of GDP-L-fucose such as amplification of GDP-D-mannose biosynthesis, regeneration of NADPH, and manipulation of the guanosine nucleotides biosynthetic pathway (Lee et al., 2009, 2011, 2012b).

In the present study, the GDP-L-fucose production system was applied for efficient production of 2-FL by introduction of the FucT2 gene from *Helicobacter pylori* into the recombinant *E. coli* able to overproduce GDP-L-fucose. Whole cell biosynthesis of 2-FL from lactose was assessed in a series of batch fermentations for recombinant *E. coli* overexpressing the necessary genes for GDP-L-fucose production and the FucT2. An EFM analysis for 2-FL production in the recombinant *E. coli* was used to compare and evaluate experimental results.

2.2   Materials and methods

2.2.1   Strains and plasmids

E. coli TOP10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] was used for genetic manipulation. *E. coli* BL21star(DE3) [F$^-$, *omp*T, *hsd*SB(r$_B$$^-m_B$$^-$), *gal*, *dcm rne131* (DE3)]

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(Invitrogen, Carlsbad, CA, USA) and JM109(DE3) [*end*A1 *gln*V44 *thi*-1 *rel*A1 *gyr*A96 *rec*A1 *mcr*B$^+$ Δ(*lac-pro*AB) e14- [F' *tra*D36 *pro*AB$^+$ *lacI*^{q} *lac*ZΔM15] *hsd*R17(r$_K$$^-m_K$$^+$) (DE3)] (NEB, Ipswich, MA, USA) were used for production of GDP-L-fucose and 2-FL. Plasmid pmBCGW containing the polycistronic *gmd-wcaG* gene cluster and *manB-manC* gene cluster was previously constructed using plasmid pETDuet-1 (Lee et al., 2009). The gene encoding FucT2 was obtained by the polymerase chain reactions (PCR) using the genomic DNA of the *Helicobacter pylori* 26695 strain (ATCC 700392) as template (Wang et al., 1999). Two PCR primers of *fucT2*_F and *fucT2*_R were used for the amplification of the *fucT2* gene. After digestion of PCR fragments of the *fucT2* gene and pCOLADuet-1 (Merck Biosciences, Darmstadt, Germany) with *Nco*I and *Sac*I, the DNA fragments were ligated to construct plasmid pHfucT2. Plasmids and primers used in this work are listed in **Table 2.1**. The constructed plasmid was confirmed by DNA sequencing. The conditions for PCR reaction, DNA manipulation, and bacterial transformation followed the descriptions in the previous study (Byun et al., 2007).

2.2.2   Batch fermentation

Batch fermentation was carried out in a 250 mL flask containing 50 mL of Lysogeny Broth (LB) medium at 25°C and pH 6.8. Agitation speed was maintained at 250 rpm. When dry cell mass reached 0.3 g/L, 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture broth. After 3 h of additional cultivation, 2.6 g/L (or 14.5 g/L) lactose was added for 2-FL production.

2.2.3 Analytical methods

Cell concentration was measured by optical density (OD) at 600 nm using a spectrophotometer (Biomate 5, Thermo, NY, USA). Overexpression of FucT2 inside the cell was analyzed by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12% polyacrylamide). After 3 h of 0.1 mM IPTG induction, cells were collected and the concentration was adjusted to around 7.2 g/L. They were resuspended in 50 mM potassium phosphate buffer (pH 7.0) and disrupted by an ultrasonic processor. After centrifugation at 15,000×g for 20 min, the supernatant (soluble fraction) and debris (insoluble fraction) were separated. Ten microliters of the soluble protein fraction (approximately 0.04 mg) and the same volume of the total and insoluble protein fractions were subjected to SDS-PAGE. Gels were stained with Coomassie brilliant blue solution and images were analyzed using a densitometer.

Concentrations of lactose, 2-FL, and acetate in batch fermentations were determined by using a high performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series) equipped with a Rezex ROA Organic Acid H^+ column (Phenomenex, Torrance, CA, USA) and a refractive index (RI) detector (Agilent, Palo Alto, CA, USA). The column was eluted with 0.01 N H_2SO_4 at a flow rate of 0.6 mL/min at 50°C.

In order to confirm 2-FL biosynthesis, culture broth at the end of the batch fermentation was collected and analyzed using a liquid chromatography/mass spectrometry (LC/MS) system. The LC (Agilent Technologies 1100 Series) was equipped with an Agilent Zorbax Eclipse ZDB-C8 (4.6x150 mm, 5 micron) column and an Agilent LC/MSD Trap XCT Plus detector. The column was eluted at a flow rate of 0.4 mL/min by the following gradient program: 95% (v/v) eluent A (15 mM ammonium acetate) and 5% eluent B (acetonitrile) for 1 min; 5% to 95%

eluent B over 6 min; 95% eluent B over 10 min. The scan range for MS was 70 - 600 mass-to-charge ratio (m/z).

2.2.4 Construction of metabolic network model for *E. coli* producing 2-FL from lactose

A metabolic network model was constructed for 2-FL producing *E. coli* that grows on lactose. The *E. coli* network was based on a model that was introduced by Stelling et al. (2002) to examine the relationship between structure and function in metabolic networks. Furthermore, the model has been used for calculating elementary flux modes in previous reports (Klamt et al., 2005; Urbanczik and Wagner, 2005). The metabolic network was composed of 108 reactions, which were involved in carbon central metabolism, amino acid synthesis, fatty acid synthesis, and biomass production. The catabolic part of the model included substrate uptake reactions, glycolysis, pentose phosphate pathway, TCA cycle, and excretion of by-products (e.g. acetate, formate, lactate, and ethanol). Previous networks were extended to include the anaplerotic reactions (e.g. malic enzyme and pyruvate oxidase) in addition to parallel pathways for initial acetate metabolism. The anabolic part of the model covers the conversion of precursors into building blocks like macromolecules and biomass. The core *E. coli* model from Stelling et al. (2002) was modified in this research to account for lactose consumption and synthesis of 2-FL. Among the reactions added for 2-FL synthesis, some minor adjustments were made to simplify the model. Lactose was assumed to break down to 2 moles of glucose because galactose can be easily converted into glucose-6-phosphate. ATP was used in place of GTP for energy transfer. As for the mass balance, it should be noted that ADP is formed whenever ATP is consumed for all the metabolic reactions in the network. The mass balance equation on ATP is therefore the

negative of the mass balance on ADP and thus the two equations are linearly dependent. Therefore, ADP can be excluded from the model in order to simplify the subsequent EFM calculation. The same is true for other cofactor pairs like NADP/NADPH and NAD/NADH. The EFM pathways in the model were estimated using METATOOL 5.1 (Pfeiffer et al., 1999; Trinh et al., 2009) with Matlab.

2.3   Results

2.3.1   Expression of α-1,2-fucosyltransferase (FucT2) in recombinant *E. coli*

The expression pattern of FucT2 was investigated during a batch fermentation of recombinant *E. coli* harboring plasmid pHfucT2. In order to maximize the expression of the soluble form of FucT2 in the recombinant *E. coli*, 0.1 mM of IPTG was used. As shown in **Fig. 2.2**, a 33 kDa protein (consistent with FucT2 (Wang et al., 1999)) was found in both soluble and insoluble fraction. While a significant amount of FucT2 was expressed in inclusion bodies, biosynthesis of 2-FL was expected because a soluble form of FucT2 was available as well.

2.3.2   Batch fermentations

From the preliminary experiments (whole cell bioconversion of 2 g/L lactose with *E. coli* BL21star(DE3) strain), we concluded that the *E. coli* BL21star(DE3) strain is not beneficial for 2-FL production because it consumed lactose for growth and maintenance instead of converting to 2-FL (data not shown). Some *E. coli* strains, such as DH5α and JM series, are known to be

unable to assimilate lactose or utilize lactose extremely inefficiently due to partial deletion of the *lac*Z gene, which codes for β-galactosidase. As such, these *E. coli* strains might be useful for 2-FL production. Hence, *E. coli* JM109(DE3) enabling overexpressing proteins under the control of *T7* promoter was chosen as an alternative host stain for 2-FL production.

2-FL production for BL21star(DE3) and JM109(DE3) was compared under batch fermentation conditions. In order to allow sufficient production of both GDP-L-fucose biosynthetic enzymes and FucT2 inside the cells, the cells were cultivated for 3 hours after 0.1 mM IPTG induction. Then, 2.6 g/L of lactose was added to initiate 2-FL production without addition of additional sugar because GDP-L-fucose can be produced from LB media (Lee et al., 2009, 2011). During the fermentations, extracellular 2-FL production (in the medium) was monitored by HPLC analysis. As a result, a small amount of 2-FL (10 mg/L) was produced in the batch fermentation of recombinant *E. coli* BL21star(DE3). Meanwhile, much higher amount of 2-FL was produced in the batch fermentation of recombinant *E. coli* JM109(DE3). About 140 mg/L of 2-FL was produced from 2.6 g/L of lactose while 0.4 g/L of lactose remained unused at the end of the fermentation (data not shown). These results suggest that the lactose concentration should be controlled at more than 0.5 g/L to maintain 2-FL production. Consequently, a yield of 60 mg 2-FL/g lactose was obtained from the batch fermentation of *E. coli* JM109(DE3) when 2.6 g/L of lactose was used. In order to obtain a higher amount of 2-FL, a batch fermentation with a higher concentration of lactose was carried out. **Fig. 2.3** shows the profiles of lactose consumption and 2-FL production in the batch fermentation of recombinant *E. coli* JM109(DE3) with 14.5 g/L lactose. The cells consumed lactose slowly but produced 2-FL constantly for 96 h. After 96 h of fermentation, the 2-FL concentration did not increase any further and lactose

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consumption stopped. As a result, a maximum 2-FL concentration of 1.23 g/L was obtained, which corresponded to an eight-fold (1.23 g/L vs. 150 mg/L) increase as compared with the previous fermentation with 2.6 g/L lactose. 2-FL yield increased to 90 mg 2-FL/g lactose when 14.5 g/L of lactose was used. This improvement might be caused from increased lactose availability inside the cell. The results of the batch fermentations are summarized in **Table 2.2**.

2.3.3   Confirmation of 2-FL biosynthesis by recombinant *E. coli* overexpressing GDP-L-fucose biosynthetic enzymes and FucT2

LC/MS analysis was performed to confirm the biosynthesis of 2-FL in the recombinant *E. coli* JM109(DE3) strain overexpressing ManB, ManC, Gmd, WcaG, and FucT2. HPLC data showed that a compound with the identical retention time to the 2-FL standard compound (**Fig. 2.4A**) was detected in the culture broth (**Fig. 2.4B**). MS scanning data (the compound with the retention time = 6.6 min) showed ion fragment of m/z 487.1, which is compatible with that of the 2-FL standard (**Fig. 2.4C**).

2.3.4   Evaluation of 2-FL yield using EFM analysis for 2-FL producing *E. coli* from lactose

In order to evaluate the efficiency of 2-FL production from lactose using the recombinant *E. coli* JM109(DE3) strain, elementary flux mode (EFM) analysis was employed to estimate a maximum theoretical yield of 2-FL from lactose. **Fig. 2.5** shows the prediction of theoretical 2-FL yield versus biomass yield for *E. coli* growing on lactose. Our experimental result from a

batch fermentation of 14.5 g/L of lactose resulted in a biomass yield of 0.1 g biomass/g lactose. This suggests that 2-FL production from lactose by the engineered *E. coli* reached 20% of the maximum 2-FL production capacity.

2.4   Discussion

In human milk, α-1,2-fucosylated oligosaccharides such as 2-FL, are known to have protective activity against pathogenic bacteria and their toxins (Chaturvedi et al., 2001; Morrow et al., 2004; Newburg et al., 2005). Hence, the availability of large amounts of α-1,2-fucosylated oligosaccharides would be useful in ready-to-use materials, drugs for fundamental investigations or therapeutic purposes. As biosynthesis of GDP-L-fucose, a key compound for biosynthesis of α-1,2-fucosylated oligosaccharides, requires a number of enzymes and cofactors such as NADPH and GTP, a whole-cell conversion approach might be more realistic for industrial production than other chemical or enzymatic approaches (Ruffing and Chen, 2006).

In previous studies, improvements of GDP-L-fucose production in recombinant *E. coli* were attempted by manipulating the pathways enabling GDP-L-fucose biosynthesis from glucose (Lee et al., 2009, 2011, 2012b). This study was undertaken to upgrade the GDP-L-fucose production system for 2-FL production through additional overexpression of FucT2. To this end, the *fucT2* gene from *H. pylori* was cloned and overexpressed in the *E. coli* BL21star(DE3) strain. While an insoluble form of FucT2 was a major expression form, an active FucT2 was also observed in the recombinant *E. coli* as shown in **Fig. 2.2**. With the aid of FucT2 overexpression, recombinant *E. coli* BL21star(DE3) could produce 2-FL in the batch fermentation with 2.6 g/L lactose. However, 2-FL yield was fairly low (4 mg 2-FL/g lactose). *E. coli* BL21star(DE3)

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seemed to assimilate lactose instead of converting to 2-FL. Most of the initially added lactose was consumed within 12 h of fermentation with a marginal growth during the fermentation (data not shown). These results suggested that *E. coli* BL21star(DE3) is not appropriate for 2-FL production.

Previously, several attempts for production of fucosylated (or sialylated) oligosaccharides from lactose have been made using the derivative of *E. coli* JM107 and JM109 since these strains are unable to produce an active β-galactosidase due to the insertion of the M15 single strand DNA into the *lacZ* gene (Dumon et al., 2001, 2004; Drouillard et al., 2006; Fierfort and Samain, 2008). In these cases, glucose (or glycerol) was used as another carbon source for GDP-L-fucose production (or CMP-*N*-acetylneuraminic acid). These nucleotide sugars are subsequently used for fucosylation (or sialylation). According to the previous reports, *E. coli* JM109(DE3) was chosen as an alternative host strain for 2-FL production. As expected, the use of *E. coli* JM109(DE3) allowed production of a considerable amount of 2-FL in the batch fermentation, corresponding to a 15-fold increase in 2-FL concentration compared with the value obtained in BL21star(DE3) strain. However, a theoretical yield of 2-FL from lactose was predicted to be 1.4 g 2-FL/g lactose when cells cannot utilize lactose for growth. Meanwhile, our experimental yield of 2-FL was only about 0.1 g 2-FL/g lactose. This result indicates that more than 90% of lactose consumed was used for other purposes such as biomass production and endogenous metabolism. Slow consumption of lactose was also observed in the batch fermentation with mixed sugars (2 g/L of lactose and 5 g/L of mannose), where we expected that lactose could be mainly used for 2-FL production as mannose might be used for cell growth. Although an enhancement of 2-FL yield (0.13 g 2-FL/g lactose) was obtained, most of the consumed lactose was not used for 2-FL

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production (data not shown). It is probable that incompletely inactivated β-galactosidase or cryptic β-galactosidase inside the cell might cause the reduction of 2-FL yield from lactose. Insertion of λ(DE3) into the genome might cause the slow assimilation of lactose, which was supported by a previous report that α-complementation of LacZ was observed with the insertion of the DE3 cassette into the genomic DNA of *E. coli* JM101 (Dumon et al., 2004).

Since slow consumption of lactose and production of biomass were observed in the batch fermentation with 2.6 g/L lactose, another batch fermentation with a high concentration of lactose (14.5 g/L) was conducted in order to obtain a higher amount of 2-FL. Generally, *E. coli* is known to produce acetate when growing on an excessive sugar even under aerobic conditions. However, JM109(DE3) strain showed no acetate production even when excessive amounts (14.5 g/L) of lactose were added as displayed in **Fig. 2.3**. It is generally known that acetate formation is accelerated when the metabolic fluxes to pyruvate exceed the capacity of the respiratory metabolism (Lee, 1996; Zhao et al., 2004). Slow consumption of lactose might not lead to acetate formation, suggesting that the lactose utilization rate by *E. coli* JM109(DE3) is not fast enough to cause acetate formation. This result could be beneficial for designing a 2-FL production process such as a fed-batch type operation since acetate formation is known to be one of the main problems occurring in fed-batch type operations of *E. coli* (Lee et al., 2011). However, low yield and productivity of 2-FL from lactose will need to be improved for industrial applications. The 2-FL production system in this study was verified by comparing the experimental 2-FL yield with the theoretical maximum yield estimated by the EFM analysis. 2-FL yield corresponded to about 20% of the theoretical maximum yield, which suggests further modifications via metabolic engineering of a host strain or optimization of fermentation

processes should be carried out for improvement of 2-FL yield. Increased FucT2 solubility and intracellular lactose availability may be considered as primary approaches for improvement of 2-FL yield.

In this study, construction of efficient 2-FL production system was attempted. The *fucT2* gene from *H. pylori* was introduced into the recombinant *E. coli* able to overproduce GDP-L-fucose and biosynthesis of 2-FL was observed in the batch fermentation with lactose. The 2-FL production system using the *E. coli* JM109(DE3) strain showed a low rate of lactose assimilation and produced a considerable amount of 2-FL in the simple batch fermentation without acetate formation. The experimental 2-FL yield corresponded to 20% of the theoretical maximum yield, which indicates that more research should be conducted. Efficient microbial 2-FL production may be useful for utilizing 2-FL as a nutraceutical compound for various applications.

2.5   Figures and tables
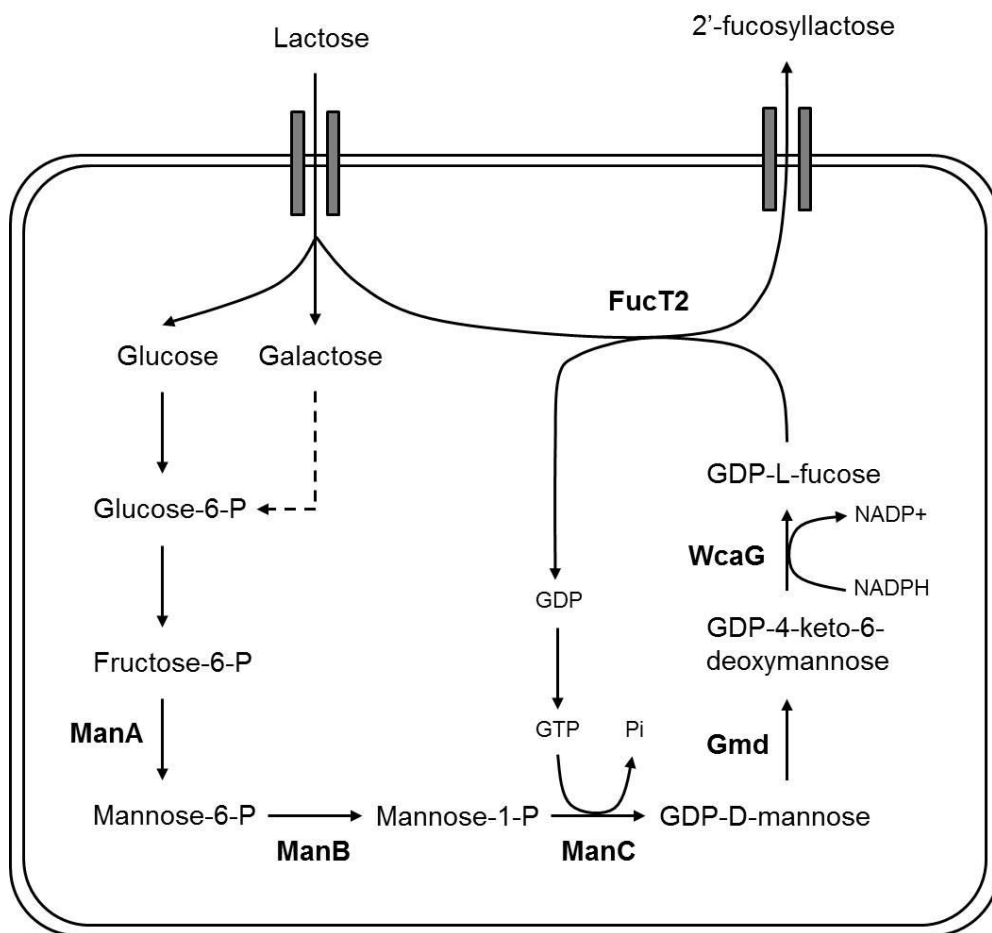
Fig. 2.1 The metabolic pathway for GDP-L-fucose and 2'-fucosyllactose (2-FL) biosynthesis in recombinant *E. coli*. The names of enzymes are abbreviated as follows; ManA, mannose 6-phosphate isomerase; ManB, phosphomannomutase; ManC, mannose 1-phosphate guanylyltransferase; Gmd, GDP-D-mannose-4,6-dehydratase; WcaG, GDP-4-keto-6-deoxymannose 3,5-epimerase 4-reductase; FucT2, α-1,2-fucosyltransferase. Pi, GDP, and GTP denote phosphate, guanosine 5'-diphosphate, and guanosine 5'-triphosphate, respectively.
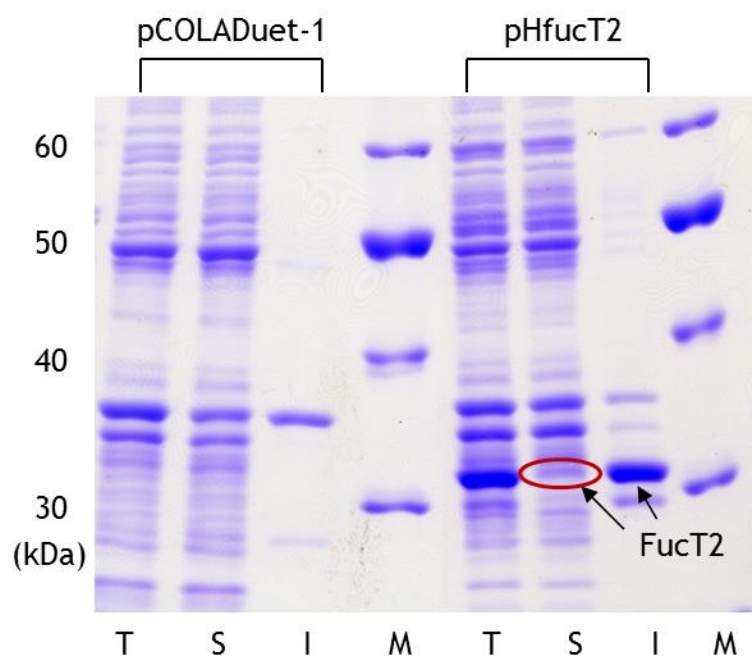
Fig. 2.2 SDS-PAGE analysis of the cell crude extract of recombinant *E. coli* BL21star(DE3) strains harboring pCOLADuet-1 and pHfucT2, respectively. Cells were harvested after 3 h of 0.1 mM IPTG induction. T, S, and I denote total, soluble and insoluble protein fractions, respectively. The arrow indicates the corresponding protein band with the estimated molecular weight of FucT2. Lane M indicates size marker.
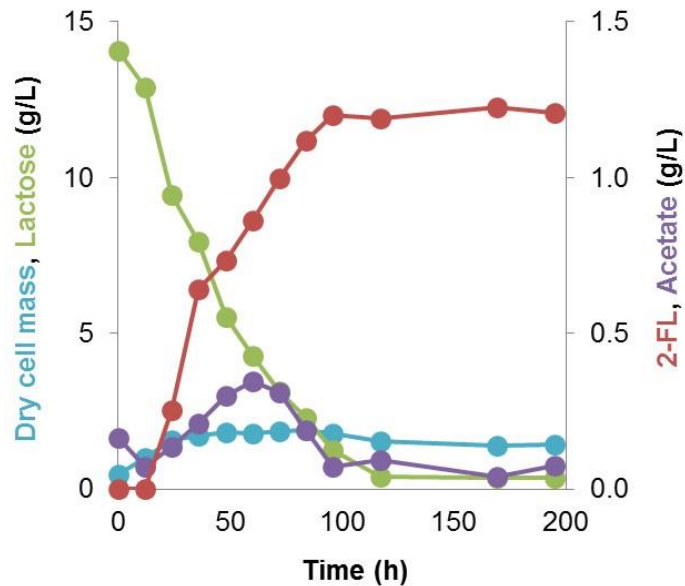
Fig. 2.3 Profile of 2-FL production in the batch fermentation of recombinant *E. coli* JM109(DE3) strains harboring plasmids pmBCGW and pHfucT2. After 3 h of 0.1 mM IPTG induction, 14.5 g/L of lactose was added for 2-FL production. Symbols denote as follows; *light blue circle*, dry cell mass; *red circle*, 2-FL concentration; *green circle*, lactose concentration; *purple circle*, acetate concentration. Measurement of cell, lactose, acetate, and 2-FL concentrations was done by three independent experiments. Symbols in the figure show the representative values of the batch fermentations.
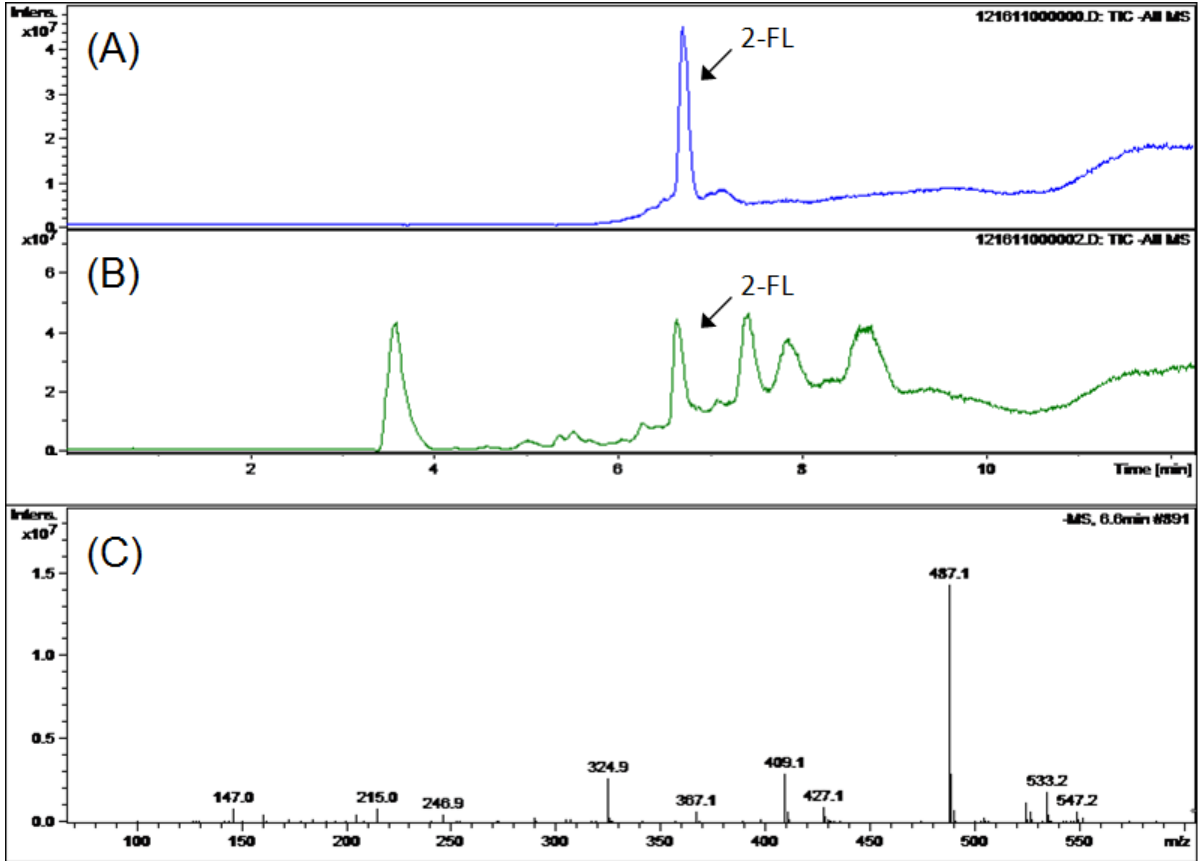
Fig. 2.4 LC/MS analysis of 2-FL biosynthesis in the batch fermentation of the recombinant *E. coli* JM109(DE3) overexpressing ManB, ManC, Gmd, WcaG, and FucT2. At the end of batch fermentation, culture broth was collected for confirmation of extracellular 2-FL production. HPLC analysis of 100 mg/L 2-FL standard solution (A), HPLC analysis of culture broth of *E. coli* JM109(DE3) harboring pmBCGW+pHfucT2 (B), and MS analysis of the compound with the retention time = 6.6 min in the culture broth of *E. coli* JM109(DE3) harboring pmBCGW+pHfucT2 (C).
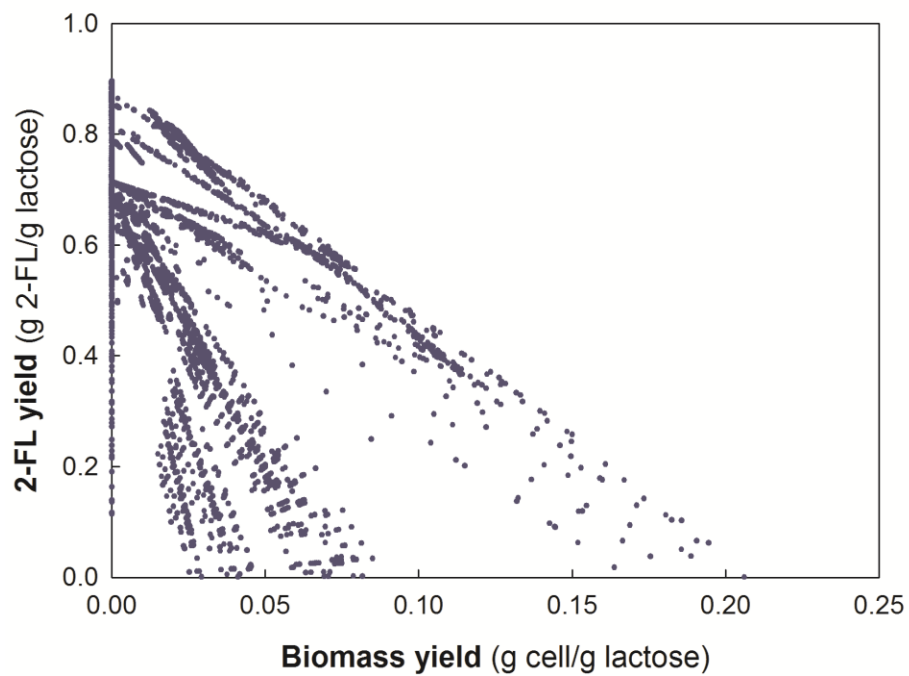
Fig. 2.5 Calculation of the theoretical maximum yield of 2-FL from lactose. Elementary flux

mode (EFM) analysis was carried out for 2-FL producing *E. coli*.

Table 2.1 Lists of primers and plasmids used in this study

Name	Sequence of PCR primers and description for plasmids	Source
PCR primers		
*fucT2*_F (*Nco*I)	5'-ACATG<u>CCATGG</u>CTTTTAAGGTGGTGCAA-3'	*H. pylori* 26695
*fucT2*_R (*Sac*I)	5'-AGTCC<u>GAGCTC</u>TTAAGCGTTATACTTTTGGGA-3'	(ATCC 700392)
Plasmids		
pETDuet-1	two T7 promoters with two MCS, pBR322 replicon (copy number ~40), Amp^r	Merck Biosciences
pCOLADuet-1	two T7 promoters with two MCS, ColA replicon (copy number 10~12), Kan^r	Merck Biosciences
pmBCGW	derived from pETDuet-1, P$_{T7}$-*manB-manC* (*Nco*I/*Sac*I)-P$_{T7}$-*gmd-wcaG* (*Nde*I/*Xho*I)-T$_{T7}$, Amp^r	Lee et al., 2009
pHfucT2	derived from pCOLADuet-1, P$_{T7}$- *fucT2* (*Nco*I/*Sac*I)-P$_{T7}$-MCS2-T$_{T7}$, Kan^r	this study

Table 2.2 Summary of batch fermentations of *E. coli* strains producing 2-FL from lactose

Strains	Plasmids	Initial lactose concentration (g/L)	Maximum dry cell mass (g/L)	Maximum 2-FL concentration (g/L)	Yield (g 2-FL/g lactose)
BL21star(DE3)	pmBCGW + pHfucT2	2.56±0.04	1.84±0.05	0.01±0.001	0.005±0.001
JM109(DE3)	pmBCGW + pHfucT2	2.55±0.02	1.17±0.05	0.15±0.014	0.06±0.005
		14.54±0.67	1.70±0.28	1.23±0.011	0.09±0.004

CHAPTER III   OPTIMIZATION OF 2'-FUCOSYLLACTOSE PRODUCTION

CONDITIONS IN ENGINEERED *ESCHERICHIA COLI*

3.1   Introduction

In the past decade, 2-FL, a human milk oligosaccharide, has been perceived as a
functional oligosaccharide owing to its interesting gut health boosting attributes (Bode, 2006,
2012). Possessing health benefits, 2-FL demand has been increasing in order to investigate its
biological functions and physicochemical properties. Possible applications include
supplementation of 2-FL in infant formula and food products.

Nevertheless, the access to 2-FL is limited due to its high cost. Currently, 2-FL is
synthesized by a chemical process. Extraction of 2-FL from human breast milk is impractical.
These limitations hinder the extension of 2-FL research (Han et al., 2012). Alternatively, the
enzymatic and biotechnological approaches to synthesize 2-FL have been developed which are
more simple and more cost-effective than existing 2-FL production methods (Albermann et al.,
2001; Baumgartner et al., 2013; Drouillard et al., 2006; Lee et al., 2012a; Wada et al., 2008). The
prerequisites to biologically synthesize 2-FL are guanosine-5'-diphosphate (GDP)-L-fucose (a
donor of L-fucose), D-lactose, and α-1,2-fucosyltransferase (FucT2) enzyme which catalyzes the
transfer reaction of L-fucose to the acceptor, D-lactose (Albermann et al., 2001). GDP-L-fucose
may be obtained via *in vivo* metabolic conversions from GDP-D-mannose (*De novo* pathway)
(Bulter and Elling, 1999) or from L-fucose (Salvage pathway) (Coffey et al., 1964). L-fucose
may be acquired extracellularly or intracellularly through catabolism of fucosylated glycans in
lysosome (Becker and Lowe, 2003). The *de novo* pathway involves two enzymatic reactions of
GDP-D-mannose-4, 6-dehydratase (GMD) and GDP-4-keto-6-deoxymannose-3, 5-epimerase-4-
reductase (WcaG or FX) responsible for converting GDP-D-mannose to GDP-L-fucose (Byun et
al., 2007; Tonetti et al., 1996). In the salvage pathway, an external L-fucose is transported into

the cytosol by L-fucose permease (FucP) while L-fucose generated in the lysosome is liberated into the cytosol by facilitated diffusion (Baumgartner et al., 2013; Jonas et al., 1990). Two enzymatic conversion steps are required to convert L-fucose to GDP-L-fucose including L-fucose kinase (FUK) and GDP-fucose pyrophosphorylase (GFPP) (Becker and Lowe, 2003). Alternately, a one-step conversion of L-fucose to GDP-L-fucose is possible by expression of the bifunctional enzyme, L-fucose kinase/L-fucose-1-phosphate guanylyltransferase (Fkp), from *Bacteroides fragilis* (Baumgartner et al., 2013).

The enzymatic synthesis of 2-FL was first reported from the *de novo* fucosylation of lactose encouraged by the FucT2 enzyme with a 65% yield of 2-FL after purification by anion-exchange chromatography and gel filtration (Albermann et al., 2001). GDP-L-fucose is also known as a precursor for the biosynthesis of colanic acid, an exopolysaccharide found in *Enterobacteriaceae* (Stevenson et al., 1996). Further attempt to produce 2-FL was then focused on eliminating the competing pathways and increasing the metabolic flux toward the pathway of the desirable product (Drouillard et al., 2006). To reserve GDP-L-fucose for 2-FL production, the enzyme which catalyzes the synthesis of colanic acid from GDP-L-fucose, colanic acid biosynthesis UDP-glucose lipid carrier transferase (WcaJ), was disrupted. β-galactosidase (LacZ) was also deactivated to secure intact lactose for 2-FL conversion by preventing the metabolism of lactose to biomass. Additionally, the positive regulator protein of the colanic acid operon, RcsA, was overexpressed to enhance the flux toward GDP-L-fucose production. By utilizing the *de novo* derived GDP-L-fucose and supplementing 15 g/L of lactose to the growth medium, the recombinant *E. coli* was able to produce 11 g/L of 2-FL extracellularly. More recently, the *de novo* and salvage approaches for GDP-L-fucose formation were coupled to intensify the

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intracellular availability of GDP-L-fucose (Baumgartner et al., 2013). The optimized recombinant *E. coli* strain, which has the *de novo* GDP-L-fucose synthesizing genes, two copies of fucosyltransferase gene, and the *fkp* gene integrated into the chromosome, produced 2-FL up to 20.28 g/L in the glycerol-limited fed-batch fermentation. Furthermore, 2-FL can be synthesized enzymatically using the glycosynthase technology (Wada et al., 2008). The α-1,2 linkage connecting L-fucose and D-galactose in the 2-FL structure can be created with the activity of 1,2-α-L-fucosynthase. The 1,2-α-L-fucosynthase can be generated from the inverting 1,2-α-L-fucosidase isolated from *Bifidobacterium bifidum* via D766G mutation. Through the enzymatic reaction comprising 10 mM β-L-fucosyl fluoride triacetate (L-fucose donor), 30 mM lactose (acceptor), and the 1,2-α-L-fucosynthase enzyme, approximately 650 µM of 2-FL can be synthesized in 2 hours at 30°C.

Previously we have established an engineered *E. coli* capable of producing 2-FL by overexpressing the *de novo* GDP-L-fucose synthesizing enzymes (*manB*, *manC*, *gmd*, and *wcaG*) from *E. coli* and the *fucT2* enzyme from *Helicobacter pylori* strain 26695 (Lee et al., 2012a). However, the variation of fermentation conditions was found to contribute greatly to the yield of the produced 2-FL. To consistently secure an efficient 2-FL production system, fermentation parameters such as culture growth temperature, concentration of isopropyl-β-D-thiogalactopyranoside (IPTG), time of the IPTG induction, and availability of lactose should be considered and optimized. In the present study, fermentation conditions used in the whole-cell biosynthesis of 2-FL by the engineered *E. coli* were investigated to determine the most appropriate environments promoting the efficient synthesis of 2-FL.

3.2   Materials and methods

3.2.1   Strain and plasmids

E. coli JM109(DE3) [*end*A1 *gln*V44 *thi*-1 *rel*A1 *gyr*A96 *rec*A1 *mcr*B$^+$ Δ(*lac-pro*AB) e14- [F' *tra*D36 *pro*AB$^+$ *lacI*^{q} *lac*ZΔM15] *hsd*R17(r$_K^-$m$_K^+$) (DE3)] (NEB, Ipswich, MA, USA) was used for production of GDP-L-fucose and 2-FL. Plasmid pmBCGW containing the polycistronic *gmd-wcaG* gene cluster and *manB-manC* gene cluster was previously constructed using plasmid pETDuet-1 (Lee et al., 2009). Plasmid pHfucT2 harboring the α1,2 *fucT2* gene from *H. pylori* strain 26695 was previously constructed using plasmid pCOLADuet-1 (Lee et al., 2012a). Plasmids used in this work are listed in **Table 3.1**.

3.2.2   Culture preparation

The engineered *E. coli* JM109(DE3) transformed with plasmids pmBCGW and pHfucT2 was grown in Lysogeny Broth (LB; sodium chloride 10 g/L, yeast extract 5 g/L, tryptone 10 g/L) supplemented with antibiotics (ampicillin 100 µg/mL and kanamycin 50 µg/mL). Antibiotics were administered to permit only the growth of *E. coli* harboring the above two plasmids necessary for 2-FL synthesis. The culture tube was routinely incubated at either 30°C for 16 h or 37°C for 12 h before starting the main fermentation of 2-FL (pre-culture).

3.2.3 Batch and fed-batch fermentation

The cell pellet of the late-exponentially grown pre-culture was used to inoculate the main fermentation. Typically, the main fermentation was performed using a 250 mL shake flask containing 50 mL working volume of LB medium, antibiotics, IPTG, and lactose. The culture flask was placed at either 25, 30, or 37°C in 300 rpm shaking incubators (Thermo Scientific, MA, USA). Small amounts of culture were sampled and analyzed every 24 hours to monitor cell growth and metabolite changes during the fermentation. All fermentation conditions were experimented in triplicates.

Fed-batch fermentation was also carried out in a 250 mL shake flask containing 50 mL working volume of the same aforementioned components as the batch fermentation. The culture flask was incubated at 25°C. Agitation speed was maintained at 300 rpm. 0.1 mM IPTG and 15 g/L lactose were added at the beginning of fermentation. As the initial/previous lactose depleted, lactose of roughly 15 g/L was replenished in the culture medium for three more times to allow a continuous accumulation of 2-FL.

3.2.4 IPTG induction experiment

IPTG is a structural mimic of lactose serving as a permanent inducer for the expressions of GDP-L-fucose biosynthetic genes and *fucT2* gene. Two concentrations of IPTG (0.1 and 0.5 mM) and two different times of induction (at the beginning simultaneously with cell inoculation and at the dry cell mass (DCM) level of 0.216 g/L) were tested in the batch fermentation.

3.2.5   Lactose concentration experiment

Different concentrations of lactose were studied to see how they affect 2-FL production.

Lactose concentrations consisting of 10, 15, 20, and 40 g/L were tested in the batch fermentation.

3.2.6   Analytical methods

Cell concentration was measured by optical density (OD) at 600 nm using a

spectrophotometer (Biomate 5, Thermo, NY, USA). Dry cell mass was calculated using an

estimate of 0.36 gram cells per OD value of 1. Concentrations of lactose, 2-FL, and acetate in

batch fermentations were determined using a high performance liquid chromatography (HPLC)

system (Agilent Technologies 1200 Series) equipped with a Rezex ROA Organic Acid H^+

column (Phenomenex, Torrance, CA, USA) and a refractive index (RI) detector (Agilent, Palo

Alto, CA, USA). The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min at

50°C.

3.3   Results

3.3.1   Effect of pre-culture and main fermentation temperatures on 2-FL production

The effect of temperature used in cultivating the engineered *E. coli* during pre-culture and

main fermentation on the ability of the cells to produce 2-FL was investigated. Pre-culture is

defined as the period of time from picking a single colony of cells to getting an exponentially-

grown culture. The purpose of pre-culture is to prepare a sufficient inoculum for starting a main

fermentation. Main fermentation is usually initiated by resuspending the cell pellet from pre-culture with a complete growth medium including (a) sugar substrate(s) and other fermentation necessities such as an inducer for protein expression and (an) antibiotic(s) for selection of the cells containing the expression plasmids.

Six combinations of two pre-culture temperatures (30 and 37ºC) and three main fermentation temperatures (25, 30, and 37ºC) were attempted and compared to determine the conditions that have the positive impact on 2-FL synthesis. As illustrated in **Fig. 3.1** and **3.2**, the difference of pre-culture temperatures did not seem to significantly influence the growth (represented in dry cell mass) of the engineered *E. coli* in the main culture regardless of the main fermentation temperature. The overall trends portrayed a slightly better growth of the cells experienced a higher pre-culture temperature (37ºC) than the cells from a lower pre-culture temperature (30ºC). Apparently, the main fermentation temperature plays an important role in cell growth. The cells grown at 25 and 30ºC in main culture generally reached a higher cell mass (above 3 g/L) than the ones grown at 37ºC no matter what temperature was used in the pre-culture.

In terms of lactose consumption, only the cells subjected to 30ºC pre-culture and later grown at 25ºC in main culture consumed lactose efficiently and were able to finish the provided lactose of about 15 g/L within 96 hours of fermentation. The cells grown at 30ºC pre-culture-30ºC main culture and 37ºC pre-culture-25 or 30ºC main culture also assimilated lactose relatively well up to 72 hours, but then slowed down the consumption and left a few grams of lactose unused at the end of the fermentation. Nevertheless, the cells exposed to 37ºC main

fermentation temperature, irrespective of pre-culture temperature, consumed lactose quickly at the beginning, but ceased to assimilate more lactose just after 24 hours of fermentation.

Another metabolite of interest, acetate is an unwanted by-product commonly excreted by *E. coli* in aerobic fermentation (Farmer and Liao, 1997). A high acetate accumulation in the medium (~over 2 g/L) adversely affects cell growth and the yield of recombinant protein production, thereby decreasing the amount of a desirable product (Landwall and Holme, 1977; Mori et al., 1979; Reiling et al., 1985). The engineered *E. coli* cultivated at 25ºC main fermentation, regardless of pre-culture temperature, produced almost no acetate throughout the fermentation. The amount of acetate generated seemed to positively correlate with the higher temperature. The cells grown at 30ºC main fermentation preceded by any pre-culture temperature secreted over 2 g/L of acetate. As the main culture temperature rose to 37ºC, an acetate buildup of over 3 g/L was observed.

With respect to 2-FL synthesis, pre-culture at 30ºC and main fermentation at 25ºC were the best conditions yielding 0.92 g/L of 2-FL. The cells from 37ºC pre-culture-25ºC main culture performed the second best with 0.82 g/L of 2-FL. While the culture grown at 30ºC in main fermentation, irrespective of pre-culture condition, produced about 0.4-0.5 g/L of 2-FL, the culture from 37ºC main fermentation barely produced any 2-FL.

In summary, the temperature combination of 30ºC pre-culture-25ºC main culture manifested the most excellent condition for 2-FL production because the engineered *E. coli* gave the highest titer of 2-FL, had better cell growth, completely fermented lactose, and accumulated the least amount of acetate.

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3.3.2   Effect of IPTG induction conditions on 2-FL production

The effect of IPTG induction on 2-FL production was studied in two aspects comprising IPTG working concentration and time of IPTG addition. IPTG, a non-metabolisable inducer, is indispensable when using the plasmid with T7 promoter in order to get the expression of your target gene. It functions to remove the attachment of a lac repressor (LacI) from the lac operator DNA sequence by causing the conformational change in the structure of LacI protein. Without the repressor, the T7 RNA polymerase protein will be able to express and bind to the T7 promoter sequence located upstream of the gene triggering transcription of the target gene (Bell and Lewis, 2000; Daber et al., 2007).

Two concentrations (0.1 and 0.5 mM) and two times of induction (at the beginning of fermentation and at Dry Cell Mass (DCM) of 0.216 g/L) of IPTG were examined. According to the best condition determined from the temperature effect experiment, 30°C pre-culture and 25°C main culture were used to grow the cells in the IPTG experiments.

Regarding cell growth, **Fig. 3.3** clearly shows that 0.1 mM concentration of IPTG led to the better cell growth compared with 0.5 mM concentration. The engineered *E. coli* rapidly grew up to DCM of 4.8 g/L in 0.1 mM IPTG conditions regardless of the time of IPTG addition. The second best growth was from the culture with 0.5 mM IPTG supplemented at DCM of 0.216 g/L. The cells produced DCM of 4.5 g/L but at a slower rate than the ones induced with 0.1 mM IPTG. However, it seemed that the addition of 0.5 mM IPTG at the beginning of fermentation negatively affected the cell growth in the early stage of the fermentation though the cells reached the same DCM of 4.5 g/L later as the ones supplemented with IPTG at DCM of 0.216 g/L.

In terms of lactose assimilation, it was obvious that the engineered *E. coli* grown in 0.1 mM IPTG added at the beginning consumed lactose faster than the rest of the tested conditions. They finished 18 g/L lactose in about 84 hours. The *E. coli* with 0.1 and 0.5 mM IPTG added at DCM of 0.216 g/L behaved similarly and completed all lactose within 96 hours. Yet, the *E. coli* with 0.5 mM IPTG added at the beginning had trouble to finish lactose and left a little amount of lactose unconsumed by the end of the fermentation.

With regard to 2-FL production, there was no difference in 2-FL synthesizing ability between supplementation of 0.1 mM at the beginning of fermentation or at DCM of 0.216 g/L. These two conditions gave about 0.72 g/L of 2-FL at the end of the day which is higher than the conditions of 0.5 mM IPTG at DCM of 0.216 g/L (0.5 g/L of 2-FL) and 0.5 mM IPTG at the beginning (0.2 g/L of 2-FL).

In regard to acetate, the induction condition of 0.5 mM IPTG at the beginning of fermentation solely had a significant quantity of acetate (~1.2 g/L) while a tiny amount of or no acetate was detected in all other conditions.

In summary, 0.1 mM IPTG induction at the beginning of fermentation resulted in the most desirable phenotypes of the engineered *E. coli* (better cell growth, more efficient lactose usage, higher 2-FL production, and no acetate formation).

3.3.3   Effect of lactose availability on 2-FL production

Referring to the previous best conditions from the effects of temperature and IPTG experiments, 30°C pre-culture, 25°C main fermentation, and 0.1 mM IPTG addition at the beginning of fermentation were consequently employed in all lactose concentration experiments.

With regard to cell growth, it seemed that there was no exact relationship between lactose concentration and cell growth (**Fig. 3.4**). The engineered *E. coli* fed with 40 g/L lactose grew the best (up to DCM 5 g/L) among all experimented lactose concentrations. The declining period of growth in 10 and 15 g/L conditions was disregarded because the lactose substrate was depleted resulting in the gradual cell death.

In relation to lactose utilization, the speed of lactose completion of the engineered *E. coli* was directly associated with the concentration of lactose. As expected, the cells were able to finish lower lactose concentration quicker than higher lactose concentration. 10, 15, and 20 g/L were completely consumed within 72, 96, and 120 hours, respectively. Still, 40 g/L condition had some remaining lactose in the culture at the end of the experimental time (168 hours).

In terms of 2-FL production, the engineered *E. coli* supplemented with 10, 15, 20, and 40 g/L lactose produced 2-FL similarly of about 1 g/L at 168 hours. Therefore, higher lactose concentration does not necessarily mean higher 2-FL production.

In summary, 10 g/L lactose concentration seemed to be the best option to produce 2-FL in a batch fermentation setting considering the amount of 2-FL produced (1 g/L) from the less amount of lactose needed (a cheaper process).

3.3.4 Fed-batch fermentation of 2-FL

To increase the 2-FL titer, the strategy of fed-batch fermentation may be useful since the higher availability of lactose in a single batch did not improve 2-FL production as tested in the previous section. To perform the fed-batch fermentation, a normal batch fermentation is first conducted with a low initial lactose concentration of ~10-15 g/L (**Fig. 3.5**). Once the initial

lactose is almost used up, more lactose is added into the culture. The addition of lactose may be repeated continually to maintain cell growth and provide sufficient substrates for 2-FL accumulation. Moreover, a higher initial cell density (DCM) of 3.6 was applied to accelerate the utilization of lactose and reduce the overall fermentation time.

In terms of cell growth, the engineered *E. coli* reached the maximum DCM of 9 g/L at 120 hours. The addition of more lactose to the culture did not further augment the cell number. Yet, the cell population maintained at above DCM of 7 g/L throughout the rest of fermentation.

In regard to lactose consumption, the rate of lactose utilization slowed down (less steep slopes) as the fermentation progressed and more lactose was supplemented. By raising the initial cell mass to ~3.6, 15 g/L of lactose was assimilated within 36 hours. However, the speed of lactose uptake declined in the second round of lactose. 48 hours were needed to finish about the same amount of lactose. By the fourth round of lactose, the cells obviously decelerated their lactose consumption and spent almost 90 hours to consume the same lactose 15 g/L.

Acetate was present during the primary stage of fermentation at low levels (less than 0.5 g/L) before 72 hours. Nonetheless, acetate vanished after this point and remained undetected throughout the rest of fermentation.

Regarding 2-FL formation, a continuous increase of 2-FL was observed as more lactose was provided to the cells. At the end of the fermentation (245 hours), the highest titer of 2-FL of 4.35 g/L was achieved.

3.4 Discussion

To secure an efficient and stable system of the whole-cell biosynthesis of 2-FL by the engineered *E. coli,* the process components related to fermentation condition and gene expression are crucial and should be carefully chosen in order to acquire the strain with the best performance. Three aspects associated with 2-FL production were investigated including pre-culture/main culture temperature, IPTG induction condition, and lactose availability.

In regard to fermentation temperature, pre-culture temperature (either 30 or 37°C) is not really a main contributing factor affecting 2-FL synthesis. The mere LB medium containing amino acids and limited carbon sources was employed to support cell growth to less than 2 g/L DCM during pre-culture. Hence, the cells in pre-culture underwent a minimal cell metabolism and the proteins related to 2-FL production were not yet induced and expressed. Rather, main fermentation temperature played an important role in the cells' ability to make 2-FL. 2-FL titer was adversely correlated with the rise of main culture temperature. At 37°C, the cell metabolism ran faster. Therefore, lactose was quickly consumed during the first 24 hours creating the strong carbon fluxes going down glycolysis and citric acid (TCA) cycle. These strong fluxes had to subsequently overflow to acetate production pathway since they overloaded what the glycolysis and TCA cycle could handle (Vemuri et al., 2006). Once acetate buildup exceeded 2 g/L, it negatively impacted cell growth, sugar uptake, and protein production (Chong et al., 2013). A similar situation of fast metabolic fluxes happened with 30°C condition but to a lesser extent. The cells grew and fermented lactose relatively well until 72 hours. Still, over 2 g/L of acetate was later generated causing the cells unable to use up all lactose during the last period of fermentation. On the other hand, a lower culture temperature like 25°C did not ensue such a high

speed of metabolism. The carbon fluxes from lactose degradation were at an appropriate level and did not inundate the central metabolic pathway. Thus, almost no acetate was produced and the cells were able to grow healthily. In respect of 2-FL formation, the lower cell metabolism at the low temperature also grants the produced protein ample time to properly fold and become a functional/soluble protein (Baneyx and Mujacic, 2004). On the contrary, proteins are produced rapidly at the higher cell metabolic rate (high temperature) but they do not have time to adequately fold and eventually aggregate to form inclusion bodies (insoluble protein) (Singh et al., 2015).

Concerning IPTG induction, IPTG concentration used to induce the recombinant protein production was clearly shown to influence 2-FL production. A higher IPTG concentration of 0.5 mM turned out unhelpful for 2-FL formation. It is likely that such the high amount of IPTG gave the cells a metabolic burden and reduced their growth rates (Kosinski et al., 1992). When they were sick, they were not able to produce 2-FL as well. Instead, more carbons were constituted as acetate, a by-product. In consideration of time of induction, the time of IPTG addition did not alter the amount of 2-FL much when exposing a low IPTG concentration (0.1 mM) to the cells. However, at a higher IPTG content (0.5 mM), the induction at the beginning of fermentation strongly diminished the cell growth rate. This could be due to the metabolic stress pushing the cells to produce lots of protein when they were trying to increase their biomass to get through the lag phase. Most researchers recommended the induction of IPTG during the exponential cell growth (DCM 0.216-0.288 g/L). At this stage of growth, the chaperones necessary for reversal of misfolded proteins are sufficiently expressed (Schlieker et al., 2002). The majority of soluble proteins can be retained benefiting the product yield. Nevertheless, we decided to move forward

with 0.1 mM IPTG induced at inoculation which generated 2-FL as good as the exponential induction condition for the convenience of fermentation experiment.

Regarding lactose availability, a higher lactose concentration provided to the growth medium did not automatically increase 2-FL production. This may be explained by the limitations of other factors, for instance, the availability of GDP-L-fucose substrate and the efficiency of fucosyltransferase, which overall made the conversion to 2-FL not so effective. We determined the low concentration of lactose (10-15 g/L) as an optimum condition for performing a batch fermentation of 2-FL since the same amount of 2-FL can be obtained as in the higher concentration of lactose and all lactose can be assimilated within a reasonable amount of time. The complete utilization of lactose would ease the subsequent purification of 2-FL.

As a direct supplementation of more lactose was not beneficial for improving a 2-FL titer, a periodic supply of lactose was attempted in a fed-batch fermentation. The fed-batch experiment was conducted using the best fermentation conditions identified previously. In addition, a higher initial cell density (DCM 3.6 g/L) was exploited to accelerate the lactose utilization. By supplying a low amount of lactose at a time, the finite lactose availability triggered the cells to continue to utilize lactose for converting to both biomass and 2-FL. Through this strategy, 2-FL titer can be enhanced as more lactose is consumed.

In conclusion, production of 2-FL from engineered *E. coli* JM109(DE3) was greatly dependent on the fermentation conditions. After subjecting the engineered *E. coli* through various experiments, the best conditions to produce 2-FL were 30ºC pre-culture-25ºC main fermentation temperature and 0.1 mM IPTG induction at the beginning of fermentation. Moreover, a low lactose concentration (10-15 g/L) seemed to be the most efficient working

concentration for a batch fermentation. A fed-batch fermentation technique was advantageous raising the titer of 2-FL to 4.35 g/L (considered over three folds of advancement from the batch fermentation). Nevertheless, a thorough optimization of production conditions may need to be performed in the future to achieve an even better yield of 2-FL.
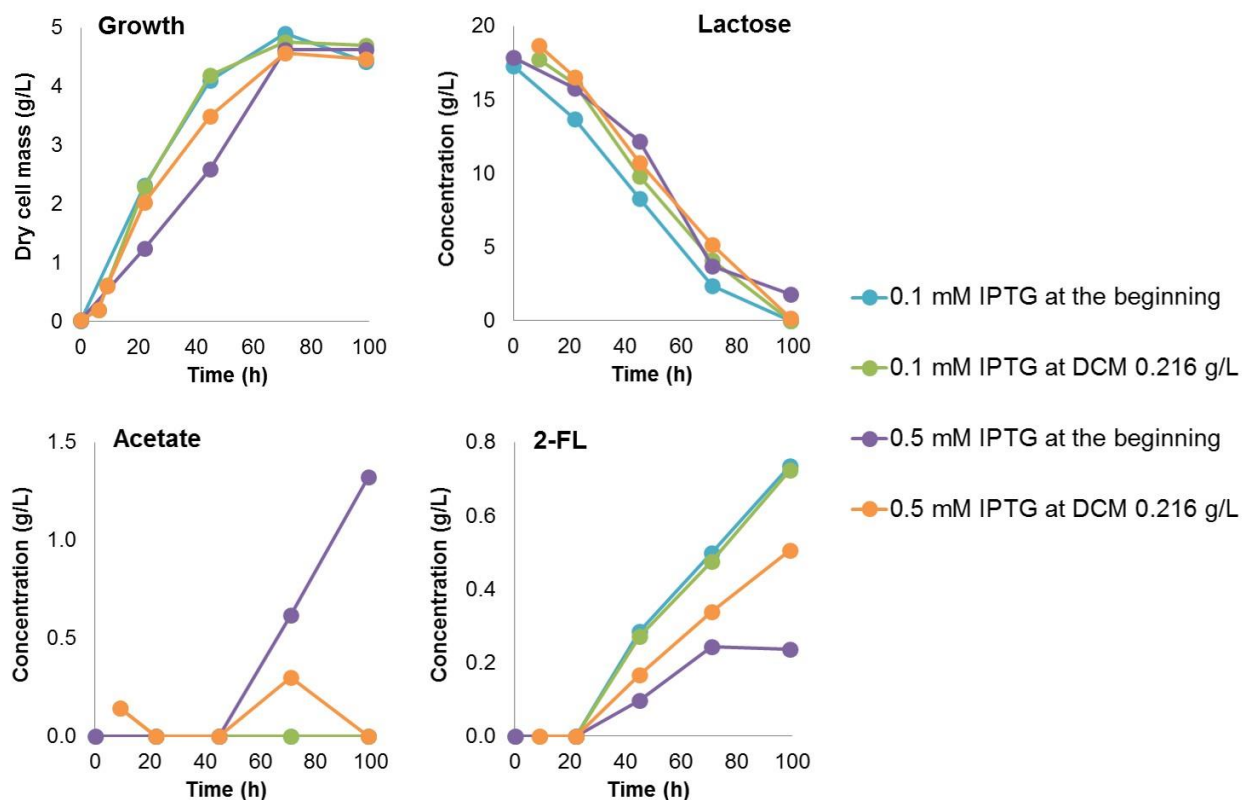
3.5 Figures and tables
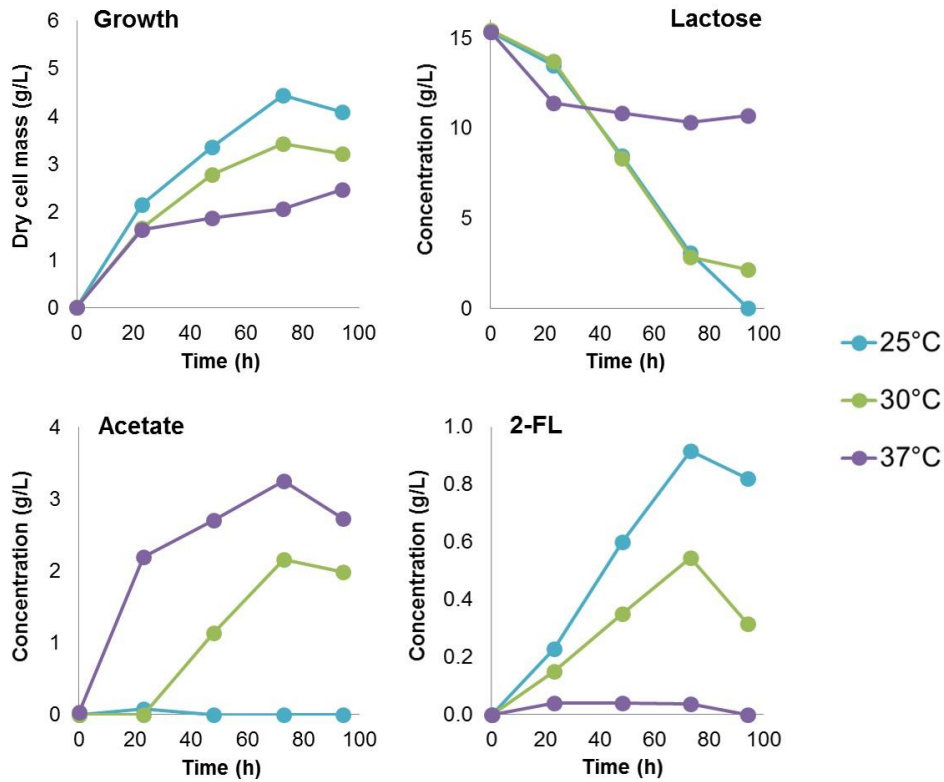
Fig. 3.1 Batch fermentation profiles of recombinant *E. coli* strain JM109(DE3) harboring

plasmids pmBCGW and pHfucT2. Effects of pre-culture and main fermentation temperatures on

2-FL production were studied. The cells were pre-cultured at 30°C. Then, they were grown at

either 25, 30, or 37°C in main fermentation. During main fermentation, cell growth, lactose

consumption, acetate production, and 2-FL production were monitored. Symbols denote as

follows; *light blue circle*, 25°C; *green circle*, 30°C; *purple circle*, 37°C.
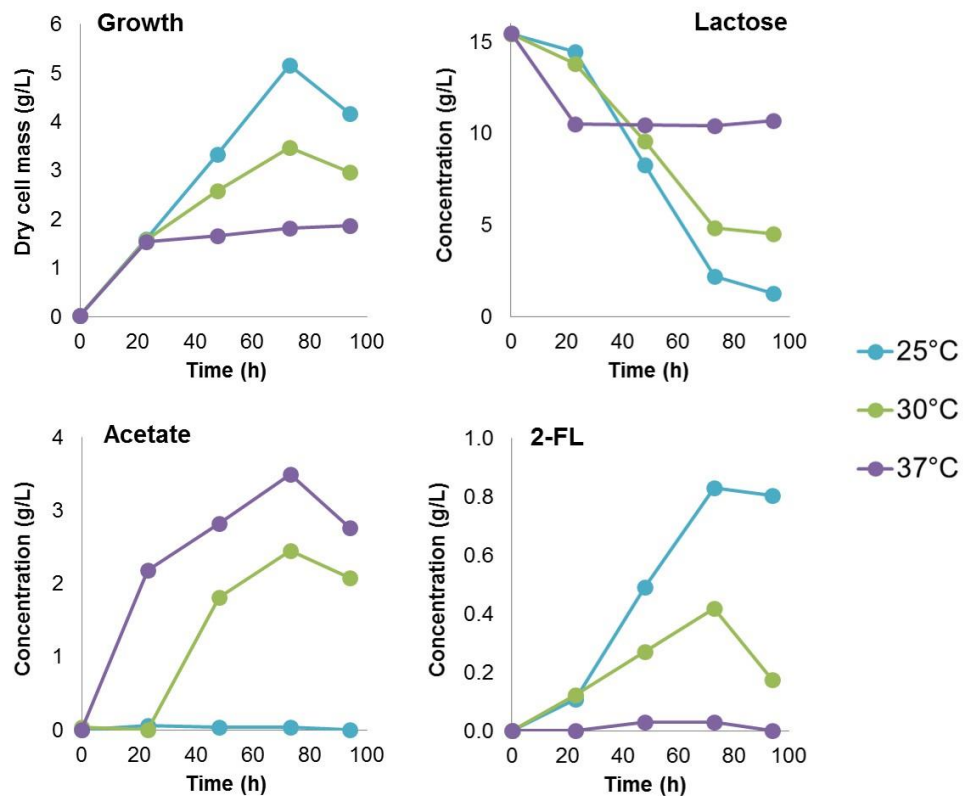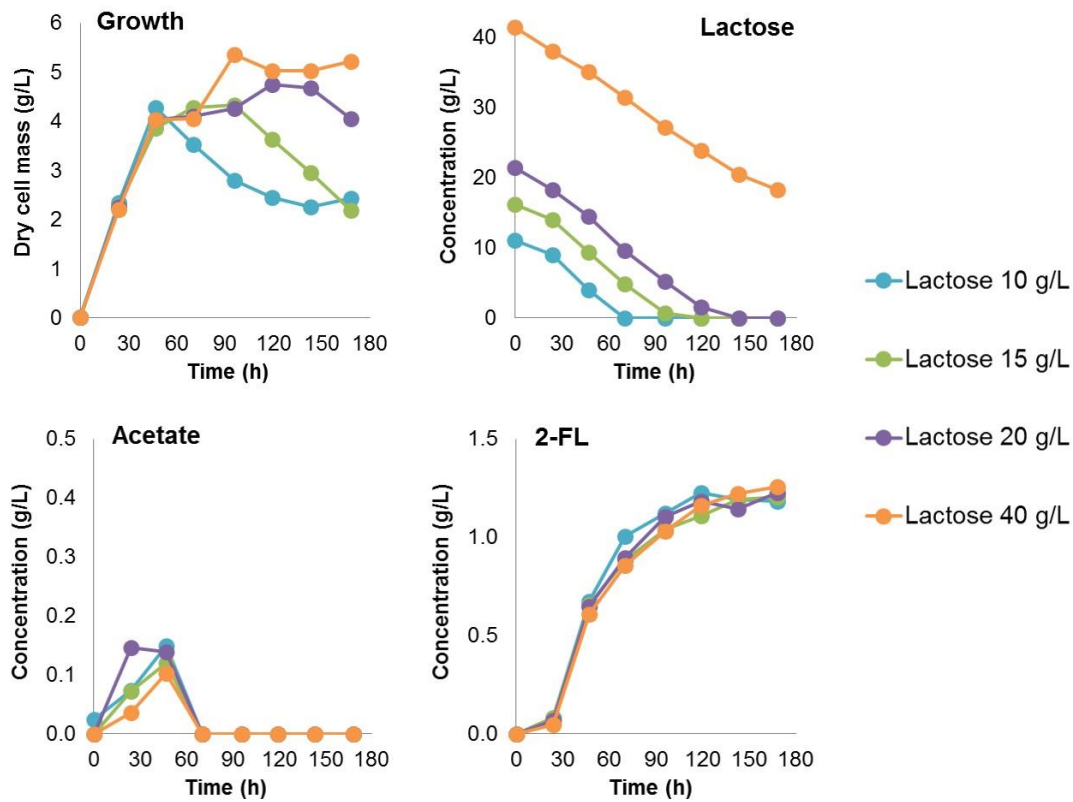
Fig. 3.2 Batch fermentation profiles of recombinant *E. coli* strain JM109(DE3) harboring

plasmids pmBCGW and pHfucT2. Effects of pre-culture and main fermentation temperatures on

2-FL production were studied. The cells were pre-cultured at 37°C. Then, they were grown at

either 25, 30, or 37°C in main fermentation. During main fermentation, cell growth, lactose

consumption, acetate production, and 2-FL production were monitored. Symbols denote as

follows; *light blue circle*, 25°C; *green circle*, 30°C; *purple circle*, 37°C.
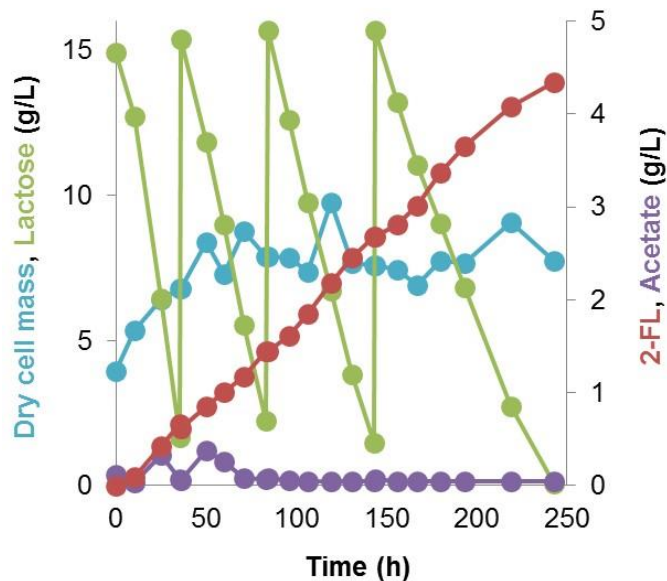
Fig. 3.3 Batch fermentation profiles of recombinant *E. coli* strain JM109(DE3) harboring plasmids pmBCGW and pHfucT2. Effect of IPTG induction on 2-FL production was studied. Two IPTG concentrations (0.1 and 0.5 mM) and two times of induction (at the beginning of fermentation and at Dry Cell Mass (DCM) of 0.216 g/L) were compared. During the fermentation, cell growth, lactose consumption, acetate production, and 2-FL production were monitored. Symbols denote as follows; *light blue circle*, 0.1 mM IPTG induction at the beginning; *green circle*, 0.1 mM IPTG induction at DCM of 0.216 g/L; *purple circle*, 0.5 mM IPTG induction at the beginning; *orange circle*, 0.5 mM IPTG induction at DCM of 0.216 g/L.

Fig. 3.4 Batch fermentation profiles of recombinant *E. coli* strain JM109(DE3) harboring

plasmids pmBCGW and pHfucT2. Effect of lactose availability on 2-FL production was studied.

Lactose concentrations of 10, 15, 20, and 40 g/L were tested. During the fermentation, cell

growth, lactose consumption, acetate production, and 2-FL production were monitored. Symbols

denote as follows; *light blue circle*, lactose 10 g/L; *green circle*, lactose 15 g/L; *purple circle*,

lactose 20 g/L; *orange circle*, lactose 40 g/L.

Fig. 3.5 Fed-batch fermentation profiles of recombinant *E. coli* strain JM109(DE3) harboring plasmids pmBCGW and pHfucT2. Optimized production conditions identified previously (pre-culture 30°C, main fermentation 25°C, 0.1 mM IPTG induction at the beginning of fermentation, and lactose 15 g/L) were employed to initiate the fermentation. More lactose (of ~15 g/L each time) was periodically fed to the culture once it depleted to allow a continuous increase of 2-FL. During the fermentation, cell growth, lactose consumption, acetate production, and 2-FL concentration were monitored. Symbols denote as follows; *light blue circle*, DCM; *green circle*, lactose; *purple circle*, acetate; *red circle*, 2-FL.

Table 3.1 Lists of plasmids used in this study

Name	Sequence of PCR primers and description for plasmids	Source
Plasmids		
pETDuet-1	two T7 promoters with two MCS, pBR322 replicon (copy number ~40), Amp^r	Merck Biosciences
pCOLADuet-1	two T7 promoters with two MCS, ColA replicon (copy number 10~12), Kan^r	Merck Biosciences
pmBCGW	derived from pETDuet-1, P$_{T7}$-*manB-manC* (*Nco*I/*Sac*I)-P$_{T7}$-*gmd-wcaG* (*Nde*I/*Xho*I)-T$_{T7}$, Amp^r	Lee et al., 2009
pHfucT2	derived from pCOLADuet-1, P$_{T7}$-*fucT2* 26695 (*Nco*I/*Sac*I)-P$_{T7}$-MCS2-T$_{T7}$, Kan^r	Lee et al., 2012a

CHAPTER IV   COMPARISON OF DIFFERENT FUCOSYLTRANSFERASES USED

IN 2'-FUCOSYLLACTOSE PRODUCTION

4.1   Introduction

Fucosylation is defined as the transfer reaction of L-fucose from its donor, GDP-L-fucose, to various acceptor molecules including oligosaccharides, glycoproteins, and glycolipids (Oriol et al., 1999). The enzymes which catalyze this reaction are known as fucosyltransferases (FucTs). Fucosylation yields the products containing a fucose unit, fucosylated glycans, which play important roles in many biological and pathological processes (Ma et al., 2006). In eukaryotes, fucosylated glycans are involved in tissue development, angiogenesis, fertilization, cell adhesion, inflammation, and tumor metastasis. Whereas in prokaryotes, fucosylation events occur less frequently, and fucosylated glycans may be associated with molecular mimicry, adhesion, colonization, and modulation of the host immune response. Fucosyltransferases can be categorized into four subfamilies by the position of fucose addition consisting of α1,2, α1,3/4, α1,6, and O-FucTs. The first three groups of FucTs are Golgi-anchored proteins (Nilsson et al., 1993, 1996) while O-FucTs are endoplasmic reticulum (ER)-localized proteins (Luo and Haltiwanger, 2005; Okajima et al., 2005).

In this study, the FucTs used for synthesis of a functional human milk oligosaccharide, 2'-fucosyllactose (2-FL), was evaluated using *Escherichia coli* as a production host. 2-FL has been reported to strengthen the gut health since it possesses a prebiotic property supporting the growth of some Bifidobacteria and prevents the colonization of pathogens (Bode, 2012). The structure of 2-FL contains a fucose unit connected to a lactose molecule. To produce 2-FL, the α1,2 type FucT is required to perform fucosylation of lactose. Normally, α1,2 FucT transfers L-fucose from GDP-L-fucose to the galactose moiety of the acceptor molecules such as N-acetyllactosamine (LacNAc) and lactose at the α1,2 position (Ma et al., 2006) (**Fig. 4.1**). α1,2

fucosylation was reported to exist in mammals such as human, rabbits, and mice, nematode (*Caenorhabditis elegans*), parasite (*Schistosoma mansoni*), and bacteria (Avent, 1997; Wang et al., 1999a; Marques et al., 2001; Zheng et al., 2002). Fucose-containing glycans resulted from α1,2 fucosylation are known to be important in many biological processes. In human, α1,2 FucTs (FUT1 and FUT2) are responsible for synthesis of fucose-containing H-blood group antigens (Avent, 1997). Fucosylated glycoconjugates produced in *C. elegans* may be associated with interactions of the nematode with its environment and serve as ligands for bacterial attachment (Zheng et al., 2008). *S. mansoni* has more than 50% fucose as their glycocalyx components of the total sugars which are thought to play a role in the parasite's life cycle and involve in the immune response and pathogenesis related to *Schistosoma* infection (Cummings and Nyame, 1996, 1999). In bacteria, several putative α1,2 FucTs have been identified in a number of strains including *E. coli*, *Salmonella enterica*, *Yersinia enterocolitica*, and *Helicobacter pylori* (Wang et al., 1999a,b, 2000; Shao et al., 2003; Reeves et al., 2006; Engels and Elling, 2014). Among these, *H. pylori* α1,2 FucTs are the most well-understood enzymes with detailed analysis of the genes, and the main enzymes used in 2-FL synthesis studies thus far (Wang et al., 1999a,b, 2000; Han et al., 2012). The α1,2 FucTs in *H. pylori* are implicated in production of fucose-containing glycoconjugates known as Lewis antigens constituted in their lipopolysaccharides (LPS). The Lewis antigens are believed to concern the pathogenesis and adaptation of this gastric pathogen to the host environment (Wang et al., 1999a).

To take a closer look at the *H. pylori* α1,2 FucTs, their *fucT* genes were described to contain a unique nucleotide arrangement (**Fig. 4.2**). The sequence consists of a poly (C) tract (Berg et al., 1997) and TAA repeats (may also be GAA or AAA) immediately after the poly (C)

sequence (Wang et al., 1999b). The changes in the repeat number of these features contribute to the variation in the genotype of *fucT* (on or off status) in different *H. pylori* strains. Despite the sequence divergence in each strain, two major genotypes can be observed in the *H. pylori* α1,2 FucTs (Wang et al., 1999b). The first type as seen in the strain UA802 has an intact/single full-length open reading frame (ORF). Therefore, the gene status is on and the full-length protein is always produced. The second type as noticed in the strain 26695 comprises two truncated ORFs as a consequence of different number of poly (C) residues (additional 2 C) from the strain UA802 which leads the initiating reading frame to encounter a TGA stop codon, and the remaining reading frame continues at about 110 basepairs further downstream where a potential start codon ATG appears (Wang et al., 1999b; **Fig 4.2B**). The production of the full-length protein by the second type *fucT* can only be possible when a perfect translation frameshift happens. Thus, the gene status in this case can be either on or off suggesting the full-length protein may not always be generated.

Aside from the *H. pylori* α1,2 FucTs, a novel α1,2 FucT encoded by the gene *wbgL* from *E. coli* strain O126 was recently characterized and employed for a successful *in vitro* synthesis of 2-FL (Engels and Elling, 2014). In the study of the substrate acceptance by WbgL, it was revealed that WbgL has a preference for the substrate with β4-linked galactose in the structure such as lactose. Therefore, it is intriguing to further explore the ability of this novel enzyme to produce 2-FL *in vivo*.

In the whole-cell biosynthesis of 2-FL using engineered *E. coli*, three sources of the α1,2 FucT (*H. pylori* UA802 FucT2, *H. pylori* 26695 FucT2, and *E. coli* O126 WbgL) were compared to identify the FucT2 leading to a high titer of 2-FL production. Several sets of fermentation

were conducted to compare the performance of the *E. coli* strains expressing different FucT2. Through a high cell density fed-batch fermentation, an enhanced 2-FL formation can be achieved in the *E. coli* expressing the best FucT2 from this study.

4.2   Materials and methods

4.2.1   Strains and plasmids

E. coli TOP10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] was used for genetic manipulation. *E. coli* JM109(DE3) [*end*A1 *gln*V44 *thi*-1 *rel*A1 *gyr*A96 *rec*A1 *mcr*B$^+$ Δ(*lac-pro*AB) e14- [F' *tra*D36 *pro*AB$^+$ *lacI*^{q} *lac*ZΔM15] *hsd*R17(r$_K$$^-m_K$$^+$) (DE3)] (NEB, Ipswich, MA, USA) and *E. coli* BL21star(DE3)Δ*lacZ* [F$^-$, *omp*T, *hsd*SB(r$_B$$^-m_B$$^-$), *gal*, *dcm rne131* (DE3) Δ*lacZ*] (Chin et al., 2015) were used for production of GDP-L-fucose and 2-FL. Plasmid pmBCGW containing the polycistronic *gmd-wcaG* gene cluster and *manB-manC* gene cluster was previously constructed using plasmid pETDuet-1 (Lee et al., 2009). Plasmid pHfucT2 harboring the gene encoding α1,2 FucT from the *H. pylori* 26695 strain was previously constructed using plasmid pCOLADuet-1 (Lee et al., 2012a).

The gene encoding α1,2 FucT from the *H. pylori* UA802 strain was obtained by the polymerase chain reactions (PCR) using the gBlocks® gene fragment of the α1,2 *fucT* ORF (Wang et al. (1999b)'s published sequence) synthesized from Integrated DNA Technologies, Inc., Coralville, IA, USA as a template. Two PCR primers of *fucT2*-UA802_F and *fucT2*-UA802_R were used for the amplification of the α1,2 *fucT* gene. After digestion of PCR

fragments of the α1,2 *fucT* gene and pCOLADuet-1 (Merck Biosciences, Darmstadt, Germany) with *Nco*I and *Sac*I, the DNA fragments were ligated to construct plasmid pHUAfucT2.

The gene encoding WbgL from *E. coli* O126 strain was obtained by PCR using the gBlocks® gene fragment of the *wbgL* ORF synthesized from Integrated DNA Technologies, Inc. as a template. Two PCR primers of *wbgL*_F and *wbgL*_R were used for the amplification of the *wbgL* gene. After digestion of PCR fragments of the *wbgL* gene and pCOLADuet-1 with *Nco*I and *Sac*I, the DNA fragments were ligated to construct plasmid pEWbgL. Primers and plasmids used in this work are listed in **Table 4.1**. The constructed plasmids were confirmed by DNA sequencing. The conditions for PCR reaction, DNA manipulation, and bacterial transformation followed the descriptions in the previous study (Byun et al., 2007).

4.2.2   Batch fermentation

Batch fermentations were carried out in a small culture tube and a 250 mL shake flask containing 5 mL and 25 mL of Lysogeny Broth (LB) medium with suitable antibiotics at 25°C, respectively. Agitation speed was maintained at 300 rpm. 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture broth at the beginning of fermentation to induce protein expression. 15 g/L lactose was usually added for 2-FL conversion. A co-substrate in addition to lactose, if any, was also tested at 15 g/L concentration.

4.2.3 Fed-batch bioreactor fermentation

Fed-batch fermentation to compare the three FucTs was performed in a 1 L bioreactor (New Brunswick Bioflo®, New Brunswick Scientific, Enfield, CT, USA). The fermentation conditions were 25°C and 500 rpm. LB medium in the presence of appropriate antibiotics was used to grow the cells. pH was controlled at 6.8 using 2 N HCl and 2 N NaOH. 0.1 mM IPTG and initial 15 g/L lactose were added at the beginning of fermentation. Once the initial lactose was almost depleted, more lactose of roughly 15 g/L was replenished twice to allow a continuous increase of 2-FL.

High cell density fed-batch fermentation was performed in a 500 mL bioreactor. The fermentation conditions were 25°C and 500-1200 rpm. A defined medium [13.5 g/L KH_2PO_4, 4.0 g/L $(NH_4)_2HPO_4$, 1.7 g/L citric acid, 1.4 g/L $MgSO_4 \cdot 7H_2O$, 10 mL/L trace element solution (10 g/L Fe(III) citrate, 2.25 g/L $ZnSO_4 \cdot 7H_2O$, 1.0 g/L $CuSO_4 \cdot 5H_2O$, 0.35 g/L $MnSO_4 \cdot H_2O$, 0.23 g/L $Na_2B_4O_7 \cdot 10H_2O$, 0.11 g/L $(NH_4)_6Mo_7O_{24}$, 2.0 g/L $CaCl_2 \cdot 2H_2O$), pH 6.8] containing 20 g/L glycerol, 0.01 g/L Thiamine·HCl, and appropriate antibiotics was used to cultivate the cells. pH was maintained at 6.8 using 28% NH_4OH throughout the fermentation. The high cell density culture was obtained through a limited feeding of either glycerol only or glycerol-mannose. The air flow rate was regulated so that a dissolved oxygen (DO) value can be maintained at above 30%. The specific growth rate of the cells was administered to stay below 0.1 by adjustment of the feeding rate. Once the dry cell mass (DCM) reached 32 g/L, 0.1 mM IPTG and about 22 g/L lactose were supplemented to permit 2-FL conversion.

4.2.4 Analytical methods

Cell concentration was measured by optical density (OD) at 600 nm using a spectrophotometer (Biomate 5, Thermo, NY, USA). Dry cell mass was calculated using an estimate of 0.36 gram cells per OD value of 1. Concentrations of lactose, mannose, glycerol, fructose, xylose, 2-FL, and acetate in batch and fed-batch fermentations were determined using a high performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series) equipped with a Rezex ROA Organic Acid H^+ column (Phenomenex, Torrance, CA, USA) and a refractive index (RI) detector (Agilent, Palo Alto, CA, USA). The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min at 50°C.

4.3 Results

4.3.1 Production of 2-FL in engineered *E. coli* JM109(DE3) expressing different fucosyltransferases in shake flask fermentation

The performance of the engineered JM109(DE3) overexpressing the GDP-L-fucose synthesizing genes and expressing different *fucT2* (one-ORF *H. pylori fucT2,* two-ORF *H. pylori fucT2,* or *E. coli fucT2* (*wbgL*)) on 2-FL production was evaluated (**Fig. 4.3**). 15 g/L lactose was supplemented in the culture medium for growth and 2-FL conversion.

In terms of cell growth, the one-ORF strain grew from DCM 0.036 to 4.9 g/L within 80 h. Within the same timeframe, the two-ORF strain multiplied a little slower to DCM 4.5 g/L

from the same initial cell concentration. The WbgL strain grew as well as the other two strains during the exponential phase, but later reached a lower DCM (3.9 g/L) than the others.

In regard to lactose consumption, the two *H. pylori* FucT2 strains behaved similarly and had almost the same rate of lactose fermentation (15 g/L was consumed within about 80 h). Interestingly, the WbgL strain assimilated lactose significantly faster than the other two strains (lactose was all finished in an estimate of 70 h).

Acetate is an unwanted by-product commonly excreted by *E. coli* in aerobic fermentation (Farmer and Liao, 1997). Acetate formation occurs as an outcome of rapid sugar consumption causing the overflow of carbon flux from acetyl-coenzyme A (CoA) to acetate instead of Tricarboxylic acid (TCA) cycle (Hollywood and Doelle, 1976). Between the *H. pylori* FucT2 strains, the one-ORF strain generated a higher acetate (0.21 g/L) than the two-ORF strain (0.08 g/L) during the fast lactose consumption period. However, acetate was re-assimilated by the cells afterward. Surprisingly, the WbgL strain did not secrete any acetate over the course of fermentation when it had the highest lactose assimilation rate.

Regarding 2-FL synthesis, the strain harboring one-ORF FucT2 produced the highest 2-FL (1.1 g/L). The two-ORF and WbgL strains produced 0.8 and 0.4 g/L of 2-FL, respectively.

4.3.2 Production of 2-FL in engineered *E. coli* JM109(DE3) expressing different fucosyltransferases in fed-batch bioreactor fermentation

The engineered JM109(DE3) overexpressing the GDP-L-fucose synthesizing genes and expressing different *fucT2* (one-ORF *H. pylori fucT2,* two-ORF *H. pylori fucT2,* or *E. coli fucT2* (*wbgL*)) was further assessed in a larger scale fermentation (1 L) to confirm the best FucT2

contributing to the highest amount of 2-FL (**Fig. 4.4**). 15 g/L lactose was supplemented in the culture medium at the beginning and continued to replenish once the previous supply was almost depleted to allow a higher accumulation of 2-FL.

In terms of cell growth, the one-ORF and two-ORF strains grew similarly to DCM 5.4 g/L. The WbgL strain grew not as well as the other two strains reaching DCM of 4.3 g/L.

In regard to lactose consumption, the two *H. pylori* FucT2 strains consumed lactose at almost the same rate throughout the fermentation. The WbgL strain displayed a slightly faster lactose assimilation rate in the initial round of lactose. However, its speed in consumption seemed to start slowing down as more lactose was added.

Regarding acetate accumulation, only a negligible amount of acetate (0.1 g/L) was detected in the two-ORF strain. No acetate was accumulated in this experiment possibly due to a highly-aerobic condition in a bioreactor which has continuous high rpm agitation and air coming in the vessel.

 For 2-FL synthesis, the strain harboring the one-ORF FucT2 is clearly the best among the three strains with a 2-FL titer of 4.2 g/L. The two-ORF and WbgL strains produced 2.2 and 2.1 g/L of 2-FL, respectively.

4.3.3   Production of 2-FL in engineered *E. coli* BL21star(DE3)Δ*lacZ* expressing the *H. pylori* one-ORF fucosyltransferase in the presence of lactose and a co-substrate

To further increase the 2-FL or any product titer in the whole-cell biosynthesis system, a high cell density fermentation technique is generally exploited. By taking advantage of a high cell concentration, the overall production of a target compound can be enhanced from the sum of

the product produced by each cell. Nevertheless, the *E. coli* JM109(DE3) may not be a good host strain for the high cell density experiment. The JM109 strain usually secretes a lot of exopolysaccharides (EPS) to the surrounding medium. When the strain is grown to a high cell density, an even larger amount of EPS produced from a numerous amount of cells can be observed. The excess amount of EPS creates a viscous growth medium leading to an inefficient oxygen transfer and acetate build-up. For these reasons, the host strain for 2-FL production was altered to *E. coli* BL21star(DE3)Δ*lacZ*. The BL21 strain has been widely employed for a successful high cell density fermentation (Lee, 1996).

To thoroughly understand the discrepancy of the new host strain, BL21, and the previous strain, JM109, in detail, the BL21 strain series naturally ferment lactose efficiently owing to a fully active *lac* operon unlike the JM109 strain. The JM109 strain is actually known to be unable to utilize lactose due to an M15 mutation (an alpha region of β-galactosidase (*lacZ*) is deleted). However, the DE3 portion in JM109(DE3) strain contains the alpha sequence of *lacZ* in addition to the T7 RNA polymerase allowing the JM109(DE3) strain to grow on lactose through the alpha complementation although at a slower rate than other natural lactose-fermenting *E. coli* (Dumon et al., 2004). In order to produce 2-FL using the BL21 strain, the BL21star(DE3) with deletion of *lacZ* gene (BL21star(DE3)Δ*lacZ*) from Chin et al. (2015) was selected. The Δ*lacZ* strain was used as a host strain in order to preserve intact lactose for 2-FL conversion. The wild-type strain would instead hydrolyze lactose and utilize it for cell growth rather than conserving it for 2-FL synthesis (Lee et al., 2012a).

It was predicted that the new host, BL21star(DE3)Δ*lacZ*, might not be able to grow on lactose as a sole carbon source because of the absence of *lacZ* gene. Consequently, a co-sugar

substrate in addition to lactose might be needed to support cell growth while lactose is mostly used to convert to 2-FL. In this fermentation, four different co-substrates comprising mannose, fructose, glycerol, and xylose were tested to find out the co-substrate which worked well with lactose to produce the highest amount of 2-FL (**Fig. 4.5**). As anticipated, the BL21star(DE3) Δ*lacZ* was not able to grow on lactose only. The best co-substrate was mannose which contributed to the highest titer of 2-FL (1.6 g/L) with the least amount of acetate. The second best co-feeding sugar was xylose which had about 0.5 g/L 2-FL. However, 2-FL was barely produced under fructose and glycerol conditions.

4.3.4   High cell density fermentation of 2-FL in engineered *E*. *coli* BL21star(DE3)Δ*lacZ* expressing the *H. pylori* one-ORF fucosyltransferase under the sole glycerol and the glycerol-mannose feeding

As mannose was discovered to be a good co-substrate benefiting 2-FL production, supplementation of mannose after IPTG induction in a high cell density fermentation was thought to enhance the GDP-L-fucose production leading to an improved 2-FL production. Mannose was not employed to support cell growth from the beginning of fermentation because a continuous feeding of a concentrated mannose solution would be relatively expensive. Instead, glycerol was decided as the main feeding sugar to bring the culture to the high cell density. Glycerol has been successfully used to achieve the high cell density culture of *E. coli* by previous researchers (Baumgartner et al., 2013; Chin et al., 2015). Moreover, the growth of *E. coli* on glycerol is generally slower than that on glucose. Acetate production can be minimized in a slower sugar metabolism condition.

In this high cell density fermentation experiments, effect of a mannose boost on 2-FL production was evaluated. **Fig. 4.6** and **4.7** display the fermentation profiles of the sole glycerol feeding and the glycerol-mannose feeding conditions, respectively. In regard to cell growth, the *E. coli* BL21star(DE3)Δ*lacZ* was able to grow under a sugar-limited feeding to the final DCM of 76.3 g/L in both conditions. Glycerol feeding was regulated in order to maintain the cell specific growth rate (µ) below 0.1. Hence, the glycerol concentration was kept close to zero throughout the fermentation to avoid the too high µ and a rapid sugar metabolism which may result in acetate production. However, the accumulation of glycerol was observed during the early exponential growth despite the careful control of glycerol feeding rate. This glycerol build-up led to acetate production of 1.2 and 0.5 g/L in the sole glycerol and glycerol-mannose conditions, respectively. After the pause of sugar feeding, acetate was re-assimilated by the cells. Once the culture reached the DCM of about 32 g/L, 0.1 mM IPTG and about 22 g/L lactose were added to the culture medium to allow the protein expression and 2-FL conversion, respectively. In the glycerol-mannose feeding, the feeding carbon source was changed from glycerol to mannose after IPTG induction. The mannose boost lasted about 30 h and the feeding sugar was switched back to glycerol. Interestingly, the *E. coli* BL21star(DE3)Δ*lacZ* in both conditions produced the same 9 g/L of 2-FL by the end of the fermentation. This result suggested that the mannose boost was not really important to achieve an enhanced 2-FL production in the high cell density fermentation. The yields of 2-FL (g 2-FL/g lactose) are 0.2 and 0.4 in the sole glycerol and glycerol-mannose conditions, respectively.

4.4   Discussion

Fucosyltransferases (FucTs) are a group of enzymes which catalyze the transfer reaction of fucose from GDP-L-fucose to various acceptor molecules such as carbohydrates, protein, and lipids. These fucose-containing glycoconjugates are described to be crucial in many biological processes mostly involved in antigen synthesis, pathogenesis, and adaptation to host enviroment of the organism. In the context of 2-FL production, FucT which transfers fucose to α1,2 position of the acceptor molecule known as α-1,2-fucosyltransferase (FucT2) is of interest. FucT2 from *Helicobacter pylori* is the most well-understood and detailedly characterized. Two major genotypes of *H. pylori* FucT2 were identified. The first genotype as seen in *H. pylori* strain UA802 contains a single full-length ORF. The second genotype as seen in *H. pylori* strain 26695 consists of two truncated ORFs. The production of a full-length protein in the second case relies solely on a perfect translation frameshift. Previously, many research groups successfully produced 2-FL by means of whole-cell biosynthesis using FucT2 from strain 26695 with two-ORF genotype (Drouillard et al., 2006; Lee et al., 2012a; Baumgartner et al., 2013; Chin et al., 2015). Despite the dependency of the full-length protein production on the perfect frameshift, over 20 g/L of 2-FL was excellently made in a fed-batch fermentation using FucT2 with two-ORF version (Baumgartner et al., 2013). Besides, the recently characterized FucT2 from *E. coli* strain O126, WbgL, is also in spotlight. 2-FL was successfully produced in the *in vitro* assay containing the crude extract of the *E. coli* expressing *wbgL* (Engels and Elling, 2014).

In this study, FucT2 from a different strain of *H. pylori*, UA802, and the novel FucT2 from *E. coli* O126 (WbgL) were compared with FucT2 from the strain 26695 for the first time in regard to 2-FL production. In shake flask fermentation of JM109(DE3) expressing *fucT2* from

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three different origins, the one-ORF strain performed the best resulting in 2-FL production of 1.1 g/L from 15 g/L lactose. The apparent advantage of the one-ORF FucT2 over the two-ORF FucT2 is that its produced protein is always functional. A greater amount of functional FucT2 protein would contribute to the more efficient conversion of lactose to 2-FL. The result suggested that the event of the perfect translation frameshift in the two-ORF genotype might happen at about 70% chance since the 2-FL made by the two-ORF strain was about 70% of the one-ORF strain. So far it is unknown about the frequency of the perfect frameshift and whether the number of the frameshift occurrence is consistent every time. With the goal to overproduce 2-FL, expression of the one-ORF FucT2 might be more reliable. For the WbgL strain, it is unclear why the strain produced the least 2-FL. The *E. coli* expressing WbgL fermented lactose the fastest. This may be explained by the high affinity of WbgL toward lactose as an acceptor as described by Engels and Elling (2014). If this is really the case, it is still confusing where exactly the consumed lactose went since it did not seem to go to biomass, 2-FL, nor acetate.

In a fed-batch fermentation in a bioreactor to compare the three FucT2s, the one-ORF FucT2 strain was proved to be the best among all the tested FucT2. The final titer of 2-FL (4.2 g/L) from this strain was two times greater than that (2.1-2.2 g/L) from the two-ORF and WbgL strains. The exact explanation for the superiority of the one-ORF strain over the other two strains cannot be concluded without the future experiments to look at the quantity and expression of the three proteins in detail.

To further improve the 2-FL or any product titer, the technique of using a high cell density culture is often selected by many researchers. The host strain to produce 2-FL had to be switched from JM109(DE3) to BL21star(DE3)Δ*lacZ* because the JM109 strain had difficulty

going to a high cell density due to the culture viscosity. Hence, the BL21 strain with deactivation of *lacZ* was chosen for the experiment in order to conserve intact lactose for 2-FL synthesis. However, the BL21star(DE3)Δ*lacZ* needed a co-sugar substrate beside lactose to grow. Mannose was found to be the best among the tested sugars yielding the highest amount of 2-FL (1.6 g/L). This is probably because mannose is the closest sugar to the synthesis of GDP-D-mannose, a precursor of GDP-L-fucose. Xylose showed a potential to be a great co-substrate in 2-FL production with the second highest concentration of 2-FL (0.5 g/L). It may be possible that xylose is a non-repressing sugar so it permits a more efficient lactose uptake than other tested sugars. In *Saccharomyces cerevisiae*, xylose has been declared to be a good carbon source to produce products other than ethanol (Kwak et al., in preparation). In fructose and glycerol conditions, 2-FL production was not noticed. Instead, a considerable amount of acetate of over 1 g/L was generated. Acetate is known to negatively affect the recombinant protein production. It is doubtful whether acetate was the true reason for no 2-FL production or the sugars themselves are the problems. If this fermentation was performed in a better aerated condition (i.e. a bioreactor), the acetate formation could have been less and some 2-FL might have beeen made. Therefore, repetition of the experiments in the future may be considered to confirm the results.

In a high cell density fed-batch fermentation with the one-ORF FucT2 strain, the mannose boost was not really helpful to increase the 2-FL production. It is possible that the boost did not last a long time (30 h) so the benefit of the boost on 2-FL synthesis was not obvious. In the future endeavor, GDP-L-fucose concentration inside the cells may be measured to determine whether the mannose addition literally increases the GDP-L-fucose concentration as compared with the condition without mannose. Although the final titer of 2-FL from the two feeding

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conditions (glycerol only and glycerol-mannose) is the same (9 g/L), the yield of 2-FL from lactose in the glycerol-mannose feeding is better (0.4 vs. 0.2 g/g). This indicated the need of less amount of lactose in the glycerol-mannose condition to get the same amount of 2-FL formation as the glycerol only condition. However, the utilization of more lactose is not a concern in reality since lactose is an inexpensive carbon source. Mannose, in fact, is much more costly than lactose.

Based on the results from this study, *H. pylori* one-ORF FucT2 (strain UA802) is the best choice to be used in constructing a 2-FL-producing *E. coli*. The genotype background with one full-length ORF guarantees the consistent production of a full-length protein leading to a better and more stable 2-FL production. The two-ORF situation depends on the uncertainty of the perfect frameshift, thus a large variation of 2-FL production may be expected. Little knowledge is known about the *wbgL* expression in *E. coli*. More research related to protein expression of these enzymes may be needed. Nevertheless, future studies should be directed toward improvement of the FucT2 protein expression in a soluble form to maximize 2-FL production as often times inclusion bodies have been a persistent problem in protein expression in *E. coli*.

4.5   Figures and tables
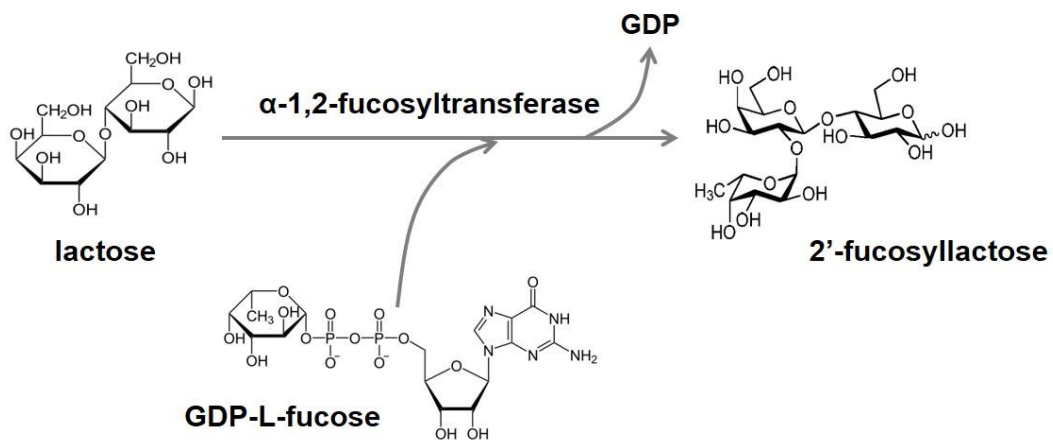
Fig. 4.1 The α-1,2-fucosyltransferase (FucT2) activity in 2'-fucosyllactose (2-FL) production.

FucT2 catalyzes the transfer reaction of L-fucose from GDP-L-fucose onto lactose at a 2'

position of the galactose unit to convert to 2-FL. The released GDP can be recycled to synthesize

more GDP-L-fucose.
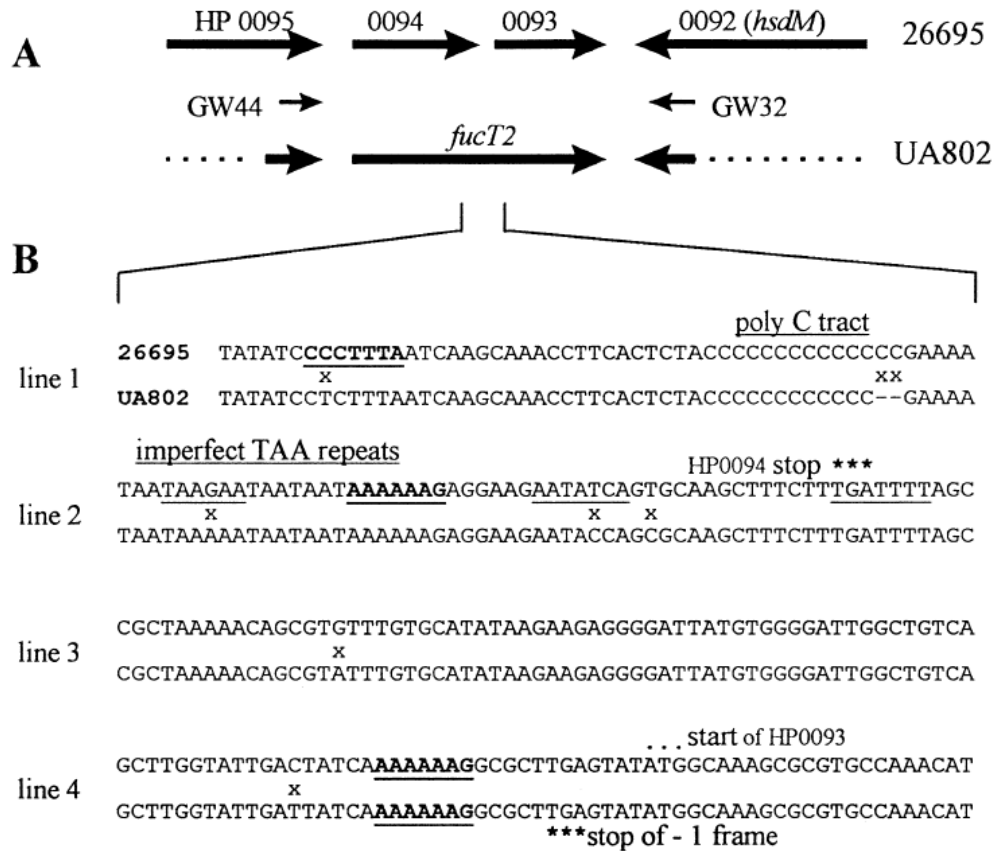
Fig. 4.2 Different open reading frame (ORF) structures of FucT2 in strains 26695 and UA802. 26695 ORF is split into two ORFs of 0094 and 0093 while UA802 ORF is complete as one full-length ORF (**A**). The hypermutable region of *fucT2* sequence. 26695 has extra 2 C bases during the poly C tract. This incident leads 26695 to encounter a TGA stop codon in the initiating reading frame. In the further downstream sequence, an ATG start codon appears which is the beginning of the remaining (second) reading frame (**B**) (Wang et al., 1999b).
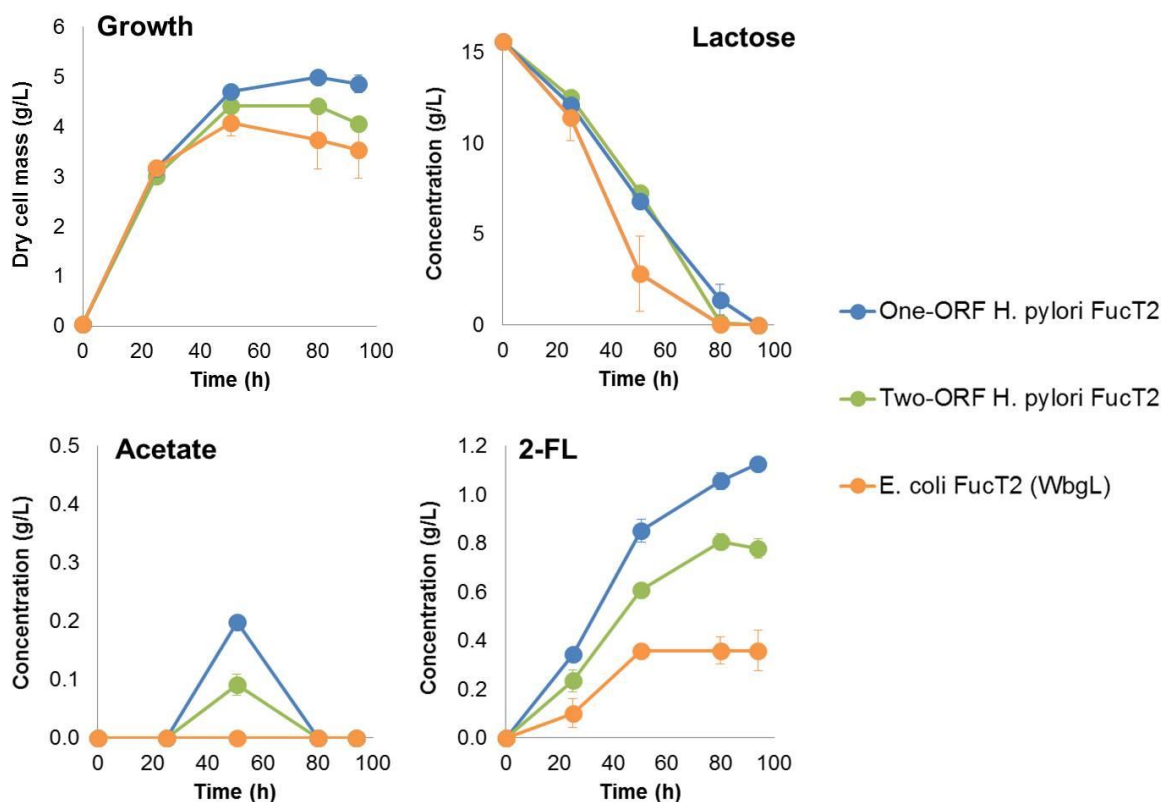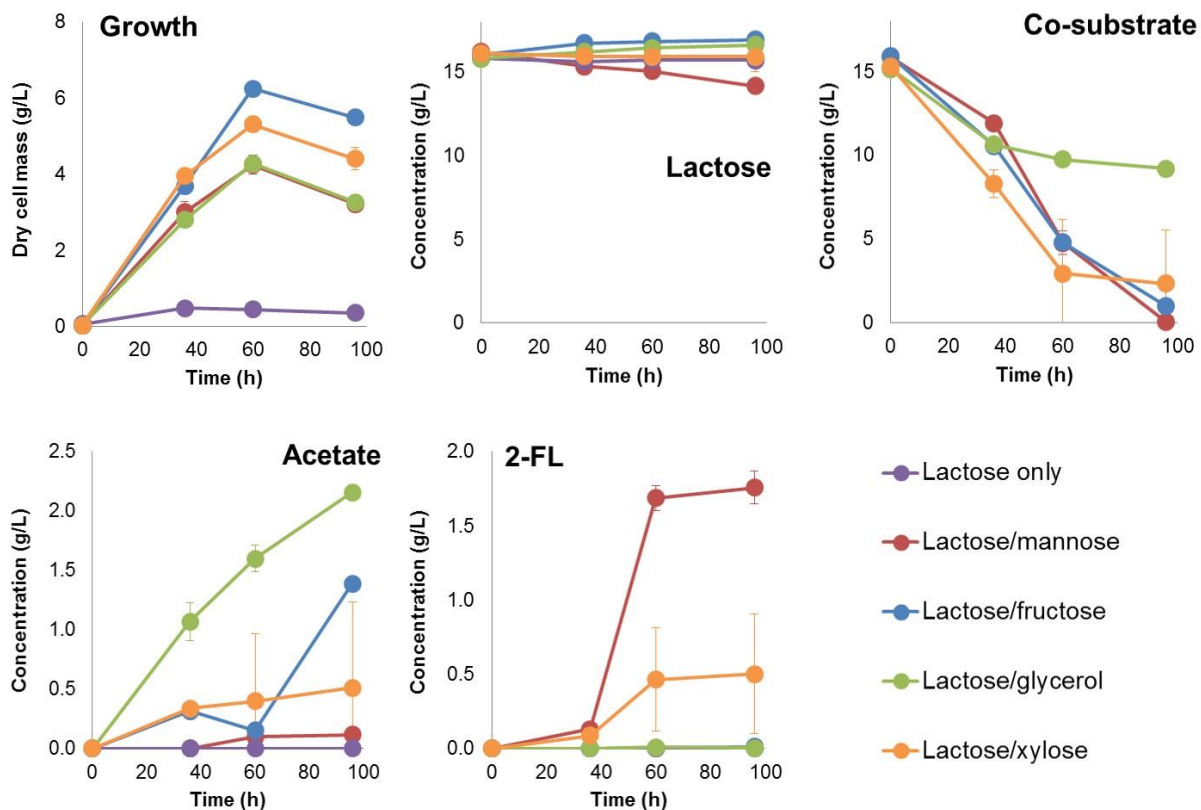
Fig. 4.3 Batch fermentation profiles of recombinant *E. coli* JM109(DE3) harboring plasmid pmBCGW and a different plasmid for FucT2 expression consisting of pHUAfucT2 (one-ORF *H. pylori* FucT2 UA802), pHfucT2 (two-ORF *H. pylori* FucT2 26695), and pEWbgL (*E. coli* O126 FucT2 (WbgL)). Fermentations were performed in shake flasks in the presence of 15 g/L lactose. 2-FL formation by *E. coli* expressing different FucT2 was compared. Line colors on the charts denote as follows; *blue*, one-ORF *H. pylori* FucT2; *green*, two-ORF *H. pylori* FucT2; *orange*, *E. coli* FucT2 (WbgL). Measurement of cell, lactose, acetate, and 2-FL concentrations was calculated from two independent experiments.
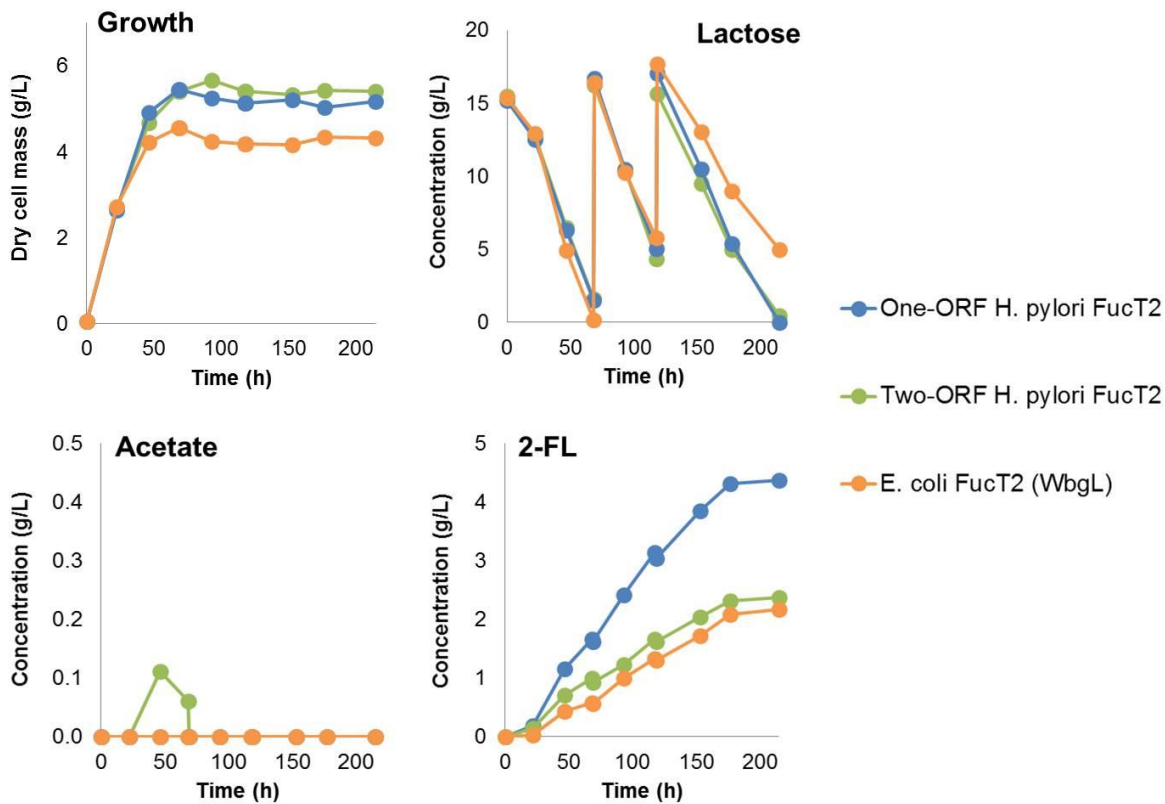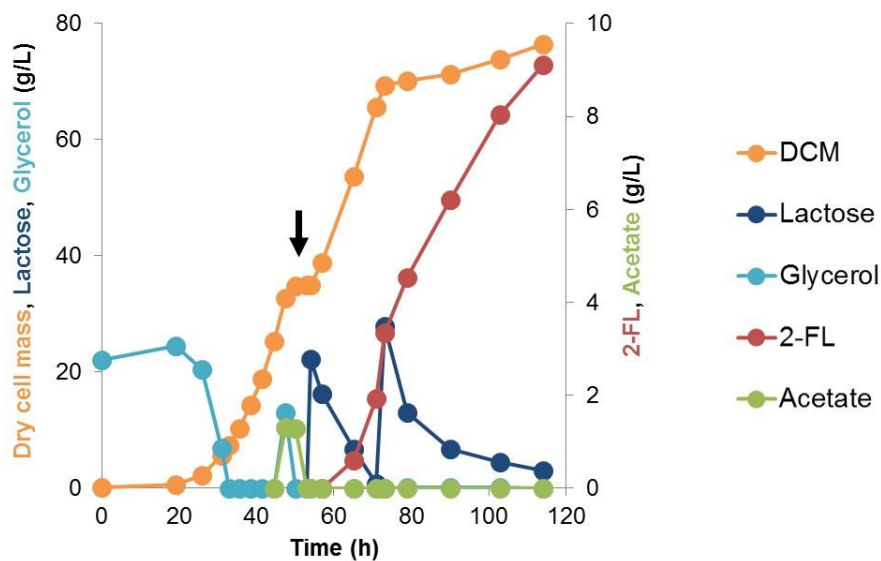
Fig. 4.4 Fed-batch fermentation profiles of recombinant *E. coli* JM109(DE3) harboring plasmids pmBCGW and a different plasmid for FucT2 expression consisting of pHUAfucT2 (one-ORF *H. pylori* FucT2 UA802), pHfucT2 (two-ORF *H. pylori* FucT2 26695), and pEWbgL (*E. coli* O126 FucT2 (WbgL)). Fermentations were performed in a 1 L bioreactor in the presence of initial 15 g/L lactose. More lactose (of ~15 g/L each round) was periodically fed to the culture once it depleted to allow a continuous increase of 2-FL. Throughout the fermentation, cell growth, lactose consumption, acetate production, and 2-FL concentration were monitored. Line colors on the charts denote as follows; *blue*, one-ORF *H. pylori* FucT2; *green*, two-ORF *H. pylori* FucT2; *orange*, *E. coli* FucT2 (WbgL).
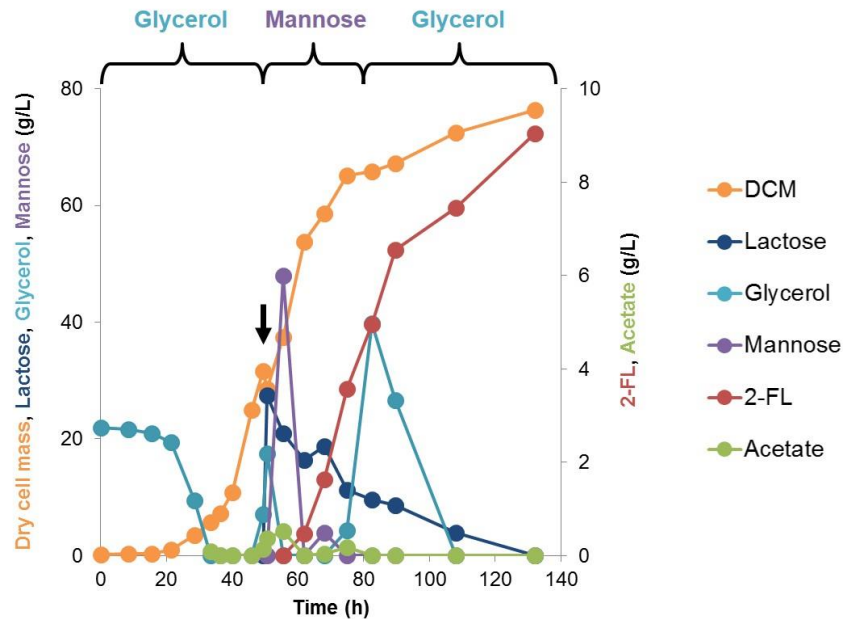
Fig. 4.5 Batch fermentation profiles of recombinant *E. coli* BL21star(DE3)Δ*lacZ* harboring

plasmids pmBCGW and pHUAfucT2 (one-ORF *H. pylori* FucT2 UA802). Fermentations were

performed in a 5 mL culture tube in the presence of 15 g/L lactose and 15 g/L of different co-

substrates. 2-FL formation in the presence of different co-substrates was compared. The co-

substrates studied included mannose, fructose, glycerol, and xylose. Line colors on the charts

denote as follows; *purple*, lactose only; *red*, lactose and mannose; *blue*, lactose and fructose;

green, lactose and glycerol; *orange*, lactose and xylose. Measurement of cell, lactose, mannose,

fructose, glycerol, xylose, acetate, and 2-FL concentrations was calculated from two independent

experiments.

Fig. 4.6 Glycerol-limited fed-batch fermentation profiles of recombinant *E. coli*

BL21star(DE3)Δ*lacZ* harboring plasmids pmBCGW and pHUAfucT2 (one-ORF *H. pylori*

FucT2 UA802). Fermentation was performed in a 500 mL bioreactor in the presence of initial 20

g/L glycerol. Glycerol-limited feeding was started after the initial glycerol was exhausted. The

feeding rate was progressively altered and controlled in order to maintain the cell specific growth

rate of below 0.1. Once the dry cell mass (DCM) reached 32, 0.1 mM IPTG and 22 g/L lactose

were added to induce protein expression and 2-FL conversion, respectively. Glycerol was solely

used to support the cell growth throughout the fermentation. Line colors on the chart denote as

follows; *orange*, DCM; *dark blue*, lactose; *light blue*, glycerol; *red*, 2-FL; *green*, acetate. A black

arrow indicated the time of IPTG and lactose addition.

Fig. 4.7 Glycerol-mannose-limited fed-batch fermentation profiles of recombinant *E. coli* BL21star(DE3)Δ*lacZ* harboring plasmids pmBCGW and pHUAfucT2 (one-ORF *H. pylori* FucT2 UA802). Fermentation was performed in a 500 mL bioreactor in the presence of initial 20 g/L glycerol. Glycerol-limited feeding was started after the initial glycerol was exhausted. The feeding rate was progressively altered and controlled in order to maintain the cell specific growth rate of below 0.1. Once the dry cell mass (DCM) reached 32, 0.1 mM IPTG and 23 g/L lactose were added to induce protein expression and 2-FL conversion, respectively. The feeding sugar was also changed from glycerol to mannose. The impact of mannose on boosting 2-FL production was interested. Mannose was used to support cell growth for about 30 h. Then, the feeding carbon source was switched back to glycerol for the rest of the fermentation. Line colors on the chart denote as follows; *orange*, DCM; *dark blue*, lactose; *light blue*, glycerol; *purple*, mannose; *red*, 2-FL; *green*, acetate. A black arrow indicated the time of IPTG and lactose addition.

Table 4.1 Lists of primers and plasmids used in this study

Name	Sequence of PCR primers and description for plasmids	Source
PCR primers		
fucT2-UA802_F (*Nco*I)	5'-TCTACAGCGGCCGC<u>CCATGG</u>GCGCCTTCAAA-3'	*H. pylori* UA802
fucT2-UA802_R (*Sac*I)	5'-TACAACGCA<u>GAGCTC</u>GCGGCCGCTCTATA-3'	
*wbgL*_F (*Nco*I)	5'-TCTACAGCGGCCGC<u>CCATGG</u>GCAGCATCATT-3'	*E. coli* O126
*wbgL*_R (*Sac*I)	5'-AGCTGTTAA<u>GAGCTC</u>GCGGCCGCTCTATA-3'	
Plasmids		
pETDuet-1	two T7 promoters with two MCS, pBR322 replicon (copy number ~40), Amp^r	Merck Biosciences
pCOLADuet-1	two T7 promoters with two MCS, ColA replicon (copy number 10~12), Kan^r	Merck Biosciences
pmBCGW	derived from pETDuet-1, P_{T7}-*manB-manC* (*Nco*I/*Sac*I)-P_{T7}-*gmd-wcaG* (*Nde*I/*Xho*I)-T_{T7}, Amp^r	Lee et al., 2009
pHfucT2	derived from pCOLADuet-1, P_{T7}-*fucT2* 26695 (*Nco*I/*Sac*I)-P_{T7}-MCS2-T_{T7}, Kan^r	Lee et al., 2012a
pHUAfucT2	derived from pCOLADuet-1, P_{T7}-*fucT2* UA802 (*Nco*I/*Sac*I)-P_{T7}-MCS2-T_{T7}, Kan^r	This study
pEWbgL	derived from pCOLADuet-1, P_{T7}-*fucT2* UA802 (*Nco*I/*Sac*I)-P_{T7}-MCS2-T_{T7}, Kan^r	This study

CHAPTER V   ATTEMPT TO PRODUCE 2'-FUCOSYLLACTOSE IN

SACCHAROMYCES CEREVISIAE

5.1   Introduction

Saccharomyces cerevisiae has a long history in fermentation and baking. It is naturally known as an ethanol producer as lected in the products derived from this yeast in food and beverage industries. By manipulation of the cell metabolism, the exploitation of this yeast can be broadened to create numerous products other than ethanol such as lactic acid (Skory, 2003) and xylitol (Hallborn et al., 1991). As a host strain to produce value-added products, the popularity of *S. cerevisiae* has been increasing owing to the ease of its cultivation, the wide availability of its genetic tools, and its Generally Recognized As Safe (GRAS) status.

One interesting area remaining unexplored in *S. cerevisiae* is production of human milk oligosaccharides (HMOs). HMOs confer many health benefits to the host including stimulating the growth of select *Bifidobacteria* (prebiotic effect), serving as receptor analogs for pathogens, and modulating immune responses (Bode, 2012). Synthesis of various HMOs has been attempted mostly in *Escherichia coli* and *Corynebacterium glutamicum* (Han et al., 2012). *S. cerevisiae,* in fact, could be a great host to produce certain HMOs such as fucosylated HMOs due to a few reasons. It has a rich intracellular pool of GDP-D-mannose since mannosylation is the major glycosylation reaction occurred in yeast (Mattila et al., 2000). As GDP-D-mannose is a substrate of GDP-L-fucose in the *de novo* pathway, this gives an advantage to produce GDP-L-fucose, a donor of L-fucose, required in fucosylation reactions. As a GRAS organism, *S. cerevisiae* secreting HMOs may replace the traditional yeast to generate functional food products without the need to purify HMOs out of the fermentation or the concern regarding enterotoxin carryover like in *E. coli*.

2'-fucosyllactose (2-FL), one of the most studied HMOs, has the most basic structure of fucosylated HMOs with one fucose unit connected to lactose on the galactose end. 2-FL has been demonstrated the protective activity against several pathogenic microorganisms such as *Campylobacter jejuni*, Enteropathogenic *E. coli*, *Salmonella enterica*, and Norovirus (Etzold and Bode, 2014; Weichert et al., 2013). A great success was achieved in whole-cell biosynthesis of 2-FL using *E. coli* with the highest titer of 20.3 g/L (Baumgartner et al., 2013). To date, no 2-FL synthesis in yeast has been recorded. As discussed previously, it would be worthwhile to enable yeast to produce 2-FL since this yeast can be employed in miscellaneous applications and provide additional health benefits and values to the products.

To construct an *S. cerevisiae* strain capable of producing 2-FL, at least three goals must be accomplished (**Fig. 5.1**). First, as a primary substrate for 2-FL synthesis, GDP-L-fucose needs to be intracellularly available. Two heterologous genes consisting of GDP-D-mannose-4, 6-dehydratase (*gmd*) and GDP-4-keto-6-deoxymannose-3, 5-epimerase-4-reductase (*wcaG*) are obliged to convert the inherent GDP-D-mannose into GDP-L-fucose (Mattila et al., 2000). Additionally, GDP-L-fucose production may be enhanced by further genetic perturbations. For instance, GDP-D-mannose pyrophosphorylase (*PSA1*) is the enzyme which catalyzes the final step of GDP-D-mannose formation. Overexpression of PSA1 was claimed to raise the cellular content of GDP-D-mannose (Janik et al., 2003). Hence, *PSA1* may be an interesting gene target for improvement of GDP-L-fucose concentration through the increase of GDP-D-mannose. Another prospective target, GDA1, which encodes for guanosine diphosphatase (GDPase), involves in the translocation of GDP-D-mannose into the golgi (**Fig. 5.2**). In normal *S. cerevisiae*, once GDP-D-mannose is produced in the cytosol, it is then transported into the golgi

to supply mannose for various mannosylation reactions. However, this migration of GDP-D-mannose to the golgi is unfavorable for GDP-L-fucose production because the cytosolic availability of GDP-D-mannose becomes insufficient for converting to GDP-L-fucose. The GDP-D-mannose golgi transporter is an antiporter type which demands the exit of GMP to the cytosol as GDP-D-mannose enters the golgi. GDA1 plays a role in converting GDP to GMP when GDP is released from GDP-D-mannose inside the golgi. Deletion of GDA1 causes an inadequacy of GMP export which subsequently leads to the 5-fold decrease of the GDP-D-mannose import into the golgi lumen (Berninsone et al., 1994). Accordingly, GDA1 knockout may be useful for GDP-L-fucose synthesis since GDP-D-mannose retains more in the cytosol.

Second, another substrate, lactose, should be transported into the cytosol where GDP-L-fucose is made. Native *S. cerevisiae* cannot metabolize nor import lactose. Further genetic modifications are then necessary to acquire the *S. cerevisiae* strain able to ingest lactose. A lactose permease from other organisms such as LacY from *E. coli*, LAC12 from *Kluyveromyces lactis* (Chang and Dickson, 1988), or CDT-1 from *Neurospora crassa* (Galagan et al., 2003) needs to be introduced into the wild-type *S. cerevisiae* in order to permit the uptake of the external lactose into the cytosol. A β-galactosidase (β-gal) which breaks down lactose into glucose and galactose is presumably needless in this situation. The intact lactose should be preserved for conversion to 2-FL. In addition, a successful production of 2-FL in *E. coli* with deactivation of the β-gal has been validated indicating the probable nonnecessity of the β-gal in the yeast system (Chin et al., 2015).

Third, the α-1,2-fucosyltransferase (FucT2) enzyme which catalyzes the transfer reaction of L-fucose from GDP-L-fucose to lactose through an α-1,2 linkage has to be expressed in the

cytosol. Since *S. cerevisiae* does not naturally perform fucosylation, an insertion of the heterologous FucT2 is unavoidable. So far, only FucT2s from *Helicobacter pylori* (Albermann et al., 2001; Baumgartner et al., 2013; Chin et al., 2015; Drouillard et al., 2006; Lee et al., 2012a) and a few *E. coli* strains (Engels and Elling, 2014; Shao et al., 2003) have been studied in the context of 2-FL biosynthesis.

In this study, the three elements anticipated to induce 2-FL synthesis were introduced in *S. cerevisiae* separately for the initial confirmation that each part was functionally expressed. First, GMD and WcaG from *E. coli* K-12 were expressed in *S. cerevisiae* to empower GDP-L-fucose production. Moreover, enhanced GDP-L-fucose production was accomplished. The effect of two mutations including PSA1 overexpression and GDA1 knockout on GDP-L-fucose production was investigated. Second, lactose transporters including CDT-1 and LAC12 were coupled with GH1-1 (a β-glucosidase which was discovered to also exhibit the β-gal activity; Liu et al., 2015 (in preparation)) and LAC4 (a β-gal from *K. lactis*; Sreekrishna and Dickson, 1985) to create the *S. cerevisiae* strain capable of assimilating lactose. Third, the FucT2 from *H. pylori* was expressed in *S. cerevisiae*. The FucT2 activity was then confirmed in the *in vitro* 2-FL synthesis assay. Although 2-FL was not yet observed when the three components were altogether incorporated in *S. cerevisiae*, the findings in this study would provide helpful information for any future endeavor related to engineering *S. cerevisiae* to produce GDP-L-fucose and 2-FL.

5.2   Materials and methods

5.2.1   Strains and plasmids

E. coli TOP10 [F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1

*ara*D139 Δ(*ara-leu*) 7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G] was used for transformation of

the constructed plasmids. *S. cerevisiae* D452-2 (*MATalpha, LEU2, HIS3, URA3, and CAN1*) was

used as a host strain to verify GDP-L-fucose production, lactose assimilation, and FucT2

expression.

For GDP-L-fucose synthesis, *gmd* and *wcaG* genes were obtained by the polymerase

chain reactions (PCR) using the genomic DNA of *E. coli* K-12 as a template. Two PCR primers

of *gmd*_F and *gmd*_R were used for the amplification of the *gmd* gene. After digestion of PCR

fragments of the *gmd* gene and pRS423GPD plasmid with *Spe*I and *Cla*I, the DNA fragments

and the plasmid were ligated to construct plasmid pRS423GPD-*gmd*. Similarly, the *wcaG* gene

was amplified by two PCR primers (*wcaG*_F and *wcaG*_R). The *wcaG* gene fragments and

pRS425GPD plasmid were digested with *BamH*I and *Hind*III, and then ligated to construct

plasmid pRS425GPD-*wcaG*.

To enable *S. cerevisiae* to assimilate lactose, two lactose transporters consisting of *cdt-1*

(MFS lactose permease; Galagan et al., 2003/cellodextrin transporter; Galazka et al., 2010) and

LAC12 (lactose permease; Chang and Dickson, 1988) genes were cloned into pRS423PGK and

pRS423GPD plasmids, respectively. pRS423PGK-*cdt-1* was constructed previously

(construction detail can be found in Oh et al., 2015 (in preparation)). *LAC12* gene was acquired

by PCR using the genomic DNA of *K. lactis* (NRRL: Y-8279) as a template. The *LAC12* gene

which was amplified by two primers (*LAC12*_F and *LAC12*_R) and pRS423GPD plasmid were treated with *Spe*I and *Sal*I, and then ligated to construct plasmid pRS423GPD-*LAC12*. In addition to the lactose transporters, two lactose-hydrolyzing genes comprising *gh1-1* (β-glucosidase; Liu et al., 2015 (in preparation)) and *LAC4* (β-galactosidase; Sreekrishna and Dickson, 1985) were cloned into pRS425PGK and pRS425GPD plasmids, respectively. pRS425PGK-*gh1-1* was constructed previously (construction detail can be found in Oh et al., 2015 (in preparation)). *LAC4* gene was attained by PCR using the genomic DNA of *K. lactis* (NRRL: Y-8279) as a template. The *LAC4* gene which was amplified by two primers (*LAC4*_F and *LAC4*_R) and pRS425GPD plasmid were digested with *Spe*I and *Sal*I, and then ligated to construct plasmid pRS425GPD-*LAC4*.

For expression of FucT2, the *fucT2* gene from *H. pylori* strain 26695 was acquired by PCR using plasmid pHfucT2 from Lee et al. (2012a) as a template. Primers (*fucT2*-26695_F and *fucT2*-26695_R) were employed to amplify the *fucT2* gene from strain 26695. The gene fragments and plasmid pRS426GPD were then treated with *BamH*I and *Cla*I, and ligated to construct plasmid pRS426GPD-*fucT2*-26695. The *fucT2* gene from a different strain of *H. pylori* (UA802) was also tested. The *fucT2* open reading frame from strain UA802 was codon-optimized for *S. cerevisiae* and synthesized using the gBlocks® service from Integrated DNA Technologies, Inc. The *fucT2* UA802 gene was then amplified by primers (*fucT2*-UA802_F and *fucT2*-UA802_R) in a PCR using the synthesized DNA as a template. The gene fragments and plasmid pRS426GPD were digested with *BamH*I and *Cla*I, and ligated to construct plasmid pRS426GPD-*fucT2*-UA802.

Primers and plasmids used in this work are listed in **Tables 5.1** and **5.2**, respectively. All constructed plasmids were confirmed by DNA sequencing.

5.2.2 Construction of *S. cerevisiae* capable of producing GDP-L-fucose using CRISPR-Cas genome-editing technique

The following procedures for genome engineering were adapted from DiCarlo et al. (2013) which describes the application of clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system in *S. cerevisiae*. Initially, the two genes necessary for GDP-L-fucose production, *gmd* and *wcaG*, were chromosomally integrated into *S. cerevisiae* D452-2. Cas9 plasmid constructed previously (Zhang et al., 2014) was transformed into the D452-2 strain. The first repair DNA of 2.2 kb including the expression cassette of GPDp-*gmd*-CYC1t was PCR amplified by primers *gmd*-donor_F and *gmd*-donor_R using plasmid pRS423GPD-*gmd* as a template. The forward and reverse primers have 62 and 65 base-overhangs which are sequences located upstream and downstream of the target integration site, respectively. The overhangs on both ends of the repair DNA are needed to provide homology for the repair DNA to fix a double-strand break occurred due to Cas9 cutting. The target site for *gmd* integration is the intergenic region between *SPB1* and *PBN1* genes. Then, the guide RNA (gRNA) plasmid containing the target sequence of the *SPB1-PBN1* site constructed previously (Lane et al., in preparation) together with the repair DNA of the *gmd* expression cassette were transformed into the Cas9-expressing D452-2 to generate the *gmd*-expressing D452-2 strain through genome integration without disrupting any native genes.

To further integrate *wcaG* into the genome, the second repair DNA of 2.1 kb including the expression cassette of GPDp-*wcaG*-CYC1t was PCR amplified by primers *wcaG*-donor_F and *wcaG*-donor_R using plasmid pRS425GPD-*wcaG* as a template. The forward and reverse primers have 39 and 42 base-overhangs which are sequences located upstream and downstream of the target integration site, respectively. The target site for *wcaG* integration is the intergenic region between *CRH1* and *HIP1* genes. Then, the gRNA plasmid containing the target sequence of the *CRH1-HIP1* site constructed previously (Xu et al., in preparation) together with the repair DNA of the *wcaG* expression cassette were transformed into the D452-2 expressing Cas9 and *gmd* to generate the D452-2 expressing *gmd* and *wcaG* (D452-2 *gmd wcaG*) without disrupting any native genes.

In the enhancement of GDP-L-fucose production experiment, the overexpression target, *PSA1* gene was acquired by PCR using the genomic DNA of D452-2 as a template. The *PSA1* gene which was amplified by two primers (*PSA1*_F and *PSA1*_R) and pRS426GPD plasmid were treated with *BamH*I and *Cla*I, and then ligated to construct plasmid pRS426GPD-*PSA1*. Next, the expression cassette of GPDp-*PSA1*-CYC1t was integrated into the chromosome through the CRISPR-Cas system. The primer pair consisting of *PSA1*-donor_F and *PSA1*-donor_R was employed to amplify the repair DNA which is the *PSA1* expression cassette of 2.2 kb using plasmid pRS426GPD-*PSA1* as a template. The forward and reverse primers both have 65 base-overhangs which are the homology immediately outside the target sequence. The site of integration for *PSA1* is the upstream region of *THI72* gene. Then, the gRNA plasmid containing the target sequence of the *THI72* site constructed previously (Zhang et al., in preparation) together with the repair DNA of the *PSA1* expression cassette were transformed into the D452-2

expressing Cas9, *gmd,* and *wcaG* to generate the D452-2 expressing *gmd*, *wcaG,* and an extra copy of *PSA1* (D452-2 *gmd wcaG PSA1*) in the genome without disrupting any native genes.

The next gene target to increase the cytosolic level of GDP-D-mannose is *GDA1*. To deactivate *GDA1,* the repair DNA of 147 bp was designed to contain a stop codon, TAA, to prevent any gene transcription followed by random 30 bases replacing the whole ORF of *GDA1* gene. The primers (Δ*GDA1*-donor_F and Δ*GDA1*-donor_R) amplifying the repair DNA have 57 base-overhangs each which are the homology immediately before and after the *GDA1* ORF. The gRNA plasmid targeting *GDA1* was constructed using the gRNA plasmid for *THI72* (Zhang et al., in preparation) as a backbone template. The primers (*GDA1*-gRNA_F and *GDA1*-gRNA_R) with the overhanging target sequences for *GDA1* were exploited to amplify the template plasmid and create a new circular plasmid pRS42K-gRNA-*GDA1*. Then, the repair DNA for Δ*GDA1* and the *GDA1* gRNA were transformed into the D452-2 expressing Cas9, *gmd,* and *wcaG* to generate the D452-2 Δ*GDA1* expressing *gmd* and *wcaG* (D452-2 *gmd wcaG* Δ*GDA1*).

To evaluate the effect of both *PSA1* and Δ*GDA1* on GDP-L-fucose synthesis, the D452-2 *gmd wcaG* strain with double mutations of *PSA1* and Δ*GDA1* was created. The repair DNA of 2.2 kb including the *PSA1* expression cassette (GPDp-*PSA1*-CYC1t) was PCR amplified using plasmid pRS426GPD-*PSA1* as a template. The primers (*PSA1*::Δ*GDA1*-donor_F and *PSA1*::Δ*GDA1*-donor_R) have 60 base-overhangs each which are the sequences instantly upstream and downstream of the *GDA1* gene. Then, the repair DNA of the *PSA1* expression cassette along with the *GDA1* gRNA were transformed into the D452-2 expressing Cas9, *gmd,* and *wcaG* to generate the D452-2 Δ*GDA1* expressing *gmd*, *wcaG,* and an additional copy of *PSA1* (D452-2 *gmd wcaG PSA1*::Δ*GDA1*) in the genome.

Primers, plasmids, and strains used in this work are listed in **Tables 5.1**, **5.2**, and **5.3**, respectively.

5.2.3 Media, culture conditions, and yeast transformation

For plasmid amplification, *E. coli* was grown overnight in lysogeny broth (LB) medium (10 g/L NaCl, 5 g/L yeast extract, and 10 g/L tryptone) containing 100 µg/mL of ampicillin at 37ºC and 250 rpm. In terms of yeast transformation, the transformants were selected using an amino acid auxotrophic marker on yeast synthetic complete (YSC) medium (MP Biomedicals, Santa Ana, CA, USA) agar plate containing 6.7 g/L Yeast Nitrogen Base, 20 g/L glucose, 20 g/L agar, and 0.69 g/L of CSM (MP Biomedicals) which comprises appropriate amino acids. In regard to Cas9 transformation, the transformants were selected using an antibiotic marker on yeast extract-peptone (YP) medium (10 g/L yeast extract and 20 g/L peptone) agar plate containing 20 g/L glucose and 20 g/L agar supplemented with appropriate antibiotics. All yeast transformations related to expression of episomal vectors and Cas9-induced genome modification were executed by the high-efficiency yeast transformation method involving the usage of lithium acetate, polyethylene glycol, and single-stranded carrier DNA as described in Gietz and Schiestl (2007). For shake flask fermentation, the engineered yeast cells were cultivated in either YSC medium with suitable amino acids and 40 g/L of a carbon source (glucose, mannose, galactose, or lactose) or YP medium with 40 g/L of the carbon source. The flasks were incubated at 30ºC and 300 rpm.

5.2.4 Fermentation experiments

The engineered yeast cells were first grown in either YSC medium with appropriate amino acids or YP medium containing 20 g/L of glucose in a 5 mL-volume culture tube to prepare inoculums for main shake flask fermentation. Cells were harvested at mid-exponential phase and inoculated in the main fermentation after a complete removal of the previous supernatant. All flask fermentation experiments were performed using 25 mL of either YSC medium or YP medium containing the tested carbon source in 125 mL flask with an initial optical density (OD) at 600 nm of the cell concentration adjusted to 1.0 (corresponding to approximately 0.3 g/L dry cell mass) under aerobic condition. A small amount of culture broth was sampled periodically during fermentation to monitor cell growth, metabolite changes, and GDP-L-fucose production. The first portion of culture broth was diluted with water to get a proper reading of the OD value representing the cell growth. The second portion of culture broth was centrifuged to separate the supernatant and the cells. The supernatant was appropriately diluted and subjected to extracellular metabolite analysis. For further intracellular GDP-L-fucose measurement, the leftover supernatant was carefully discarded. Then, the cell pellet was washed twice and resuspended with water of the same volume as the culture prior to centrifugation. The cell suspension was vigorously vortexed in the presence of glass beads for about 40 min to break the cells. Finally, the cell suspension was adequately centrifuged, and the cell lysate was subjected to GDP-L-fucose analysis.

5.2.5 Fucosyltransferase activity assay (*In vitro* synthesis of 2-FL)

Yeast cells grown to mid-log phase at 30ºC in YSC medium with 20 g/L of glucose and appropriate amino acids were harvested by centrifugation at 4,000 rpm and 4ºC for 5 min. The cell pellet was washed with cocktail buffer (25 mM Tris-HCl pH 7.5, 1 mM Dithiothreitol (DTT), and one cOmplete tablet (a protease inhibitor; F. Hoffmann-La Roche Ltd.) per 50 mL buffer) and resuspended in the same buffer. The cell suspension was vortexed vigorously in the presence of glass beads for 30 sec and then rested on ice for 30 sec. The process was repeated back and forth for the total time of 20 min. The cell suspension was centrifuged at 14,000 rpm and 4ºC for 10 min to completely remove cell debris. The supernatant is the crude extract expected to contain the intracellular enzymes including the *fucT2* in the engineered yeast cells.

The fucosyltransferase assay was conducted to confirm the functional expression of FucT2 in *S. cerevisiae*. The assay reaction of 100 µL comprises 1.5 mM GDP-L-fucose, 2 mM lactose, 50 µL of the crude extract prepared formerly, 1 mM DTT, 1 U alkaline phosphatase, and an assay buffer (25 mM Tris, 150 mM NaCl, 10 mM $MnCl_2$, and 5 mM $CaCl_2$, pH 7.5). The reaction solution was incubated statically at 30°C. Samples were taken every 2-3 hours until GDP-L-fucose and lactose depleted. Usually, 3 µL of the solution was drawn from the reaction tube and diluted 100 times with water. The diluted reaction was subjected to heating at 95°C for 5 min to halt the enzyme activity. Then, the sample was centrifuged at a high speed for 1 min to eliminate any potential debris after heating. The supernatant was submitted for analysis of GDP-L-fucose, lactose, and 2-FL through chromatographic methods.

5.2.6   Analytical methods

Cell concentration was measured as the optical density (OD) value at 600 nm using a spectrophotometer (Biomate 5, Thermo, NY, USA). Concentrations of glucose, mannose, galactose, and lactose in fermentation experiments were determined using a high performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series) equipped with the refractive index detector using a Rezex ROA-Organic Acid H^+ (8%) column (Phenomenex Inc., Torrance, CA, USA). The column was eluted with 0.005 N of H_2SO_4 at a flow rate of 0.6 mL/min at 50ºC. GDP-L-fucose concentration was measured using the HPLC system (Beckman Coulter System Gold composed of 508 Autosampler, 126 Solvent module, and 168 Detector) set up with the diode array detector using a CAPCELL PAK C18 MG column (250×4.6 mm, Shiseido, Tokyo, Japan). The column was eluted at a flow rate of 0.6 mL/min with 98% (v/v) of 20 mM triethylamineacetate at pH 6.0 and 2% of acetonitrile. GDP-L-fucose was monitored by absorbance at 254 nm.

5.3   Results

To empower *S. cerevisiae* to produce 2-FL, at least three aspects of genetic modification need to be executed including introduction of *gmd* and *wcaG* enzymes required to convert GDP-D-mannose to GDP-L-fucose, incorporation of a lactose transporter needed to bring lactose into the cytosol, and introduction of *fucT2* catalyzing the transfer reaction of L-fucose from GDP-L-fucose onto lactose.

5.3.1 Production of GDP-L-fucose in engineered *S. cerevisiae*

Plasmids pRS423GPD-*gmd* and pRS425GPD-*wcaG* for expressions of GMD and WcaG, respectively, were transformed into *S. cerevisiae* D452-2. The D452-2 transformants harboring *gmd* and *wcaG* genes were tested for GDP-L-fucose production in a shake flask fermentation in the presence of 20 g/L of the three different carbon sources (glucose, mannose, and galactose) (**Fig. 5.3**). Glucose and mannose seemed to be better substrates leading to higher GDP-L-fucose concentrations (about 1-1.5 mg product per 1 g cell weight). However, a galactose culture only produced about half of the amount of GDP-L-fucose (about 0.5 mg product per 1 g cell weight) produced in glucose and mannose conditions. Additionally, expressions of both GMD and WcaG are necessary to acquire GDP-L-fucose. Single expression of GMD or WcaG is not sufficient to gain GDP-L-fucose (data not shown).

5.3.2 Effects of *PSA1* overexpression and *GDA1* knockout on GDP-L-fucose production

To enhance GDP-L-fucose synthesis in *S. cerevisiae*, further genetic perturbations may be required. The concentration of GDP-D-mannose is a crucial factor impacting GDP-L-fucose formation as GDP-D-mannose is a direct substrate to produce GDP-L-fucose. Thus, any genetic modification which can increase the GDP-D-mannose pool is anticipated to benefit GDP-L-fucose production. *PSA1* and *GDA1* are among interesting gene targets because they relate to GDP-D-mannose synthesis and the consumption pathway of GDP-D-mannose, respectively. In this part of study, construction of *S. cerevisiae* capable of producing GDP-L-fucose using CRISPR-Cas genome-editing approach was demonstrated. The expression cassettes of *gmd* and

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wcaG genes were integrated into the chromosome of *S. cerevisiae* D452-2 as a basal strain. Then, an additional copy of *PSA1* gene was integrated into the basal strain to examine the effect of *PSA1* overexpression. Next, *GDA1* gene was deleted from the genome of the basal strain to explore the impact of *GDA1* knockout. The combined effects of the two mutations (*PSA1* and Δ*GDA1*) were also investigated.

GDP-L-fucose production was evaluated in D452-2 *gmd wcaG* with different mutations in a shake flask fermentation in the presence of 40 g/L mannose (**Fig. 5.4**). As expected, the parental D452-2 was not able to produce any GDP-L-fucose. The D452-2 *gmd wcaG* basal strain produced 2.2 mg of GDP-L-fucose per 1 g cell weight. In the basal strain with an extra copy of *PSA1* in the genome, 1.9 mg of GDP-L-fucose per 1 g cell weight was synthesized. An enhanced GDP-L-fucose production was noticed in the basal strain with *GDA1* knockout. 3.5 mg of GDP-L-fucose per 1 g cell weight was observed. However, no synergistic effect was detected in the basal strain with both *PSA1* and *GDA1* modifications. A similar amount of GDP-L-fucose formation of 3.6 mg per 1 g cell compared to the strain with *GDA1* deletion only was observed. In summary, *PSA1* overexpression did not have a positive effect on GDP-L-fucose improvement, but disruption of *GDA1* contributed to about 50% advancement in GDP-L-fucose formation.

5.3.3 Construction of *S. cerevisiae* capable of assimilating lactose

S. cerevisiae wild-type does not assimilate lactose. Therefore, introduction of a lactose permease and a β-galactosidase into *S. cerevisiae* is necessary to attain lactose metabolism. Two candidates for a lactose transporter (LAC12 from *K. lactis* and CDT-1 from *N. crassa*) and two candidates for a lactose-hydrolyzing enzyme (LAC4 from *K. lactis* and GH1-1 from *N. crassa*)

were paired up in four possible combinations and assessed for their lactose-utilizing ability in *S. cerevisiae* D452-2 (**Fig. 5.5**). When 40 g/L lactose was provided to the cells in a shake flask fermentation, all four strains were able to consume lactose ranging from 9-17 g/L. LAC4 paired with any tested lactose transporter seemed to result in a better lactose consumption (at least 15 g/L). GH1-1, an original β-glucosidase, was proved to also have a β-galactosidase activity. When GH1-1 was matched with CDT-1, lactose was more efficiently assimilated than GH1-1 matched with LAC12 (14.6 vs. 9.6 g/L).

5.3.4   Functional expression of FucT2 in *S. cerevisiae*

FucT2 from *H. pylori* strain 26695 has been exploited extensively in 2-FL production in *E. coli* (Albermann et al., 2001; Baumgartner et al., 2013; Chin et al., 2015; Drouillard et al., 2006; Lee et al., 2012a). Hence, it was selected as the first candidate in search of FucT2 which can functionally express in *S. cerevisiae*. In addition, FucT2 from a different strain of *H. pylori* (UA802) was chosen as the second candidate. FucT2 UA802 enzyme was characterized early at the same time as FucT2 26695 (Wang et al., 1999a,b), but has not been widely experimented in the context of 2-FL synthesis. Plasmids pRS426GPD-*fucT2*-26695 and pRS426GPD-*fucT2*-UA802 were constructed and transformed into *S. cerevisiae* D452-2 individually. The *in vitro* synthesis of 2-FL was assayed using a standard GDP-L-fucose, lactose, and the crude extract of D452-2 expressing *fucT2*. Interestingly, the findings indicated the functional expression of FucT2 UA802 in D452-2, but not FucT2 26695. After 12 h of the reaction, 2-FL was detected in the assay containing the FucT2 UA802 lysate. The amount of the produced 2-FL equaled 0.4 mM from the initial substrates of 1.5 mM GDP-L-fucose and 2 mM lactose.

5.4   Discussion

S. cerevisiae is a GRAS and beneficial microorganism in the food industries. It has been largely employed in alcoholic beverage fermentations and baked products. To further add value to this yeast, *S. cerevisiae* can be genetically modified to produce health-promoting products. 2-FL is a human milk oligosaccharide which may strengthen a gut health by supporting the growth of Bifidobacteria and preventing the attachment of pathogens. Therefore, it may be worthwhile to extend the ability of *S. cerevisiae* to produce a functional compound such as 2-FL since 2-FL can be incorporated in the products created by this yeast and provides health benefits to consumers.

To construct *S. cerevisiae* capable of producing 2-FL, the primary to-do tasks consist of introduction of *gmd* and *wcaG* to convert the inherent GDP-D-mannose to GDP-L-fucose, internalization of lactose into the cytosol for 2-FL conversion, and expression of *fucT2* which catalyzes the synthesis of 2-FL. In the first task, expression of *gmd* and *wcaG* permitted the production of GDP-L-fucose with the highest level of production in glucose as a carbon source. Mannose which came the second in the amount of the produced GDP-L-fucose was initially thought to be better than glucose. Mannose is closer to GDP-D-mannose in the metabolic pathway, but apparently a bigger proportion of carbon fluxes from mannose must have gone back to fructose-6-phosphate and the central metabolism rather than proceeding to the production of GDP-D-mannose. Nevertheless, mannose may be more suitable to use in the yeast strain intended for 2-FL production than glucose. Glucose may inhibit the uptake of lactose which is required to be internalized for 2-FL conversion. Glucose is the preferred sugar by the yeast so the uptake of other sugars is normally repressed during glucose metabolism. For future studies, other

sugar sources may be attempted. Mannose is relatively expensive and may not be a great choice for a large-scale fermentation. Xylose, for example, is cheap and has been researched to be superior to glucose to produce products other than ethanol (Kwak et al., in preparation).

In an effort to improve GDP-L-fucose production through further genetic perturbations, the effects of *PSA1* overexpression and *GDA1* deletion on GDP-L-fucose formation were explored. Overexpression of *PSA1* which catalyzes the last step of GDP-D-mannose synthesis did not have a positive effect on GDP-L-fucose enhancement. On the other hand, deletion of *GDA1* which involves in the golgi import of GDP-D-mannose led to an increase in GDP-L-fucose production by 50%. Although a previous study reported an elevated level of GDP-D-mannose through *PSA* overexpression (Janik et al., 2003), it is likely that the surplus GDP-D-mannose was not utilized for GDP-L-fucose synthesis, but for production of other mannose-containing compounds. Many types of mannosyltransferases exist for different purposes which compete for GDP-D-mannose and take away most of GDP-D-mannose from GDP-L-fucose production. However, disruption of these competitive pathways may not be very helpful because these enzymes localize in the golgi but GDP-L-fucose synthesis takes place in the cytosol. Also, it is difficult to knock out some mannosyltransferases since most of the knockouts can be lethal to the cells. Thus, an indirect gene target like *GDA1* is more plausible in this situation since its deletion is not detrimental to the cell viability (Dean et al., 1997). Deletion of *GDA1* improves GDP-L-fucose formation because more GDP-D-mannose is available in the cytosol for GDP-L-fucose conversion. Owing to the insufficiency of GMP in the GDA1 null mutant, a significant portion of GDP-D-mannose cannot travel to the golgi so it accumulates more in the cytosol.

The second task to complete toward 2-FL production in *S. cerevisiae* is internalization of lactose into the cytosol. To achieve this goal, expression of a lactose transporter is unavoidable. Ultimately, an intact lactose is desired for 2-FL conversion. Therefore, expression of a β-galactosidase is unfavorable. A co-expression of FucT2 and the lactose transporter may be sufficient to bring lactose in the cells since the activity of FucT2 may promote the import of lactose required to produce 2-FL. In the engineered *E. coli* without the β-galactosidase, 2-FL can be made successfully in the presence of only the lactose permease (LacY) and FucT2 (Chin et al., 2015). However, it is uncertain whether the same situation can be expected in yeast. In this study, the lactose permease was tested together with the β-galactosidase to prepare for the worst case that expressions of the lactose permease and FucT2 are not enough to bring in lactose. The combination of LAC12 and GH1-1 caused the least amount of lactose consumed by the cells (10 g/L) as compared with three other combinations which performed similarly to each other (15-17 g/L). The possible explanation for LAC12-GH1-1 may be because GH1-1 is not originally the true β-galactosidase and so it may not work as well when paired with the lactose permease from a different species. Nevertheless, the LAC12-GH1-1 pair may be the best option to choose in the event that the β-galactosidase activity is needed since its lactose metabolism is minimal suggesting that more intact lactose is available for 2-FL production.

The third task required to produce 2-FL in *S. cerevisiae* is expression of FucT2. FucT2 from *H. pylori* strain 26695 has been successfully employed to produce 2-FL in *E. coli* since 2001 (Albermann et al., 2001). However, a slight worry regarding the negative result by *fucT2* 26695 in *S. cerevisiae* arose due to the genetic characteristic of this gene as described in Wang et al. (1999a). The FucT2 26695 sequence has consecutive 14 C bases and imperfect TAA repeats

in the middle of the gene causing the split of the sequence into two ORFs. In order to produce a functional protein, the FucT2 26695 relies solely on the perfect translational frameshift which does not always happen. According to this fact, it was doubtful about the functional expression of FucT2 26695 in *S. cerevisiae*. Another candidate of FucT2 from *H. pylori* strain UA802 was then evaluated. The sequence of FucT2 UA802 is complete with a single full-length ORF which should give a constant production of functional protein. As anticipated, FucT2 UA802 was functionally expressed in *S. cerevisiae* encouraging the synthesis of 2-FL, but not FucT2 26695.

As the three elements necessary for 2-FL production in *S. cerevisiae* were confirmed separately, the combination of the three parts was attempted in one strain. Unfortunately, an *in vivo* 2-FL production in *S. cerevisiae* has not yet succeeded so far. It is predicted that some of the produced GDP-L-fucose may have been transported through a non-specific sugar nucleotide transporter into the golgi and L-fucose may have been fucosylated with an unknown acceptor molecule. Thus, the cytosolic GDP-L-fucose may not be enough for 2-FL production. Still, more investigations to better understand the yeast sugar nucleotide system are needed to verify this hypothesis and determine what is missing to produce 2-FL in *S. cerevisiae*.

5.5   Figures and tables
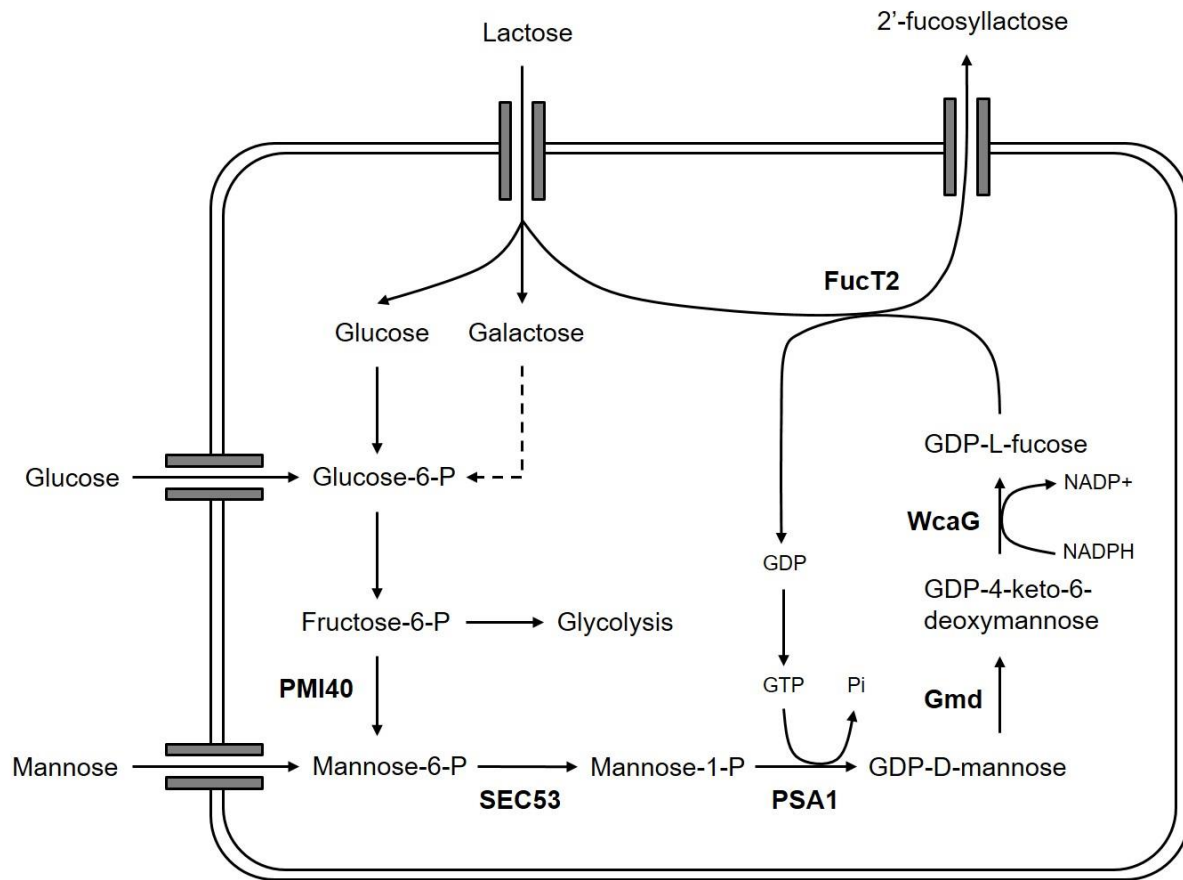
Fig. 5.1 Scheme to produce 2'-fucosyllactose (2-FL) in *S. cerevisiae*. *S. cerevisiae* innately

produces GDP-D-mannose as a mannose donor for various mannosylation reactions. In order to

enable this yeast to produce 2-FL, at least three elements need to be accomplished. First, an

intracellular GDP-L-fucose production must be achieved. This requires a heterologous

expression of GDP-D-mannose-4,6-dehydratase (Gmd) and GDP-4-keto-6-deoxymannose 3,5-

epimerase 4-reductase (WcaG) which converts the inherent GDP-D-mannose to GDP-L-fucose.

Second, lactose must be transported to the cytosol. The native *S. cerevisiae* cannot assimilate

lactose. Therefore, an introduction of a lactose transporter from other organisms is necessary. A

lactose-hydrolyzing enzyme which breaks down lactose into glucose and galactose may also be needed. Third, α-1,2-fucosyltransferase (FucT2) must be functionally expressed in *S. cerevisiae*. An introduction of the heterologous FucT2 is required. These three components are fundamental to construct the *S. cerevisiae* capable of producing 2-FL. Furthermore, GDP-D-mannose pyrophosphorylase (PSA1) may be an interesting overexpression target to increase the intracellular pool of GDP-D-mannose. The higher availability of GDP-D-mannose may lead to an enhanced GDP-L-fucose synthesis. The other enzymes shown in the diagram are abbreviated as follows; PMI40, mannose-6-phosphate isomerase; SEC53, phosphomannomutase. Pi, GDP, and GTP denote phosphate, guanosine 5'-diphosphate, and guanosine 5'-triphosphate, respectively.
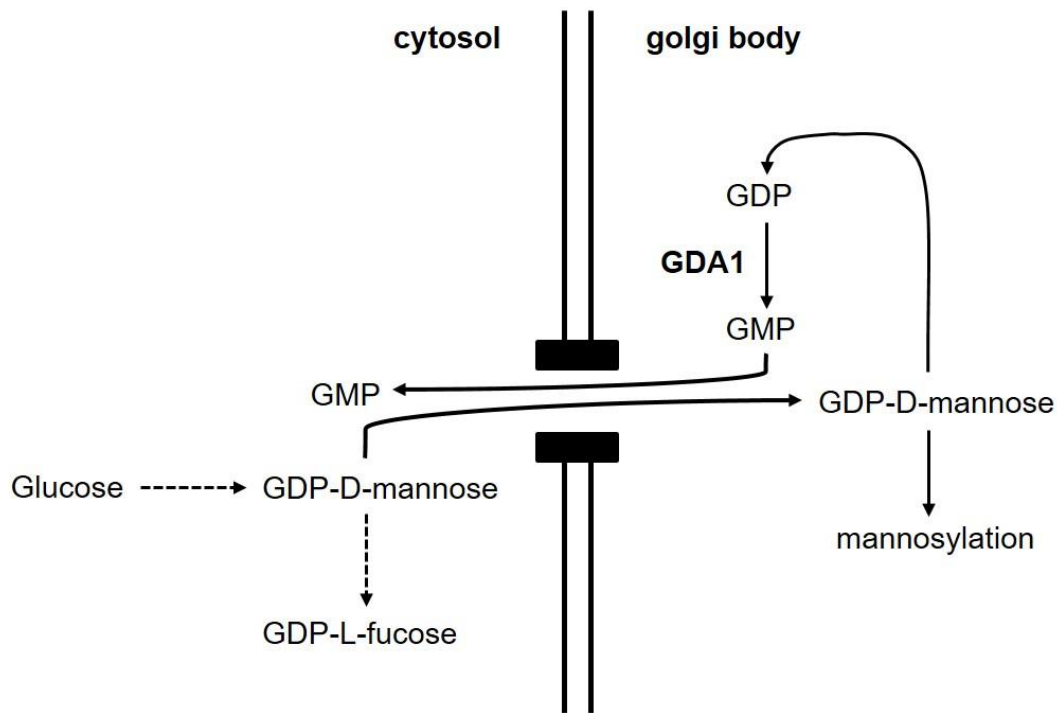
Fig. 5.2 Fate of GDP-D-mannose in *S. cerevisiae.* Once GDP-D-mannose is produced in the

cytosol, it is then transported into the golgi body through an antiporter. GDP-D-mannose

proceeds as a substrate for many kinds of mannosylation reactions and releases GDP. Guanosine

diphosphatase (*GDA1*) catalyzes the conversion of free GDP to GMP. As GMP travels back to

the cytosol through the same antiporter, more GDP-D-mannose is allowed to enter the golgi.

With a goal to produce GDP-L-fucose, the translocation of GDP-D-mannose into the golgi is

undesirable. Berninsone et al. (1994) reported that *GDA1* knockout mutant had a 5-fold

reduction in the rate of GDP-D-mannose entry into the golgi. Hence, GDA1 may be a good

deletion target to enhance GDP-L-fucose production since *GDA1* knockout results in a higher

availability of cytosolic GDP-D-mannose needed to convert to GDP-L-fucose.
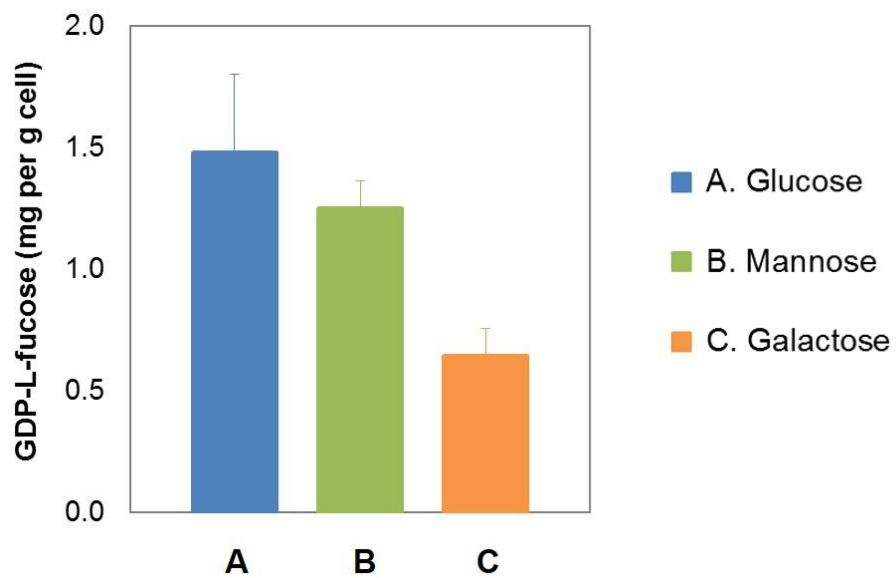
Fig. 5.3 GDP-L-fucose production by *S. cerevisiae* D452-2 harboring plasmids pRS423GPD-*gmd* and pRS425GPD-*wcaG* from 20 g/L sugar. The amount of GDP-L-fucose produced by the engineered *S. cerevisiae* was compared in three different sugar substrates. Colors of the bar chart denote as follows; *blue*, glucose; *green*, mannose; *orange*, galactose.
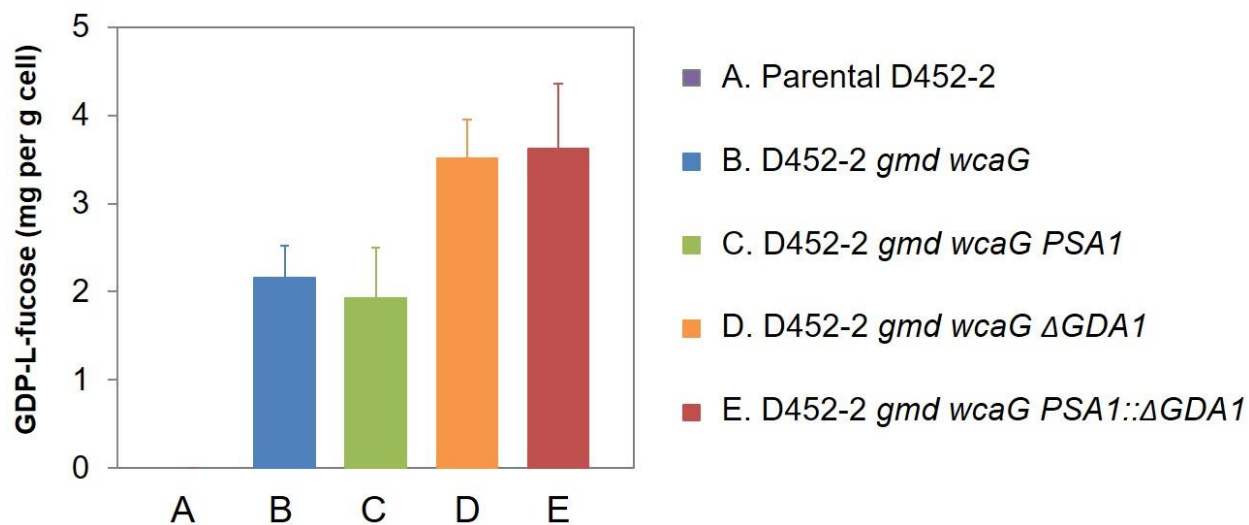
Fig. 5.4 GDP-L-fucose production by *S. cerevisiae* D452-2 with different genetic perturbations

from 40 g/L mannose. The D452-2 with *gmd* and *wcaG* integrated in the genome is the basal

strain to produce GDP-L-fucose. Effects of *PSA1* overexpression and *GDA1* deletion in the basal

strain on enhancement of GDP-L-fucose were examined. Colors of the bar chart denote as

follows; *purple*, parental D452-2; *blue*, D452-2 *gmd wcaG*; *green*, D452-2 *gmd wcaG PSA1*;

orange, D452-2 *gmd wcaG ΔGDA1*; *red*, D452-2 *gmd wcaG PSA1::ΔGDA1*.
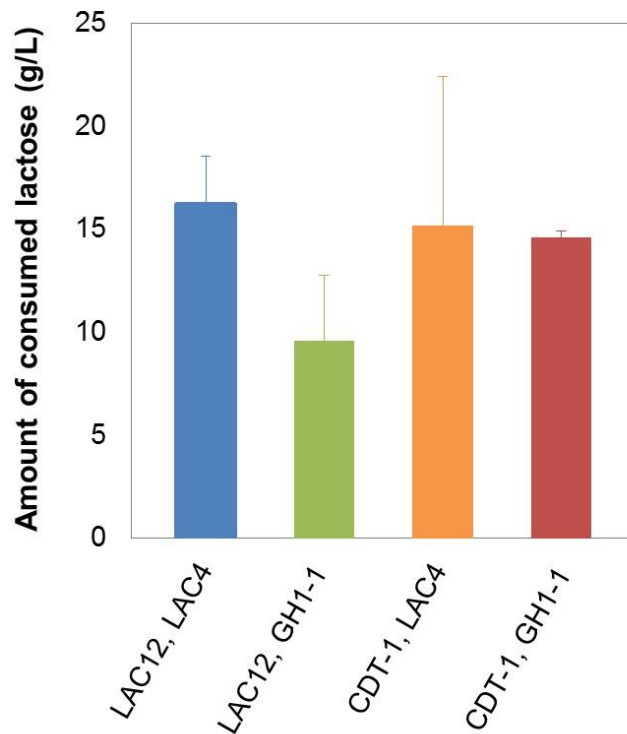
Fig. 5.5 Lactose consumption by *S. cerevisiae* D452-2 harboring different combinations of a lactose transporter (pRS423GPD-*LAC12* or pRS423PGK-*cdt-1*) and a lactose-hydrolyzing enzyme (pRS425GPD-*LAC4* or pRS425PGK-*gh1-1*). This experiment was conducted with the initial lactose of 40 g/L. The abbreviation and source of the enzymes are as follows; LAC12, lactose permease from *K. lactis*; CDT-1, lactose permease/cellodextrin transporter from *N. crassa*; LAC4, β-galactosidase from *K. lactis*; GH1-1, β-glucosidase from *N. crassa*. Colors of the bar chart denote different sets of protein expression as follows; *blue*, LAC12 and LAC4; *green*, LAC12 and GH1-1; *orange*, CDT-1 and LAC4; *red*, CDT-1 and GH1-1.
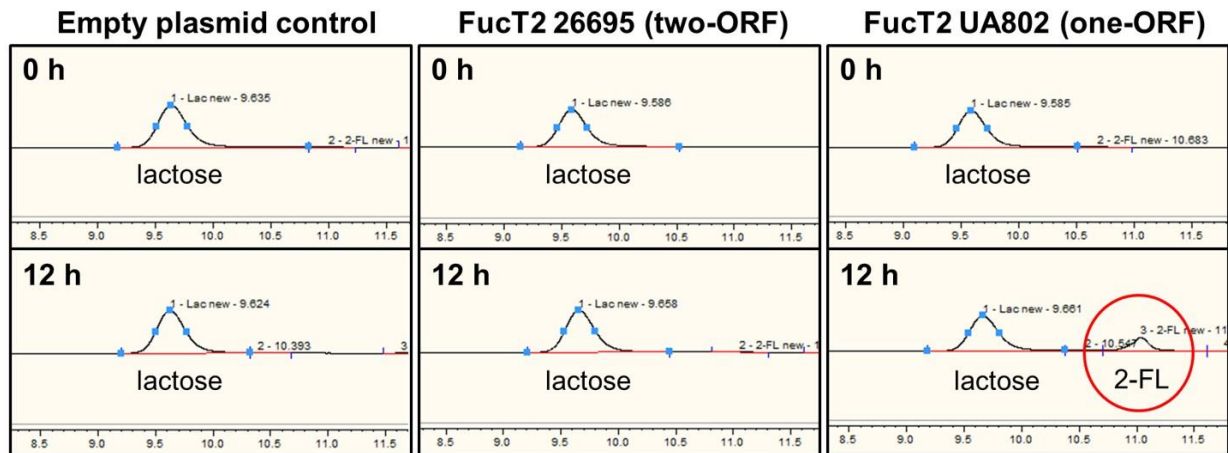
Fig. 5.6 The high performance liquid chromatography (HPLC) results of the *in vitro* test of 2-FL synthesis by *S. cerevisiae* D452-2 harboring pRS426GPD (control), pRS426GPD-*fucT2*-26695, and pRS426GPD-*fucT2*-UA802. The functional expression of *fucT2* in D452-2 was investigated by adding the crude extract of the D452-2 expressing different *fucT2* in the assay reaction comprising GDP-L-fucose and lactose. Production of 2-FL was analyzed after 12 h of the assay. Empty plasmid control, FucT2 26695 (two-ORF), and FucT2 UA802 (one-ORF) denote plasmid pRS426GPD without fucosyltransferase (*fucT2*) insertion, pRS426GPD with *fucT2* from *H. pylori* strain 26695 which has the sequence in two ORFs, and pRS426GPD with *fucT2* from *H. pylori* strain UA802 which has a single full ORF, respectively.

Table 5.1 List of primers used in this study

Name	Sequence of PCR primers (restriction sites are underlined)	Source
*gmd*_F (*Spe*I)	5'-TCTAGAGCGGCCGC<u>ACTAGT</u>GCCACCATGTCAAAAGTCGCTCTCATCAC-3'	*E. coli* K-12
*gmd*_R (*Cla*I)	5'-TCTAGAGCGGCCGC<u>ATCGAT</u>TTATGACTCCAGCGCGATCG-3'	
*wcaG*_F (*BamH*I)	5'-TCTAGAGCGGCCGC<u>GGATCC</u>GCCACCATGAGTAAACAACGAGTTTTTATTGC-3'	*E. coli* K-12
*wcaG*_R (*Hind*III)	5'-TCTAGAGCGGCCGC<u>AAGCTT</u>TTACCCCCGAAAGCGGTCTT-3'	
*LAC12*_F (*Spe*I)	5'-TCTAGAGCGGCCGC<u>ACTAGT</u>GCCACCATGGCAGATCATTCGAGCAG-3'	*K. lactis* Y-8279
*LAC12*_R (*Sal*I)	5'-TCTAGAGCGGCCGC<u>GTCGAC</u>TTAAACAGATTCTGCCTCTG-3'	
*LAC4*_F (*Spe*I)	5'-TCTAGAGCGGCCGC<u>ACTAGT</u>GCCACCATGTCTTGCCTTATTCCTGAGAAT-3'	*K. lactis* Y-8279
*LAC4*_R (*Sal*I)	5'-TCTAGAGCGGCCGC<u>GTCGAC</u>TTATTCAAAAGCGAGATCAAACTC-3'	
fucT2-26695_F (*BamH*I)	5'-AATGCC<u>GGATCC</u>AAAACTTTTAAGGTGGTGCAA-3'	*H. pylori* 26695
fucT2-26695_R (*Cla*I)	5'-AATGCC<u>ATCGAT</u>TTAAGCGTTATACTTTTGGGA-3'	
fucT2-UA802_F (*BamH*I)	5'-TCTACAGCGGCCGC<u>GGATCC</u>GCCACCATGGCCTTTAAGGTCGTCC-3'	*H. pylori* UA802
fucT2-UA802_R (*Cla*I)	5'-TATAGAGCGGCCGC<u>ATCGAT</u>GGCATTATACTTTTGAGAC-3'	
*PSA1*_F (*BamH*I)	5'-TCTAGAGCGGCCGC<u>GGATCC</u>GCCACCATGAAAGGTTTAATTTTAGTCGGTGG-3'	*S. cerevisiae* D452-2
*PSA1*_R (*Cla*I)	5'-TCTAGAGCGGCCGC<u>ATCGAT</u>TCACATAATAATAGCTTCCTTTGGAAC-3'	

Table 5.1 (cont.)

Name	Sequence of PCR primers	Source
gmd-donor_F	5'-TGATTAATTCTATGTATACATAATATATCTATTGC TTTCTTTTCCTTTATTTTCAATATTATcagctatgaccatgatta cgccaagcgcgc-3'	*E. coli* K-12
gmd-donor_R	5'-CGTATCGCAAAGAAGCATCACAAGAAAAAGTAG TAACAAAGAGCATTATTTTTCCATTCCCTTGTgccaggg ttttcccagtcacgacgttgtaa-3'	
wcaG-donor_F	5'-AACCTCGAGGAGAAGTTTTTTTACCCCTCTCCA CAGATCcaggaaacagctatgaccatg-3'	*E. coli* K-12
wcaG-donor_R	5'-TAATTAGGTAGACCGGGTAGATTTTTCCGTAAC CTTGGTGTCtgtaaaacgacggccagt-3'	
PSA1-donor_F	5'-TGCCTTATTAATTTATATGGAAGACGAGATAATT CATTAATTAGTCTACTCGACTAGATGAAATAcagctat gaccatgattacgccaag-3'	*S. cerevisiae* D452-2
PSA1-donor_R	5'-AGAAATGAATTCTATTATGATAGCGAATGCAAA ATATTCGTCCACATTCTTTTATCTATTGTCTTggttttcc cagtcacgacgttgtaa-3'	
Δ*GDA1*-donor_F	5'-GCGAACAGTTAAGGGTCCTCTCGAGAAGAAACA TTAAGACATCATCGCACAAAAAACtaatggcaaacagtcca aggtacgcttacaccg-3'	*S. cerevisiae* D452-2
Δ*GDA1*-donor_R	5'-CCGCAATCTCCTCTTCCTTAATATACATGGGACG AAAAGCGGTGTCCATGTTCTTATcggtgtaagcgtaccttgga ctgtttgccatta-3'	
PSA1::Δ*GDA1*-donor_F	5'-TTGGCGAACAGTTAAGGGTCCTCTCGAGAAGAA ACATTAAGACATCATCGCACAAAAAACcagctatgacca tgattacgccaagcgcgc-3'	*S. cerevisiae* D452-2
PSA1::Δ*GDA1*-donor_R	5'-TCACCGCAATCTCCTCTTCCTTAATATACATGGG ACGAAAAGCGGTGTCCATGTTCTTATgccagggttttccca gtcacgacgttgtaa-3'	
GDA1-gRNA_F	5'-TTTGAATGAATTGAACGATTgttttagagctagaaatagcaag-3'	*S. cerevisiae* D452-2
GDA1-gRNA_R	5'-AATCGTTCAATTCATTCAAAgatcatttatctttcactgcgga-3'	

Table 5.2 List of plasmids used in this study

Name	Description of plasmids	Source
pRS423GPD	*HIS3*, *GPD* promoter, *CYC1* terminator, 2 μ origin, and Amp^r	Christianson et al., 1992
pRS425GPD	*LEU3*, *GPD* promoter, *CYC1* terminator, 2 μ origin, and Amp^r	Christianson et al., 1992
pRS426GPD	*URA3*, *GPD* promoter, *CYC1* terminator, 2 μ origin, and Amp^r	Christianson et al., 1992
pRS423PGK	*HIS3*, *PGK1* promoter, *CYC1* terminator, 2 μ origin, and Amp^r	Christianson et al., 1992
pRS425PGK	*LEU3*, *PGK1* promoter, *CYC1* terminator, 2 μ origin, and Amp^r	Christianson et al., 1992
pRS423GPD-*gmd*	pRS423GPD harboring *gmd* gene from *E. coli* K-12	This study
pRS425GPD-*wcaG*	pRS425GPD harboring *wcaG* gene from *E. coli* K-12	This study
pRS423GPD-*LAC12*	pRS423GPD harboring *LAC12* gene from *K. lactis* Y-8279	This study
pRS425GPD-*LAC4*	pRS425GPD harboring *LAC4* gene from *K. lactis* Y-8279	This study
pRS423PGK-*cdt-1*	pRS423PGK harboring *cdt-1* gene from *N. crassa*	Oh et al., in preparation
pRS425PGK-*gh1-1*	pRS425PGK harboring *gh1-1* gene from *N. crassa*	Oh et al., in preparation
pRS426GPD-*fucT2*-26695	pRS426GPD harboring *fucT2* gene from *H. pylori* 26695	This study
pRS426GPD-*fucT2*-UA802	pRS426GPD harboring *fucT2* gene from *H. pylori* UA802	This study
pRS426GPD-*PSA1*	pRS426GPD harboring *PSA1* gene from *S. cerevisiae D452-2*	This study

Table 5.2 (cont.)

Name	Description of plasmids	Source
pRS41N-Cas9	A single-copy plasmid harboring Cas9 with a nourseothricin marker	Zhang et al., 2014
pRS41H-Cas9	A single-copy plasmid harboring Cas9 with a hygromycin B marker	Zhang et al., in preparation
pRS42H-gRNA-*SPB1-PBN1*	A multicopy plasmid containing the gRNA for *SPB1-PBN1* site with a hygromycin B marker	Lane et al., in preparation
pRS42K-gRNA-*CRH1-HIP1*	A multicopy plasmid containing the gRNA for *CRH1-HIP1* site with a geneticin marker	Xu et al., in preparation
pRS42K-gRNA-*THI72*	A multicopy plasmid containing the gRNA for *THI72* site with a geneticin marker	Zhang et al., in preparation
pRS42K-gRNA-*GDA1*	A multicopy plasmid containing the gRNA for *GDA1* site with a geneticin marker	This study

Table 5.3 List of strains used in this study

Name	Description of strains	Source
D452-2	*S. cerevisiae*, *MATα*, *LEU2*, *HIS3*, *URA3*, and *CAN1*	Hosaka et al., 1992
D452-2 *gmd wcaG*	D452-2 with *gmd* and *wcaG* genes integrated in the genome	This study
D452-2 *gmd wcaG PSA1*	D452-2 *gmd wcaG* with an additional copy of *PSA1* gene in the genome	This study
D452-2 *gmd wcaG* Δ*GDA1*	D452-2 *gmd wcaG* with *GDA1* gene deleted in the genome	This study
D452-2 *gmd wcaG PSA1*::Δ*GDA1*	D452-2 *gmd wcaG* with an additional copy of *PSA1* gene in place of *GDA1* gene deleted in the genome	This study

CHAPTER VI   **SUMMARY AND FUTURE STUDIES**

6.1   Summary

In chapter II, the *E. coli* capable of producing 2-FL was first constructed. Two plasmids comprising pmBCGW harboring the enzymes in the pathway to GDP-L-fucose (*manB*, *manC*, *gmd*, and *wcaG*) and pHfucT2 harboring the α-1,2-fucosyltransferase (*fucT2*) from *H. pylori* strain 26695 were transformed into *E. coli* JM109(DE3) and BL21star(DE3). Fermentations of the engineered *E. coli* were performed on lactose as a sole carbon source. The slow lactose-consuming JM109(DE3) strain utilized more lactose for 2-FL conversion whereas the fast lactose-consuming BL21star(DE3) strain utilized most lactose for cell growth. JM109(DE3) was chosen as a host strain for 2-FL production. In the batch fermentation with 14.5 g/L lactose, the engineered JM109(DE3) was able to produce 1.2 g/L of 2-FL.

In chapter III, the fermentation conditions used in 2-FL production by the engineered JM109(DE3) were optimized. The optimized batch fermentation conditions leading to the highest amount of 2-FL include preculture at 30ºC-main culture at 25ºC, 0.1 mM IPTG induction at the beginning of fermentation, and 10-15 g/L lactose concentration. The optimized production conditions were applied in a fed-batch fermentation. Four rounds of 15 g/L lactose were used for cell growth and 2-FL conversion in the fermentation. A final 2-FL titer of 4.3 g/L was achieved.

In chapter IV, three different *fucT2* enzymes (*fucT2* from *H. pylori* 26695, *fucT2* from *H. pylori* UA802, and *fucT2* from *E. coli* O126 (*wbgL*)) were compared to identify the *fucT2* leading to the highest amount of 2-FL. The engineered JM109(DE3) with *fucT2* from *H. pylori* UA802 produced the highest amount of 2-FL (4.2 g/L) in the fed-batch fermentation. The high cell density culture fed-batch fermentations (glycerol only and glycerol-mannose feeding) were performed in an attempt to further increase the 2-FL titer. In this high cell density culture

experiment, the host strain for 2-FL production was changed to BL21star(DE3)Δ*lacZ* because JM109(DE3) had difficulty growing to the high cell density due to the EPS-derived culture viscosity. The BL21star(DE3)Δ*lacZ* expressing the best *fucT2* from *H. pylori* UA802 was able to produce 9 g/L of 2-FL at the end of the fermentation in both glycerol only and glycerol-mannose conditions. Addition of mannose was not helpful to obtain an enhanced 2-FL production though it was shown previously to be a good co-substrate with lactose to get the highest amount of 2-FL.

In chapter V, the host to produce 2-FL was moved from *E. coli* to *S. cerevisiae*. Although a successful 2-FL production in *S. cerevisiae* was not attained, the minimum three elements required to produce 2-FL in *S. cerevisiae* were demonstrated. First, *gmd* and *wcaG* genes need to be introduced to produce GDP-L-fucose from the inherent GDP-D-mannose. Second, a lactose transporter is required to bring lactose into the cytosol. Third, *fucT2* gene which can functionally express in *S. cerevisiae* needs to be introduced. Moreover, *GDA1* gene involved in the transport of GDP-D-mannose into the golgi was identified as a deletion target to improve GDP-L-fucose production in *S. cerevisiae*.

6.2   Future studies

In regard to production of 2-FL in engineered *E. coli,* improvement of the *fucT2* expression in a soluble fraction should be performed. As shown on the SDS-PAGE in chapter II, most of the *fucT2* was expressed in inclusion bodies. If more *fucT2* can be expressed in a soluble form, 2-FL production should be enhanced tremendously. Examples of future attempts include promoter and induction system alteration, and co-expression of *fucT2* with molecular chaperones. Furthermore, new gene targets to increase the intracellular supply of GDP-L-fucose

should be identified. In the consumption pathway of GDP-L-fucose, many genes implicated in the colanic acid operon may be perturbed. Concerning EPS production by JM109(DE3), the mechanism of EPS secretion, the composition of EPS, and the control of EPS may be investigated to improve 2-FL production in JM109(DE3). About *fucT2* enzyme, a large survey of *fucT2* from more sources may be conducted. Protein expressions of different *fucT2* should be compared to better understand their activities and select the best *fucT2* possible to enhance 2-FL synthesis.

In regard to production of GDP-L-fucose and 2-FL in engineered *S. cerevisiae, GDA1* gene may be thoroughly analyzed to understand how its deletion actually affects the cytosolic concentration of GDP-D-mannose and enhances GDP-L-fucose synthesis. Other ways to further increase the cytosolic concentration of GDP-D-mannose should be investigated. Enhancement of GDP supply used in GDP-L-fucose synthesis may be attempted by overexpression of enzymes in guanosine nucleotides biosynthetic pathways. The competing pathways for GDP-D-mannose may be eliminated. Some mannosyltransferases which are not lethal to the cells may be disrupted (more deletion targets). Moreover, labeling experiments to check where the carbons from GDP-D-mannose go to should be conducted as the data may be useful for future genetic modification and strain improvement.

CHAPTER VII   REFERENCES

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