Efficient biosynthesis of difucosyllatose(DFL) by engineered *Escherichia coli*

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ABSTRACT

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Human milk oligosaccharides (HMOs) are essential bioactive components that perform various physiological functions, such as shaping the gut microbiota, inhibiting the adhesion of pathogenic bacteria, and modulating the immune system. Among them, difucosyllactose (DFL) exhibits superior bioactivity due to its unique structure containing two fucose residues, making it a promising candidate for use as a functional food ingredient and pharmaceutical material. In this study, a metabolic engineering strategy was developed to enable DFL production using *Escherichia coli.* To enhance the accumulation of the donor molecule GDP-L-fucose, the genes *waaF* and *wcaJ*, which are involved in competing biosynthetic pathways, were deleted. In addition, to prevent the degradation of the acceptor molecule lactose, the *lacZ* gene was also deleted. To synthesize the precursor

2'-fucosyllactose (2'-FL), two α-1,2-fucosyltransferases —*WbgL* and *FucT2*—were introduced, and their expression was systematically evaluated under various constitutive promoter conditions to identify the optimal configuration. Subsequently, a dual fucosylation pathway was constructed by introducing *Hp3/4FT,* an α-1,3/4-fucosyltransferase that works in conjunction with *WbgL* to facilitate DFL biosynthesis while minimizing the formation of the byproduct 3-FL. To assess the potential for enhanced enzyme expression and catalytic performance, both wild-type and truncated variants of *Hp3/4FT* were compared in terms of DFL productivity. Initial fermentation using the engineered strain exhibited low DFL titers. To address this limitation, the biosynthesis of 2'-FL was enhanced by replacing the promoter, and fermentation conditions were further optimized. Additionally, a mutated variant of *Hp3/4FT* was introduced to improve enzyme expression and overall DFL production.

CONTENTS

LIST OF FIGURES

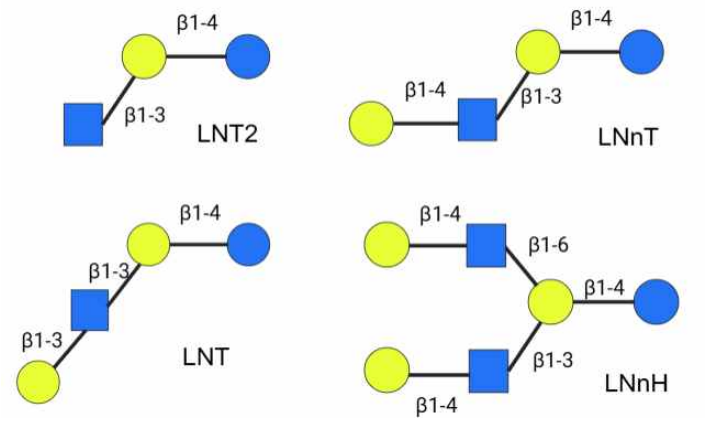
LIST OF TABLES

1. Introduction

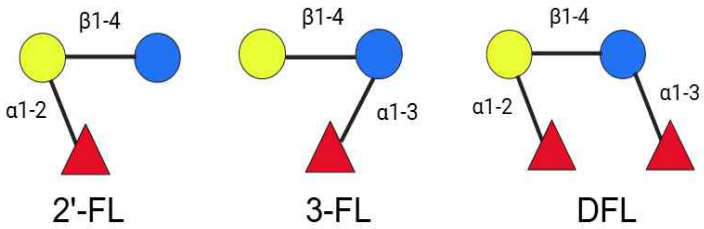
Human milk oligosaccharides (HMOs) are the third most abundant solid component in human milk, following lipids and lactose. They play a crucial role in supporting healthy infant growth and immune system development (Li et al. 2022). Recently, HMOs have garnered significant attention in the fields of infant nutrition and pharmaceutical applications due to their diverse physiological functions. These include acting as essential prebiotics that shape the gut microbiota, preventing the adhesion of pathogens and viruses, selectively promoting the growth of beneficial gut bacteria, modulating immune responses, and contributing to neural development (Baumgartner et al. 2013, Yu et al. 2018, Sun et al. 2023).

HMOs are classified into three major types based on structural and functional characteristics: neutral HMOs, fucosylated HMOs, and sialylated HMOs (Zhu et al. 2023). Over 200 structurally distinct HMOs have been identified to date (Lu et al. 2021). Fucosylated HMOs contain one or more L-fucose residues linked to the terminal position of lactose. Representative examples include 2'-fucosyllactose(2'-FL), 3-fucosyllactose(3-FL), and difucosyllactose (DFL), which have been extensively studied (Fig. 1). 2'-FL is the most abundant component, making up approximately 30% of the total HMO content in human milk.

(a) Neutral non-fucosylated HMOs



(b) Fucosylated HMOs



(c) Sialylated HMOs

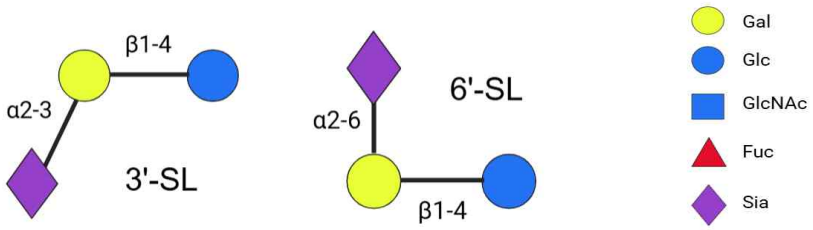


Fig. 1. Structures of core human milk oligosaccharides (HMOs).

The schematic representation illustrates the core structures of human milk oligosaccharides (HMOs), (a)classified into neutral non-fucosylated HMOs, (b)fucosylated HMOs, and (c)sialylated HMOs.

The target molecule of this study, DFL, exhibits a unique structure. It contains two fucose residues linked to the C2 position of galactose via an α-1,2 bond and to the C3 position of glucose via an α-1,3 bond (Thurl et al. 2017, Craft and Townsend 2019). Due to this structural feature, DFL demonstrates superior bioactivity compared to other fucosylated oligosaccharides.

In particular, DFL has been reported to exhibit significant antimicrobial activity, effectively inhibiting the growth and proliferation of Group B *Streptococcus* (GBS). This contributes to the prevention of bacterial infections in newborns (Liang et al. 2024). DFL also exerts protective effects against pathogenic viruses and bacteria. It further promotes the selective proliferation of beneficial bacteria such as *Bifidobacteria,* supporting the maintenance of intestinal microbial balance (Zhu et al. 2024). These physiological functions are significant not only for infants but also for adult health (Liu et al. 2018). Recent studies have highlighted that fucosylated oligosaccharides, including DFL, may support long-term immune system development and health maintenance by shaping the gut microbiota.

Conventional methods for obtaining HMOs include direct extraction from human milk, chemical synthesis, and enzymatic synthesis. Direct extraction is unsuitable for industrial production due to ethical concerns and the limited availability of human milk as a raw material (Seydametova et al. 2019). Chemical synthesis involves complex procedures and toxic reagents, leading to

environmental and economic concerns. Enzymatic synthesis also faces limitations in large-scale production due to the high cost of GDP-L-fucose and the low catalytic activity of α-1,2-fucosyltransferase (Palur et al. 2023).

As an alternative to overcome these challenges, microbial biosynthesis strategies have recently gained significant attention. This approach enables the efficient production of precursors from inexpensive carbon sources. Required enzymes are expressed intracellularly through recombinant techniques, allowing both cost-effectiveness and high productivity (Chin et al. 2016). *Escherichia coli* is particularly considered a promising host for HMO production due to its rapid growth rate, ease of genetic manipulation, and compatibility with various fermentation processes (Li et al. 2021). Recent studies have shown that manipulating specific genes in *E. coli*—such as *wcaJ, lacZ,* and *lon*—can significantly increase GDP-L-fucose accumulation. In addition, optimized expression of α-1,2- and α-1,3-fucosyltransferases enhances HMO productivity (Lee et al. 2021, Lin et al. 2022).

In this study, we applied multiple metabolic engineering strategies to enhance DFL production. *Escherichia coli* possesses an endogenous de novo pathway for the synthesis of GDP-L-fucose. This pathway began with mannose-6-phosphate and proceeded through a series of enzymatic reactions involving *ManA, ManB, ManC, Gmd,* and *WcaG* to produce GDP-L-fucose

(Wang et al. 2019). In wild-type *E. coli*, GDP-L-fucose is mainly consumed for the biosynthesis of capsular polysaccharides or other cell wall components. The strain also lacks fucosyltransferase genes required for the production of fucosylated oligosaccharides such as 2'-FL and DFL. As a result, it is unable to synthesize the desired compounds (Lu et al. 2021).

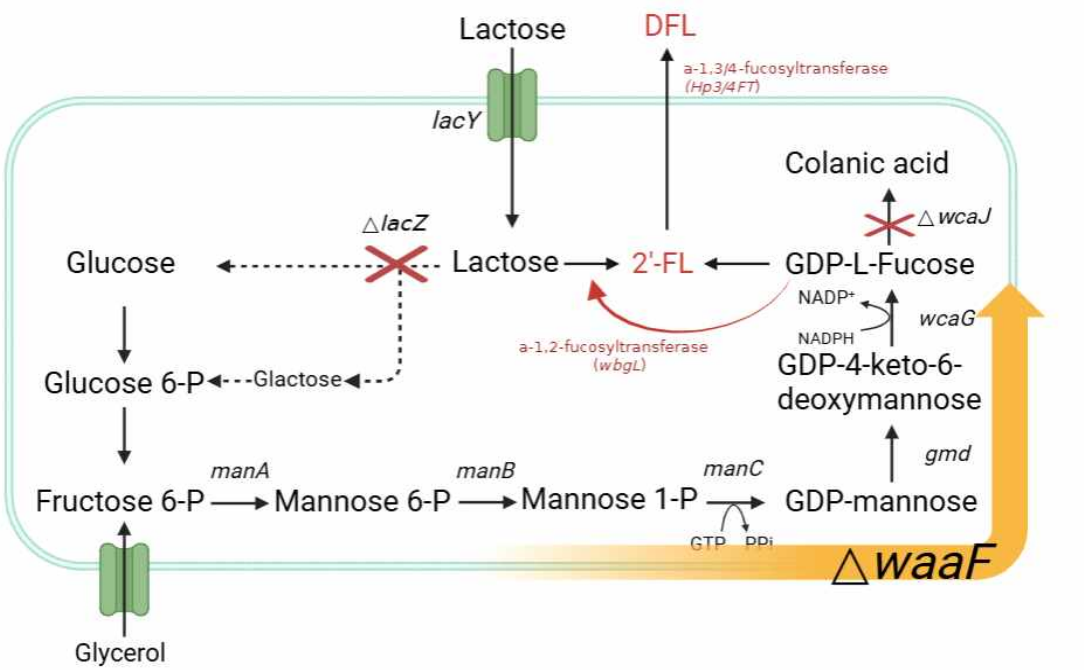


Fig. 2. Pathway Optimization Strategy for Efficient DFL Biosynthesis in Engineered *E. coli*

The metabolic pathway of *E. coli* was redesigned for DFL biosynthesis by applying strategies to enhance precursor accumulation (deletion of *waaF, wcaJ*, and *lacZ*) and optimize fucosyltransferase expression (introduction of *wbgL* and *Hp3/4FT*). The schematic diagram illustrates the comprehensive approach used to achieve efficient DFL production.

To address this issue, we established a multi-step metabolic engineering strategy focusing on precursor supply and enzyme expression optimization (Fig. 2). First, to maximize the accumulation of GDP-L-fucose and lactose, we deleted the *waaF, wcaJ*, and *lacZ* genes, thereby enhancing the availability of precursors. To construct the enzyme system required for DFL biosynthesis, we introduced *wbgL* as the α-1,2-fucosyltransferase and *Hp3/4FT* as the α-1,3/4-fucosyltransferase.

In addition, to increase the enzyme activity, we explored optimal expression combinations by introducing various promoters (T7, J23100, J23108, J23117). Despite constructing the initial biosynthetic pathway for DFL production, including the supply of GDP-L-fucose and the expression of key fucosyltransferases, the overall productivity remained unsatisfactory. To address this limitation, we adopted a multi-pronged strategy (1) enhancing 2'-FL precursor supply via promoter replacement, (2) optimizing fermentation conditions to improve metabolic efficiency, and (3) introducing a Y131D variant of *Hp3/4FT* to increase catalytic performance. Through these integrated strategies, this study aims to establish a robust and economically feasible microbial platform for the industrial production of DFL.

2. Materials and Methods

2.1 Strains and Media

The strains used in this study are summarized in Table 1. For plasmid construction, *E. coli* Turbo (Invitrogen, MA, USA) was employed, while *E. coli* JM109(DE3) (Promega, Madison, WI, USA) was used as the host strain for DFL production. The *E. coli* Turbo strain was cultured at 37° C in Luria-Bertani(LB) medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl). After plasmid introduction, antibiotics (kanamycin 50㎍/mL, ampicillin 100㎍/mL, streptomycin 50㎍/mL) were added to maintain plasmid stability and facilitate selection. Most *E. coli* strains used in this study were cultured at 37° C, which is the optimal growth temperature for *E. coli*. For transformation experiments using the CRISPR-Cas9 system, the *E. coli* JM109(DE3) strain harboring the *pCas* plasmid was utilized. To maintain the stability of the *pCas* plasmid, the strain was cultured at 30° C in LB medium supplemented with kanamycin(50㎍/mL). This lower temperature was chosen to minimize the potential loss of the pCas plasmid, which may occur at higher temperatures(37° C). Subsequent plasmid introduction was performed using electroporation.

2.2 Plasmids

The plasmids used in this study are summarized in Table 2. In this study, plasmid constructs were generated primarily using the pTarget and pCOLADuet-1, pACYCduet-1 vectors to facilitate genetic manipulation and expression of genes involved in the DFL biosynthetic pathway. The pTarget plasmid contains a high copy number origin of replication, enabling efficient replication within *E. coli*. Therefore, it was primarily used for CRISPR-Cas9 based gene deletion and genome integration. This vector was utilized to construct gene editing plasmids containing sgRNA expression cassettes and homology arms. The pTarget plasmid confers streptomycin resistance as an antibiotic marker. In contrast, the pCOLADuet-1 and pACYCduet-1 vectors were used for the expression of heterologous genes introduced via transformation, primarily for cloning to verify the expression of the α-1,2-fucosyltransferase (*wbgL*) gene. These vectors confer kanamycin and chloramphenicol resistance as antibiotic markers.

2.3 Reagents

All reagents and materials used in this study were handled according to the manufacturer's instructions, and unless otherwise specified, they were stored and used at room temperature or under

the recommended conditions. Solid media were prepared by adding agar to LB Broth at a final concentration of 2% (w/v), and agarose required for electrophoresis was obtained from Agarose LE Master (Biosesang). Additionally, two different PCR systems were used depending on the purpose of DNA amplification. For general gene amplification, TOPsimple™ PCR PreMIX-Forte (Enzynomics, Korea) was employed, while reactions requiring high-precision amplification utilized Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, USA). Plasmid purification was performed using the APrep™ Plasmid Mini Kit (APBIO, Korea). For PCR product purification, the APrep™ PCR DNA Kit(APBIO, Korea) from the same manufacturer was used. Other reagents used in the experiments are specified in the respective methods.

2.4. Gene cloning

In this study, various plasmids were designed and constructed according to their intended purposes, including gene deletion, genome integration, and heterologous gene expression. The genes used for cloning were *lacZ, waaF, wcaJ, wbgL, fucT2*, and *Hp3/4FT.* For CRISPR-Cas9-based gene editing, the N20 sequences of the sgRNAs corresponding to each target gene were designed according to the following criteria.

The sgRNA for the *lacZ* gene was designed based on a previous study(Liang et al. 2024). and the N20 sequence was inserted into

the sgRNA expression site of the pTarget vector using the F_sg_*lacZ* / R_sg_*lacZ* primers. For other genes, the N20 sequences were designed using the gRNA design tool provided by the ATUM website(https://atum.bio). The genomic sequence of *E. coli* JM109(DE3) was referenced, and the gRNA target sites were selected based on the proximity to the NGG PAM sequence. The plasmid constructs included homology arms to facilitate homologous recombination within the CRISPR-Cas9 system. These homology arms were derived from the upstream and downstream genomic sequences of each target gene, with approximately 1kb for *lacZ* and *waaF*, and around 200bp for *wcaJ*. The homology arms were either amplified by PCR or synthesized by LnCBio Korea when necessary.

The heterologous genes *wbgL, fucT2*, and *Hp3/4FT* were redesigned to optimize codon usage for efficient expression in *E. coli* JM109(DE3). The optimized sequences were synthesized by LnCBio Korea and subsequently used for cloning. The constructed inserts were cloned into vectors using the Gibson Assembly method. The completed plasmids were subjected to Sanger sequencing by Macrogen Korea to verify the accuracy of the inserted genes and homology arms. Only plasmids with confirmed nucleotide sequences were used for subsequent experiments.

All relevant primer information is summarized in Table 3.

2.5 Genome editing via CRISPR-Cas9 system

The gene deletion and introduction experiments conducted in this study were based on the two-plasmid CRISPR-Cas9 system proposed by Jiang et al.(2015). To construct an efficient DFL producing strain, unnecessary metabolic pathways were eliminated, and the flux of the precursor GDP-L-fucose was redirected towards the target product synthesis pathway by sequentially deleting the *lacZ, waaF*, and *wcaJ* genes. Before performing CRISPR-Cas9 experiments, the JM109(DE3) strain was prepared for pCas plasmid introduction. The strain was pre-cultured overnight at 37° C in LB medium, followed by main culture until the optical density at 600nm(OD_{600}) reached 0.8. At this point, the cells were collected and prepared as fresh competent cells for electroporation. Transformation was conducted using a BIO-RAD MicroPulser with a 0.2cm cuvette under a voltage of 2.5kV.

The strain harboring the pCas plasmid was cultured in LB medium containing kanamycin. To induce pCas expression, L-arabinose was added to the main culture at a final concentration of 0.1mM. Subsequently, the pTZ plasmid was introduced to induce *lacZ* gene cleavage by Cas9. The transformed colonies were cultured at 30° C on LBKS medium, and gene deletion was verified by colony PCR using the F_cp_*lacZ* and B_cp_*lacZ* primers. Colonies confirmed to have the *lacZ* gene deleted were cultured in the presence of isopropyl β-D-1-thiogalactopyranoside(IPTG) 0.5mM to remove

the pTarget plasmid. Subsequently, the pCas plasmid was cured by culturing at 37° C. The same strategy was successfully applied to delete the *waaF* and *wcaJ* genes.

The introduction of the α-1,2-fucosyltransferase gene(*wbgL*) for 2'-FL production, the α-1,3/4-fucosyltransferase gene (*Hp3/4FT*) for DFL production, and the *fucT2* gene to compare 2'-FL production under different promoters was also performed using the same CRISPR-Cas9 based strategy as described previously.

2.6 Fluorescence Analysis of GFP Expression

GFP signal measurement was performed according to the protocol described by (Drew et al. 2006). Transformed JM109(DE3) strains harboring the pETduet EGFP plasmid with different promoters were pre-cultured overnight at 37° C in 5mL of LB medium. The overnight culture was diluted 50-fold into fresh 5mL LB medium and cultured at 37° C. When the optical density OD_{600} reached 0.4-0.5, IPTG was added to a final concentration of 0.5mM, and the culture was incubated at 25° C for 4hours. After induction, cells were harvested by centrifugation at 15,000rpm for 2minutes, washed with DW, and resuspended. The resuspended cells were transferred to a 96-well plate, and GFP signals were measured using a Microplate Reader(SpectraMax M5, Molecular Devices, USA) with excitation at 485nm and emission at 512nm.

2.7 Fermentation

The engineered *Escherichia coli* JM109(DE3) strains for 2'-FL and DFL production were inoculated in 5mL of LB medium and pre-cultured overnight at 37° C. The following day, the cells were harvested and adjusted to an initial OD_{600} of 0.1. The adjusted culture was then inoculated into 25mL of LB medium supplemented with lactose 2g/L and glycerol 5g/L and dispensed into a 250mL flask. All flasks were cultured at 25° C and 250 rpm to induce the expression of α-1,2-fucosyltransferase. For strains harboring the pCW or pCF plasmids, IPTG was added at a final concentration of 0.1mM to induce expression via the T7 promoter, along with kanamycin supplementation. In addition to batch fermentation, fed-batch cultivation was performed by supplementing glycerol at 5g/L when depletion was observed during culture. Fermentation was carried out for a total of 72hours, with samples collected every 24hours. The samples were centrifuged at 15,000rpm to remove the cells, and the supernatant was collected and stored at -20° C.

2.8 Analytical Methods

The growth of *Escherichia coli* cells was assessed by monitoring the OD_{600} using an OPTIZEN™ NanoQ Series Microvolume UV-VIS Spectrophotometer(KLAP Co., Ltd., Korea). HPLC analysis was performed using a Rezex ROA-Organic Acid H^+(8%) column

(Phenomenex Inc.Torrance,CA) equipped with a Refractive Index Detector(Agilent Technologies, Germany). The mobile phase consisted of 0.005mM sulfuric acid (H2SO4), with a flow rate of 0.6mL/min and a column temperature of 50° C. The sample injection volume was set to 5㎕L. The concentrations of lactose, glycerol, 2'-FL, and DFL were quantified using the external standard method, based on standard curves prepared with each respective standard compound. The standard materials were obtained from Louisville, KY, USA, and used for analysis.

Table 1. Strains

Strains	Relevant description	Reference
E. coli Turbo	F' proA+B+ lacIq △*lacZ*M15 / *fhuA2* △(lac-proAB) glnV galK16 galE15 R(zgb-210::Tn10)TetS endA1 thi-1 △(hsdS-mcrB)5	NEB (Ipswich, MA,USA)
E. coli JM109(DE3)	F′ traD36 proAB+ lacIq ⊿(*lacZ*)M15/ ⊿(lac-proAB),glnV44e14-gyrA96recA1	NEB (Ipswich, MA,USA)
JM109(DE3)-pCas	*E. coli* JM109(DE3)+harboring pCas	This study
JM109(DE3)-pET	*E. coli* JM109(DE3)+harboring pET	This study
JM109(DE3)-pES	*E. coli* JM109(DE3)+harboring pES	This study
JM109(DE3)-pEM	*E. coli* JM109(DE3)+harboring pEM	This study
JM109(DE3)-pEW	*E. coli* JM109(DE3)+harboring pEW	This study
△Z	*E. coli* JM109(DE3)△*lacZ*	This study
△ZJF	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ*	This study
△ZJF 02	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23100-*wbgL*	This study
△ZJF 03	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23108-*wbgL*	This study

Table 1. Strains (continued)

Strains	Relevant description	Reference
△ZJF 04	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23117-*wbgL*	This study
△ZJF 05	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23100-*FucT2*	This study
△ZJF 06	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23108-FucT2	This study
△ZJF 07	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23117-FucT2	This study
△ZJF 08	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23100-wbgL aslA-hemY::P_J23100-Hp3/4FT	This study
△ZJF 09	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* ompW-yciE::P_J23100-wbgL aslA-hemY::P_J23100-Hp3/4FT TO	This study
△ZJF 10	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* +harboring pTW	This study
△ZJF 11	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* +harboring pTF	This study
△ZJF 12	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* +harboring pCW	This study
△ZJF 13	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* +harboring pCF	This study

Table 1. Strains (continued)

Strains	Relevant description	Reference
△ZJF 14	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* atpI-rsmG::P_J23100-truncated *Hp3/4FT*(Y131D) harboring pAW	This study
△ZJF 15	*E. coli* JM109(DE3)△*lacZ*△*waaF*△*wcaJ* atpI-rsmG::P_J23100-*Hp3/4FT* harboring pAW	This study

Table 2. Plasmids

Strains	Relevant description	Reference
pCas	repA101(Ts) kan Pcas-cas9 ParaB-Red lacIq Ptrc-sgRNA-pMB1, KanR	Addgene (#62655)
pTarget	pMB1 aadA sgRNA, AmpR, Constitutive expression of sgRNA without donor editing template DNA	(Lee et al., 2021)
pCOLAduet-1	Two T7 promoters with two MCS, ColA, replicon, and KanR	(Lee et al., 2021)
pETduet-1	Two T7 promoters with two MCS, pBR322, replicon, and AmpR	(Lee et al., 2021)
pACYCduet-1	Two T7 promoters with two MCS,P15A,replicon, and CmR	(Lee et al., 2021)
pTZ	Derivate from pTarget, contain sgRNA targeting to *lacZ*	This study
pTF	Derivate from pTarget, contain sgRNA targeting to *waaF*	This study
pTJ	Derivate from pTarget, contain sgRNA targeting to *wcaJ*	This study
pTWS	Derivate from pTarget, contain sgRNA targeting to ompW-yciE locus for PJ23100-*wbgL* integration	This study
pTWM	Derivate from pTarget, contain sgRNA targeting to ompW-yciElocus for PJ23108-*wbgL* integration	This study
pTWS	Derivate from pTarget, contain sgRNA targeting to ompW-yciE locus for PJ23117-*wbgL* integration	This study
pTFS	Derivate from pTarget, contain sgRNA targeting to ompW-yciE locus for PJ23100-*FucT2* integration	This study

Table 2. Plasmids (continued)

Strains	Relevant description	Reference
pTFM	Derivate from pTarget, contain sgRNA targeting to ompW-yciE locus for PJ23108-*FucT2* integration	This study
pTFW	Derivate from pTarget, contain sgRNA targeting to ompW-yciE locus for PJ23117-*FucT2* integration	This study
pTH	Derivate from pTarget, contain sgRNA targeting to aslA-hemY locus for PJ23100-*Hp3/4FT* integration	This study
pTHT	Derivate from pTarget, contain sgRNA targeting to aslA-hemY locus for PJ23100-truncated *Hp3/4FT* integration	This study
pTHX	Derivate from pTarget, contain sgRNA targeting to atpI-rsmG locus for PJ23100-*Hp3/4*FT integration	This study
pTHY	Derivate from pTarget, contain sgRNA targeting to atpI-rsmG locus for PJ23100-*Hp3/4FT*(Y131D) integration	This study
pTW	Derived from pCOLAduet-1, PT7-*wbgL,* and KanR	This study
pTF	Derived from pCOLAduet-1, PT7-*FucT2*, and KanR	This study
pCW	Derived from pCOLAduet-1, PTrc-*wbgL*, and KanR	This study
pCF	Derived from pCOLAduet-1, PTrc-*FucT2*, and KanR	This study

Table 2. Plasmids (continued)

Strains	Relevant description	Reference
pAW	Derived from pACYCduet, PTrc-*wbgL*, and CmR	This study
pET	Derived from pETduet-1, pT7-EGFP, and KanR	This study
pES	Derived from pETduet-1, PJ23100-EGFP, and KanR	This study
pEM	Derived from pETduet-1, PJ23108-EGFP, and KanR	This study

Table 3. Primers

3.1 Primer sequences for gRNA 변경

Name	Sequence (5’-3’)
F_sg_*lacZ*	CGGGTGAACTGATCGCGCAGgttttagagctagaaatagcaagtt
R_sg_*lacZ*	CTGCGCGATCAGTTCACCCGactagtattatacctaggactgagc
F_sg_*waaF*	GGTTATCAGGTGGTTCTGTTgttttagagctagaaatagcaagtt
R_sg_*waaF*	AACAGAACCACCTGATAACCactagtattatacctaggactgagc
F_sg_*wcaJ*	TGGTTAGGGTTTGAAGTGGTgttttagagctagaaatagcaagtt
R_sg_*wcaJ*	ACCACTTCAAACCCTAACCAactagtattatacctaggactgagc
F_sg_ ompW-yciE	CGTAATATACGGGGTCAATAgttttagagctagaaatagcaagtt
R_sg_ ompW-yciE	TATTGACCCCGTATATTACGactagtattatacctaggactgagcta
F_sg_a slA-hemY	TCTGGCGCAGTTGATATGTAgttttagagctagaaatagcaagtt
R_sg_ aslA-hemY	TACATATCAACTGCGCCAGAactagtattatacctaggactgagcta
F_sg_ atpI-rsmG	AAAGTCGCAATTGTATGCACgttttagagctagaaatagcaagtt
R_sg_ atpI-rsmG	GTGCATACAATTGCGACTTTactagtattatacctaggactgagc

Table 3. Primers(continued)

3.2 Primer sequences for Gene deletion

Name	Sequence (5’ -3’)
F_VECTOR	agaagcttagatctattacc
B_VECTOR	gcaggtcgactctagagaat
Deletion of lacZ	
F_del_*lacZ*	cgagtcggtgctttttttgaattctctagagtcgacctgcCTGGAGGCTGAAGTTCAGATGTGCGGCGAG
R_del_*lacZ*	aactcgagtagggataacagggtaatagatctaagcttctTTTCCATCAGTTGCTGTTGACTGTAGCGGC
F_cp_*lacZ*	ACAAATCAGCGATTTCCATGTTGCC
R_cp_*lacZ*	GAAACCGTCGATATTCAGCCATGTG
Deletion of *waaF*	
F_del_*waaF*	aaagtggcaccgagtcggtgctttttttgaattctctagagtcgacctgcGGCAGATCTGACAAATCTGC
R_del_*waaF*	gctgcacatgaactcgagtagggataacagggtaatagatctaagcttctGGAAGGAATCTGTGCGAACC
F_cp_*waaF*	CGCGTGAAGGCCATAAAGGCAGCAT
R_cp_*waaF*	ACGGCGTGCTGCTGTTTTGCAATAT

Table 3. Primers(continued)

3.2 Primer sequences for Gene deletion

Deletion of *wcaJ*	
F_del_*wcaJ*	aaagtggcaccgagtcggtgctttttttgaattctctagagtcgacctgcTT GAGCAGGCGGTTAACCCC)
R_del_*wcaJ*	gctgcacatgaactcgagtagggataacagggtaatagatctaagcttctTT TTATCGCTCATGGCGCGG)
F_cp_*wcaJ*	TTTTTTCACGTAAGCTCATA
R_cp_*wcaJ*	GGCTAATAACAGGAACAACG

Table 3. Primers(continued)

3.2 Primer sequences for Gene Integration

Name	Sequence (5’-3’)
ompW-yciE:: wbgL	
F_vec_*wbgL*	agaagcttagatctattacc
R_vec_*wbgL*	gcaggtcgactctagagaat
F_Ins_*wbgL*	ctttttttgaattctctagagtcgacctgcTACGCCTCGATCCGTGGGTG
R_Ins_*wbgL*	gggataacagggtaatagatctaagcttctAGAAATTTTTAATTCGCTCT
F_cp_*wbgL*	GATCATGGCAAAGAGGCAGGGCTTTCCGAT
R_cp_*wbgL*	GGTATATTCCCTTCTGATGAAATAGTCAAA
aslA-hemY:: *Hp3/4FT*	
F_vec_Truncated_ *Hp3/4FT*_ aslA-hemY	agaagcttagatctattacc
R_vec_Truncated_ *Hp3/4FT*_ aslA-hemY	gcaggtcgactctagagaat
F_Ins_ *Hp3/4FT*_ aslA-hemY	ctttttttgaattctctagagtcgacctgcTTTATCCAGAAAATGAATTG
R_Ins_ *Hp3/4FT*_ aslA-hemY	gggataacagggtaatagatctaagcttctCCGTTAAAACTAAAAACAGC
F_cp_*Hp3/4FT*	TGCGTGCGGTGGCAGTTGAC
R_cp_*Hp3/4FT*	ACTAAGGCGAACATAAGAGA

Table 3. Primers(continued)

3.2 Primer sequences for Gene Integration

Name	Sequence (5’-3’)
atpI-rsmG:: Hp3/FT	
F_vec_ *Hp3/4FT*_ atpI-rsmG	GGATCCTCGACTAGCATAACCCCTTGGGGCCTC TAAACGG
R_vec_ *Hp3/4FT*_ atpI-rsmG	CGAATTCGGATCCTGGCTGTGGTGATGATGGTG ATGGCTG
F_Ins_Truncated_ *Hp3/4FT*(Y131D)	TCACCACAGCCAGGATCCGAATTCGATGTTCCA GCCTCTGTTAGACGCTT
R_Ins_Truncated_ *Hp3/4FT*(Y131D)	AAGGGGTTATGCTAGTCGAGGATCCTCAGGTAG CCTTAGACAGAAGTCTT
F_Ins_*Hp3/4FT*	TCACCACAGCCAGGATCCGAATTCGATGTTCCA GCCGCTGCTCGACGCTT
R_Ins_*Hp3/4FT*	AAGGGGTTATGCTAGTCGAGGATCCTTATTTAC GGACCCACCGACGTATT
F_cp_*Hp3/4FT*	CTCTCTGAACGATATGGTGAGCTGGTGCCACCA TCTTCCT
R_cp_*Hp3/4FT*	AAGGGGTCTTTGAGGCTGAACAGCAATCCACTT GCTATCA

Table 3. Primers(continued)

3.3. Primer sequences for Promoter replacement

Name	Sequence (5’-3’)
F_vec_*wbgL*	GGATCCTCGACTAGCATAACCCCTT
R_vec_*wbgL*	TTAAAAACGATATCCTGCTGAGAAC
F_mp/wp_*wbgL*	GTTCTCAGCAGGATATCGTTTTTAActgacagctag ctcagtcctaggtataatg
R_mp/wp_*wbgL*	GCCCCAAGGGGTTATGCTAGTCGAGGATCCTCA GCATGAACTGTGCTTATCCACA
F_vec_*FucT2*	GGATCCTCGACTAGCATAACCCCTTGGGGCCTC TAAACGG
R_vec_*FucT2*	CGAATTCGGATCCTGGCTGTGGTGATGATGGTG ATGGCTG
F_sp/mp/wp _*FucT2*	TCACCACAGCCAGGATCCGAATTCGATGGCCTT CAAGGTTGTACAGATAT
R_sp/mp/wp _*FucT2*	AAGGGGTTATGCTAGTCGAGGATCCCTAGGCAT TGTACTTCTGGCTCTTG

Table 3. Primers(continued)

3.4. Primer sequences for Plasmid expression

Name	Sequence (5’-3’)
F_Trc_pCOLA /pACYC	TTGACAATTAATCATCCGGCTCGTATAATGGGAATTGTGAGCGGATAACAATTCCCCTGTAGAAATAATTTTGTTTAACT
R_Trc_pCOLA /pACYC	CATTATACGAGCCGGATGATTAATTGTCAAATTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGAGATCCCGGACAC

3. Result

3.1 Metabolic Engineering Strategies for Enhancing Precursor Availability

3.1.1. Host Strain Selection: *E. coli* BL21(DE3) vs. JM109(DE3)

To ensure the stability of precursor supply for fucosylated oligosaccharide biosynthesis, two *E. coli* strains with different lactose consumption rates, BL21(DE3) and JM109(DE3), were compared. BL21(DE3) rapidly consumes lactose regardless of induction, which can lead to the rapid depletion of precursors during the early stages of cultivation, thereby limiting the productivity of fucosylated products. In contrast, JM109(DE3) is a β-galactosidase-deficient strain harboring a mutation in the *lacZ* gene, allowing lactose uptake while inhibiting its metabolism, thereby maintaining a stable lactose concentration over an extended period (Baumgartner et al. 2013).

In this study, the lactose consumption patterns of the two strains were analyzed under identical conditions using lactose as the sole carbon source. The results showed that BL21(DE3) consumed most of the lactose within approximately 10hours, whereas JM109(DE3) exhibited a slower consumption pattern, utilizing lactose over a period of more than 24hours (Fig. 3). These results indicate that JM109(DE3) can stably supply lactose as a precursor for an extended period, which is advantageous for the accumulation of GDP-L-fucose and fucosylated oligosaccharides.

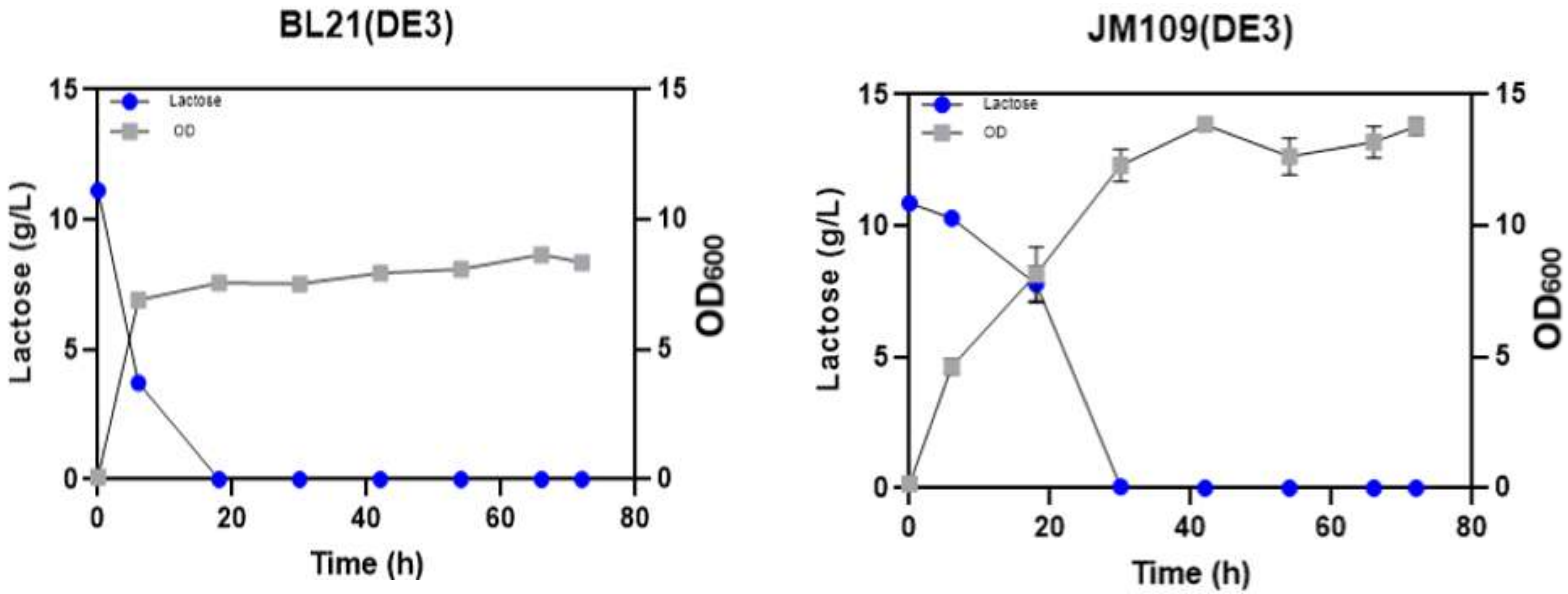


Fig. 3. Comparison of lactose consumption and cell growth between *E. coli* BL21(DE3) and JM109(DE3) (Supplementary with 10g/L of lactose). Symbols: Lactose (blue circle), OD600 (gray square). Error bars represent standard deviations from three independent experiments.

3.1.2. Disruption of Competing Pathways: *waaF* and *wcaJ* Deletion

In *E. coli*, endogenously synthesized GDP-L-fucose is primarily utilized for the biosynthesis of cell wall polysaccharides such as lipopolysaccharide(LPS) and colanic acid(CA). Since these pathways competitively consume GDP-L-fucose required for fucosylated oligosaccharide biosynthesis, blocking these pathways can be an effective strategy to enhance precursor accumulation and productivity. In this study, to block these competing metabolic pathways, the *waaF* gene involved in LPS synthesis and the *wcaJ* gene encoding the initiation enzyme for colanic acid biosynthesis were selectively deleted.

The *waaF* gene encodes ADP-heptose:LPS heptosyltransferase II, which catalyzes the addition of a heptose residue to the core oligosaccharide of LPS(Nakao et al. 2012). In contrast, the wcaJ gene encodes UDP-glucose-1-phosphate transferase, an enzyme that initiates the biosynthesis of colanic acid by directly utilizing GDP-L-fucose as a substrate(Stevenson et al. 1996). Therefore, these genes represent critical nodes within the primary metabolic pathways that consume GDP-L-fucose.

Gene deletions were achieved using the CRISPR-Cas9 system. Recombinant strains were screened by antibiotic selection, and the successful deletion of each gene was verified by colony PCR analysis. Genetic confirmation was obtained by detecting the expected amplicon size corresponding to each targeted deletion

(Fig. 4B). In addition, phenotypic changes associated with gene deletions were observed. Notably, the strain with the *waaF* gene deletion exhibited a sticky colony morphology distinct from the wild-type when cultured on LB medium(Fig. 4A), which is interpreted as an alteration in cell surface properties due to a defect in the LPS core structure. According to previous studies, the deletion of the *waaF* gene destabilizes the LPS structure, thereby activating colanic acid synthesis via the RcsCDB signaling pathway, resulting in increased cell surface stickiness (Ren et al. 2016).

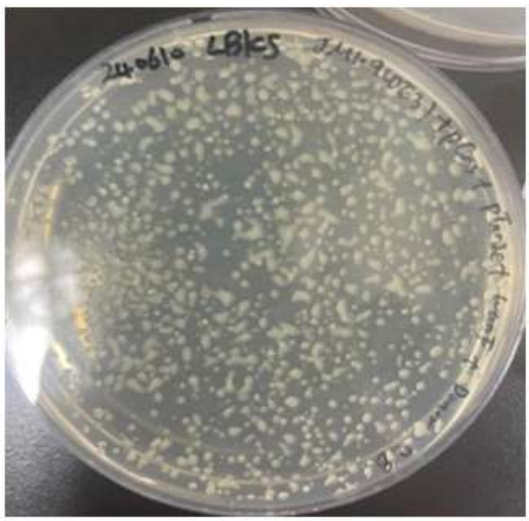


Fig. 4A. Sticky colony phenotype of *E. coli* JM109(DE3) on LBKS plate after *waaF* gene deletion.

The colonies exhibit a mucous and cohesive appearance, suggesting altered extracellular polysaccharide production. The plate was incubated at 30° C for 24 hours.

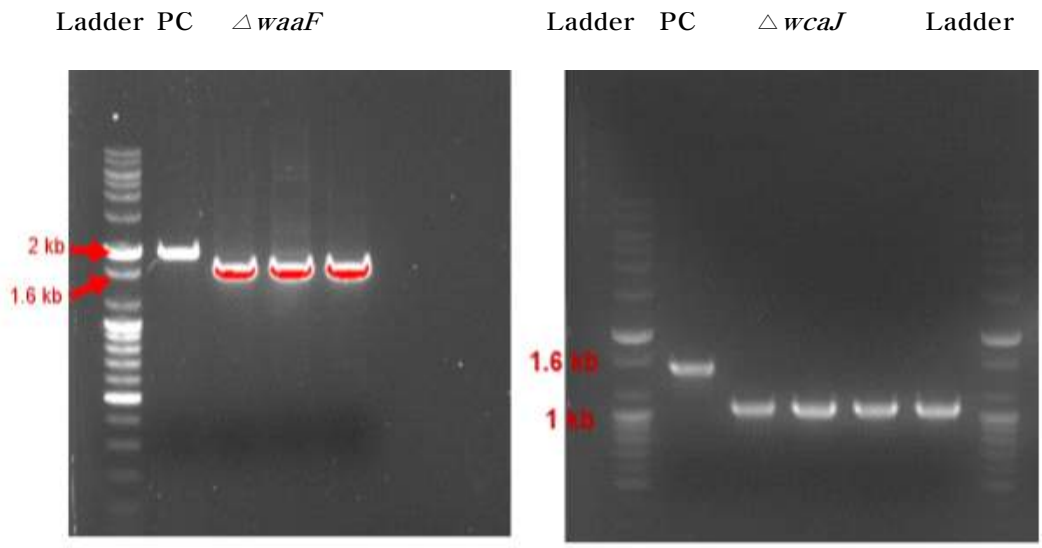


Fig. 4B. PCR verification of *waaF* and *wcaJ* gene deletions in *E. coli* JM109(DE3).

PCR analysis confirming the deletion of *waaF*(1.6kb) and *wcaJ*(1.0kb) genes in *E. coli* JM109(DE3). The positive control(PC) represents the wild-type strain, showing the respective band sizes of 1.9kb(*waaF*) and 1.4kb(*wcaJ).*

3.1.3. Prevention of Lactose Degradation: *lacZ* Deletion

In 2'-FL production, lactose serves as an essential substrate. However, *E. coli* utilizes lactose for cell growth by breaking it down into glucose and galactose via β-galactosidase, which is encoded by the *lacZ* gene. This pathway limits the availability of lactose required for 2'-FL synthesis, thereby posing a disadvantage in terms of precursor preservation. Lee et al. (2021) reported that residual β-galactosidase activity under lactose-based co-fermentation conditions excessively hydrolyzed lactose,

resulting in most of the lactose being consumed for cell growth, with a significantly low conversion rate to 2'-FL. Therefore, eliminating β-galactosidase activity is expected to enable more efficient utilization of lactose as a precursor for 2'-FL production (Lee et al. 2021).

Based on this strategy, the *lacZ* gene was deleted using the CRISPR-Cas9 system, and the deletion was confirmed by colony PCR(Fig. 5a). The expected size of the PCR product was successfully amplified, indicating that the *lacZ* deletion was successfully achieved at the genetic level. Subsequently, the engineered strain was subjected to a 72hour shake-flask batch fermentation, and the fermentation broth was analyzed. In the *lacZ*-deficient strain, almost no lactose consumption was observed in the medium, whereas the parental strain harboring the *lacZ* gene exhibited rapid lactose depletion under the same conditions (Fig. 5b). These results indicate that the removal of the *lacZ* gene effectively preserves the precursor, contributing to the stability of 2'-FL production during prolonged fermentation.

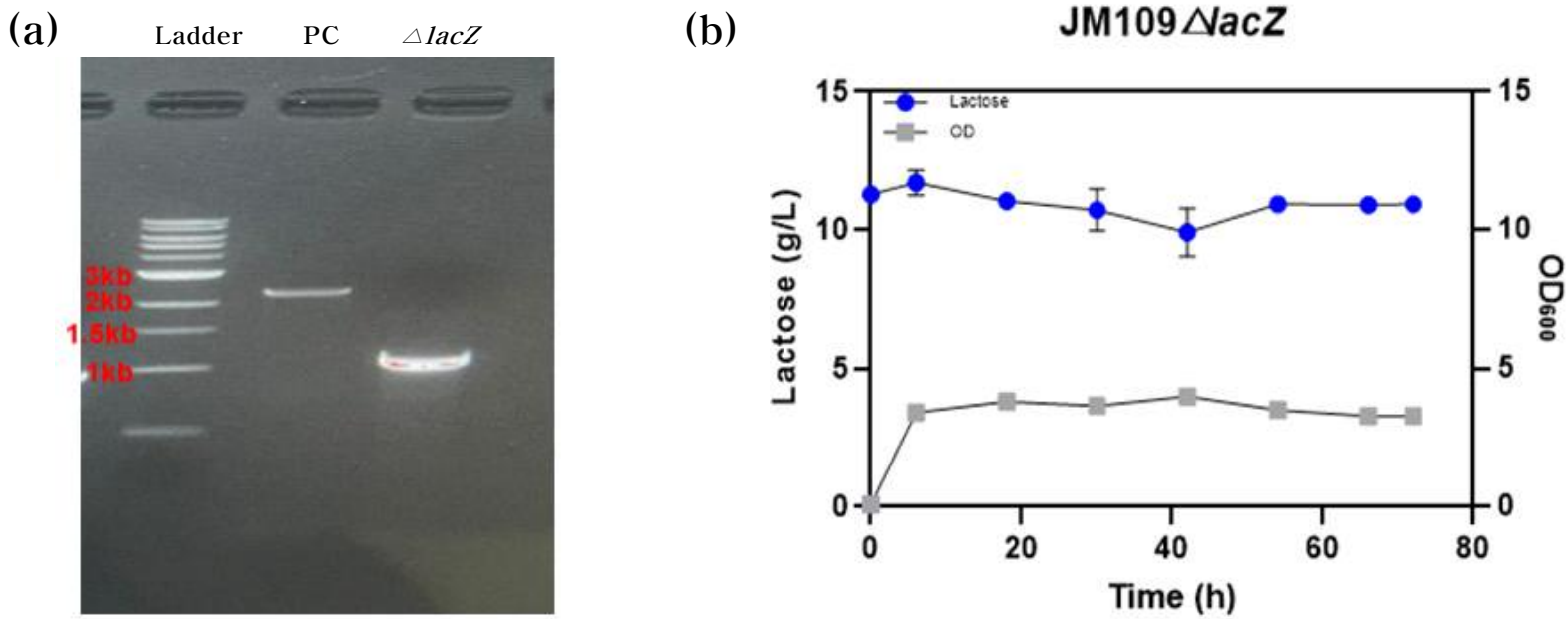


Fig. 5. Verification of *lacZ* gene deletion and batch fermentation profile of *E. coli* JM109(DE3)⊿*lacZ*.

(a) PCR analysis confirming the deletion of the *lacZ* gene(1.2kb) in *E. coli* JM109(DE3). The positive control (PC) represents the wild-type strain carrying the pCas plasmid(2.3kb). (b) Lactose consumption and cell growth profile of *E. coli* JM109(DE3) ⊿*lacZ* in batch fermentation. (Supplementary with 10g/L of lactose). Symbols: Lactose (blue circle), OD600 (gray square). Error bars represent standard deviations from three independent experiments.

3.2. Fucosyltransferase Expression and Optimization

3.2.1. Enhanced 2'-FL Production through Promoter Engineering

Although α-1,2-fucosyltransferase is a highly active enzyme, issues such as growth inhibition, protein misfolding, or inclusion body formation can arise depending on the expression level. In particular, the T7 promoter induces high transcriptional activity; however, when the transcription rate exceeds the protein folding rate, it can lead to improper folding of heterologous proteins and accumulation in insoluble forms(Thomas and Baneyx 1996, Ren et al. 2016). Therefore, selecting a promoter with a regulated expression strength is essential to achieve stable and functional expression of α-1,2-fucosyltransferase.

In this study, the relative expression efficiencies of various promoters with different strengths were quantitatively assessed using an EGFP reporter system. Four promoters (T7, J23100, J23108, and J23117) were selected and individually cloned into the pETDuet-1 vector containing the EGFP gene. The resulting constructs were transformed into *E. coli* JM109(DE3). The fluorescence intensity of each strain was then measured using a microplate reader. Among the tested promoters, T7 exhibited the highest fluorescence intensity, followed by J23100, J23108, and J23117 in descending order (Fig. 6).

Based on the results of the promoter strength comparison experiment using the EGFP reporter system, two types of α-1,2-fucosyltransferase genes—*wbgL* from *Escherichia coli* O126:H27 and *FucT2* from *Helicobacter pylori* were integrated into the ompW-yciE intergenic site of the *E. coli*

JM109(DE3) chromosome under three constitutive promoters (J23100, J23108, J23117). The resulting strains were then evaluated for actual 2'-FL production.

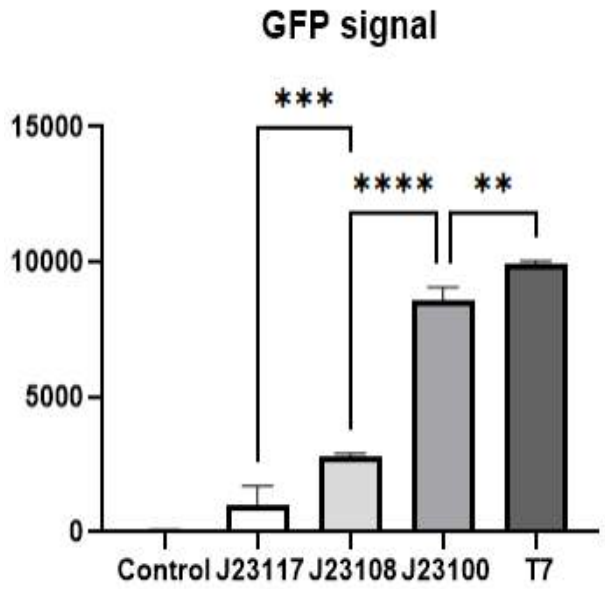


Fig. 6. Relative fluorescence intensity of EGFP expressed under different promoters in *E. coli* JM109(DE3)

EGFP signal intensity measured under different promoters J23117, J23108, J23100, T7. The control represents a plasmid without the EGFP gene. Statistical significance is indicated by asterisks. Error bars represent standard deviations from three independent experiments.

In this study, considering the limitations of conventional plasmid-based expression systems—such as inducer and antibiotic dependency, metabolic burden, and plasmid loss—as well as the potential for protein misfolding and inclusion body

formation during T7 promoter-driven overexpression (Baumgartner et al. 2013), we established a more stable chromosomal expression system utilizing constitutive promoters that do not require inducers. Similar to the results of the EGFP reporter experiment, the use of the J23100 promoter resulted in the highest 2'-FL production at 0.258g/L. This was followed by the J23108 promoter at 0.193 g/L and the J23117 promoter at 0.179g/L, showing a decreasing trend in 2'-FL production in the order of J23100, J23108, and J23117. However, the overall differences in production were not significant, suggesting that while promoter strength may influence transcription levels, its impact on actual metabolite production can be limited (Fig. 7).

Under the same promoter conditions, the strain expressing *wbgL* consistently exhibited higher 2'-FL production compared to the strain expressing *FucT2*. This difference is likely attributable to variations in enzyme conversion efficiency or expression stability. Based on these results, the △ZJF 02 strain, which demonstrated the highest 2'-FL productivity, was selected as the host strain for the introduction of α-1,3-fucosyltransferase. This strain was subsequently employed for metabolic engineering strategies aimed at enhancing DFL biosynthesis.

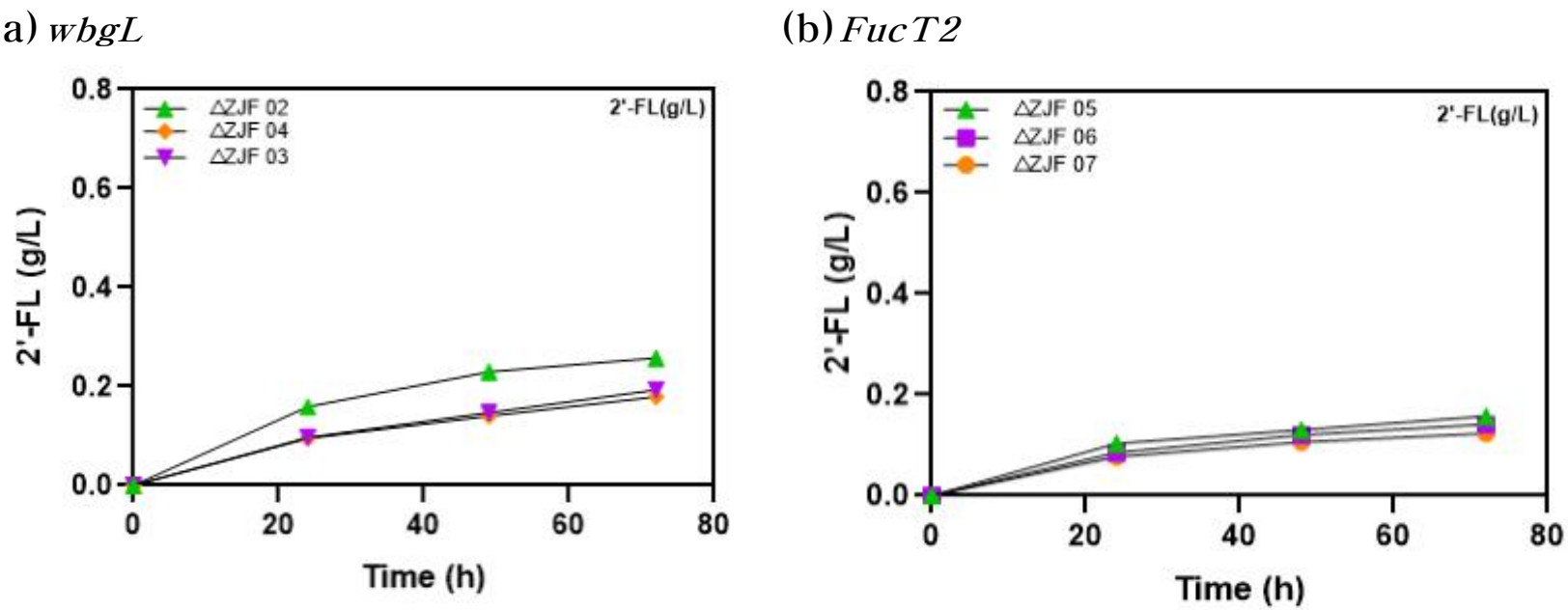


Fig. 7. Batch fermentation profiles of 2'-FL production by engineered *E. coli* JM109(DE3) strains expressing *wbgL* and *FucT2* under different promoters.

(Supplementary with 2g/L of lactose, 5g/L Glycerol). (a)Symbols: △ZJF 02 (green triangle), △ZJF 03 (Purple inverted triangle), △ZJF 04 (Orange diamond). (b)Symbols: △ZJF 05 (green triangle), △ZJF 06 (Purple square), △ZJF 07 (Orange circle).

3.2.2. Introduction of α-1,3/4-Fucosyltransferase

In this study, the *Hp3/4FT* gene from Helicobacter pylori and its truncated variant were selected as α-1,3/4-fucosyltransferases and integrated into the chromosome. Unlike conventional *Hp3/4FT* exhibits low activity toward non-fucosylated oligosaccharides(e.g., lactose) but selectively acts on α-1,2-fucosylated acceptors such as 2'-FL. This substrate specificity makes it a suitable enzyme for the selective biosynthesis of DFL, as it minimizes the formation of byproducts such as 3-FL. Previous studies have reported that the truncated form of *Hp3/4FT* exhibits superior expression stability and enzyme activity compared to the full-length version, contributing to enhanced DFL production efficiency (Zhu et al. 2024).

Therefore, in this study, the *Hp3/4FT* gene and the truncated *Hp3/4FT* gene were individually integrated into the aslA-hemY intergenic site under the control of the J23100 promoter. The presence of DFL was quantitatively analyzed by HPLC after 72 hour batch fermentation. HPLC analysis of the strain expressing truncated *Hp3/4FT* demonstrated the presence of DFL, as indicated by the sample peak observed at a retention time(RT) of 3.52min, which corresponded to the RT of the DFL standard. (Fig. 9); however, the final yield was only approximately 0.15g/L, indicating that the overall productivity remained low. In the strain expressing truncated *Hp3/4FT*, only DFL was detected, and 2'-FL

was depleted, whereas in the strain expressing *Hp3/4FT*, only 2'-FL was detected with no trace of DFL (Fig. 8). This difference suggests that the truncated form exhibits catalytic activity contributing to DFL synthesis. In contrast, the full-length *Hp3/4FT*, due to relatively low enzyme activity under the same conditions, appears unable to convert 2'-FL to DFL when the 2'-FL concentration is limited.

These results indicate that the expression of α-1,3/4-fucosyltransferase alone is insufficient to achieve high efficiency DFL biosynthesis, highlighting that the biosynthesis and supply of 2'-FL represent major bottlenecks in productivity. In fact, even in the strain expressing truncated *Hp3/4FT*, 2'-FL was rapidly depleted, while the DFL yield remained limited. Therefore, to enhance DFL production efficiency, it is essential to improve precursor accumulation by genetically regulating the 2'-FL biosynthetic pathway.

Accordingly, in the next phase of this study, metabolic engineering strategies were implemented to strengthen the 2'-FL biosynthesis pathway.

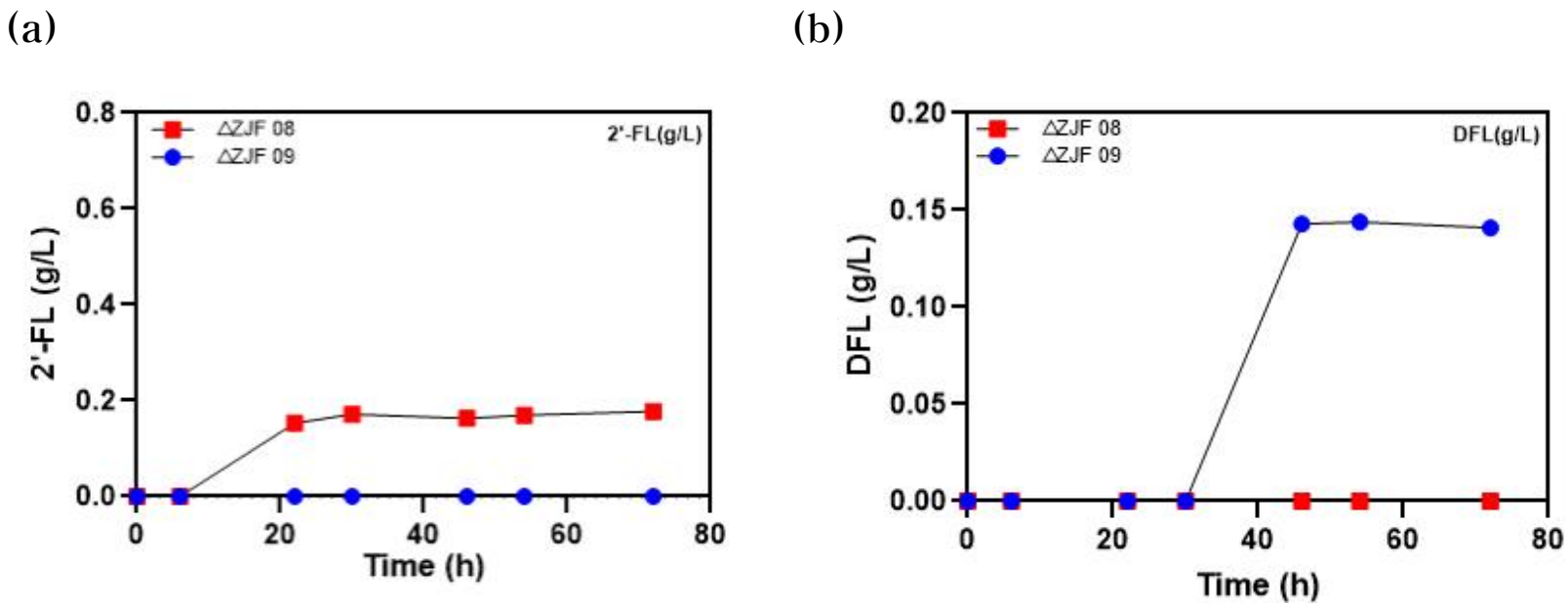


Fig. 8. Comparative batch fermentation profiles of engineered *E. coli* △ZJF 08 and △ZJF 09 strains.

(Supplemented with 2g/L lactose and 5g/L glycerol.) (a) 2'-FL production profiles of △ZJF 08 (red square) and △ZJF 09 (blue circle) strains. (b) DFL production profiles of △ZJF 08 (red square) and △ZJF 09 (blue circle) strains.

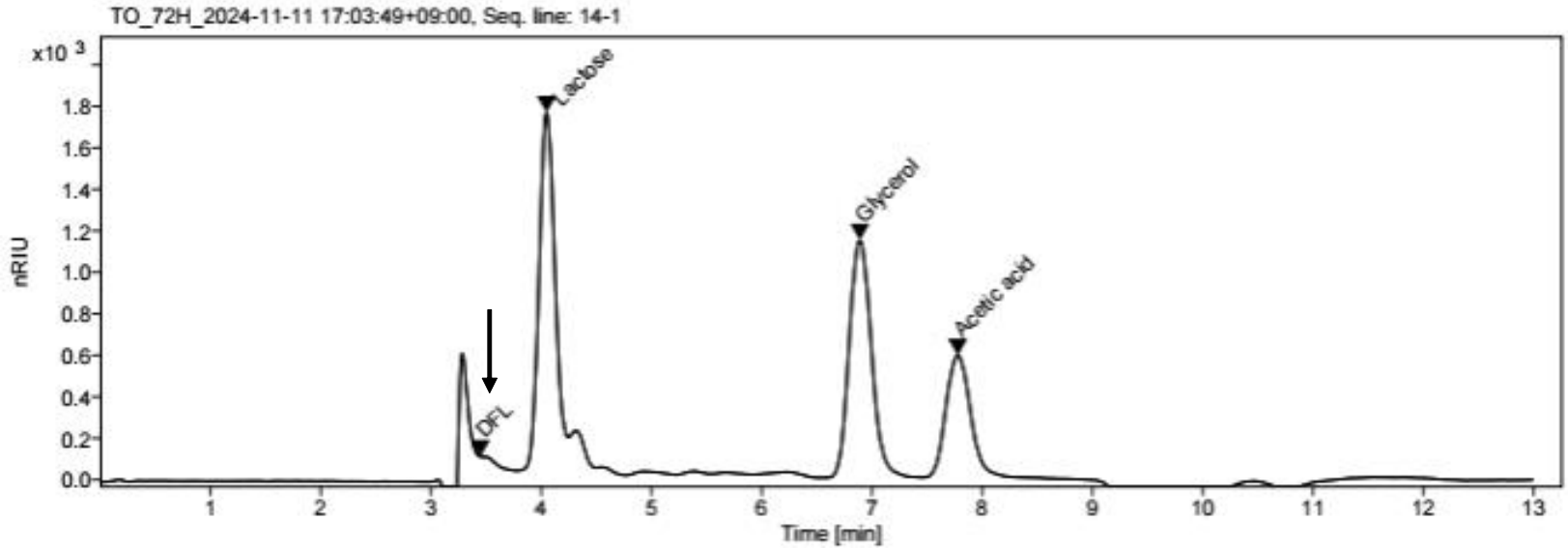


Fig. 9. HPLC analysis of DFL production in engineered *E. coli* △ZJF 09 strains.(Retention time(RT): 3.52min)
Cells were collected at 72hours after 0.1mM IPTG induction in batch fermentations. The chromatogram shows the HPLC profiles of DFL production from recombinant *E. coli* strains. The analysis was performed using a refractive index detector (RID).

3.3. Strategies for Enhancing DFL Production

The key strategies employed in this study to enhance DFL production are summarized as follows. First, the expression level of α-1,2-fucosyltransferase was regulated to increase the supply of the precursor, 2'-FL. Second, fed-batch fermentation was introduced in place of conventional batch fermentation to improve production efficiency. Third, a mutant form (Y131D) of the Hp3/4FT gene was introduced to enhance the enzymatic activity of α-1,3/4-fucosyltransferase. In the following sections, the rationale for each strategy and the corresponding experimental results are described in detail.

3.3.1. Enhancement of 2'-FL Supply via Trc Promoter Replacement

Based on the previous results, this study hypothesized that regulating the expression level of α-1,2-fucosyltransferase could increase the supply of 2'-FL, thereby enhancing DFL productivity. According to previous studies, the Trc promoter is effective in maintaining a balance between functional enzyme expression and precursor production during α-1,2-fucosyltransferase expression. In fact, Lee et al.(2021) reported that the Trc promoter-based expression system is advantageous for increasing 2'-FL production compared to the T7 promoter.

To experimentally validate the previously established hypothesis, the relative effects of the T7 and Trc promoters on the accumulation of 2'-FL were evaluated. For this purpose, △ZJF 10-13 strains were constructed. After batch fermentation, the 2'-FL production levels of each strain were analyzed. The experimental results showed that the 2'-FL concentration of the △ZJF 12 strain reached a maximum of 0.7g/L (Fig. 10), which was significantly higher than that of the △ZJF 10 strain, which produced approximately 0.4g/L of 2'-FL. Similarly, the △ZJF 13 strain exhibited a 2'-FL production of approximately 0.4g/L, representing more than a twofold increase compared to the △ZJF 11 strain, which produced about 0.2g/L (Fig. 11). These results suggest that the expression of α-1,2-fucosyltransferase under the control of the Trc promoter can maintain functional enzyme expression without imposing excessive expression burdens, thereby effectively enhancing 2'-FL production. In both genes, the Trc promoter condition consistently led to increased production, indicating that strengthening precursor accumulation through Trc promoter regulation can serve as a key strategy to improve DFL production efficiency.

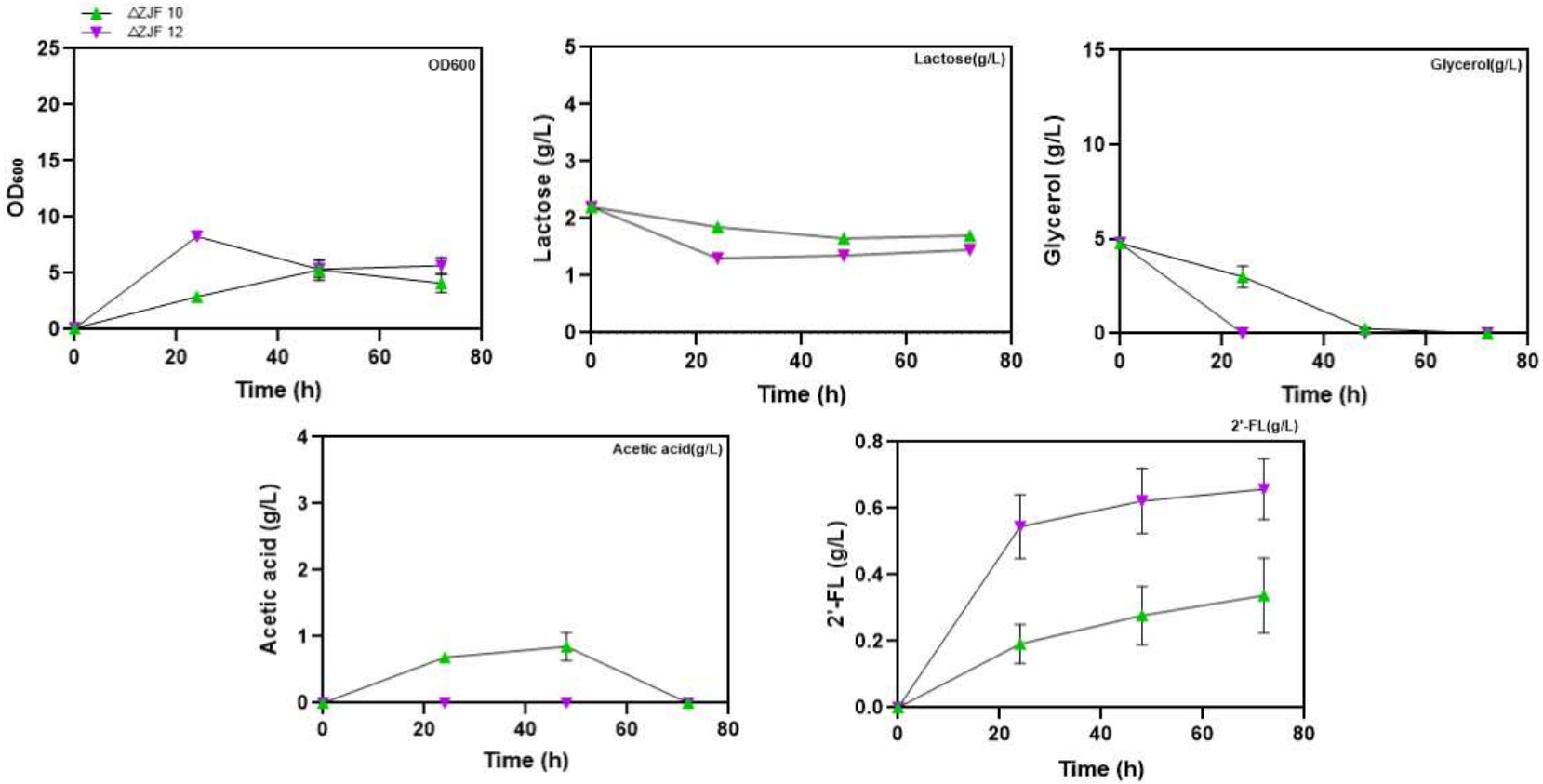


Fig. 10. Batch Fermentation Analysis of *E. coli* △ZJF 10 and △ZJF 12 Strains: Effect of T7 vs. Trc Promoters.

(Supplementary with 2g/L lactose and 5g/L glycerol.) Symbols: △ZJF 10(Green triangle), △ZJF 12(Purple inverted triangle)

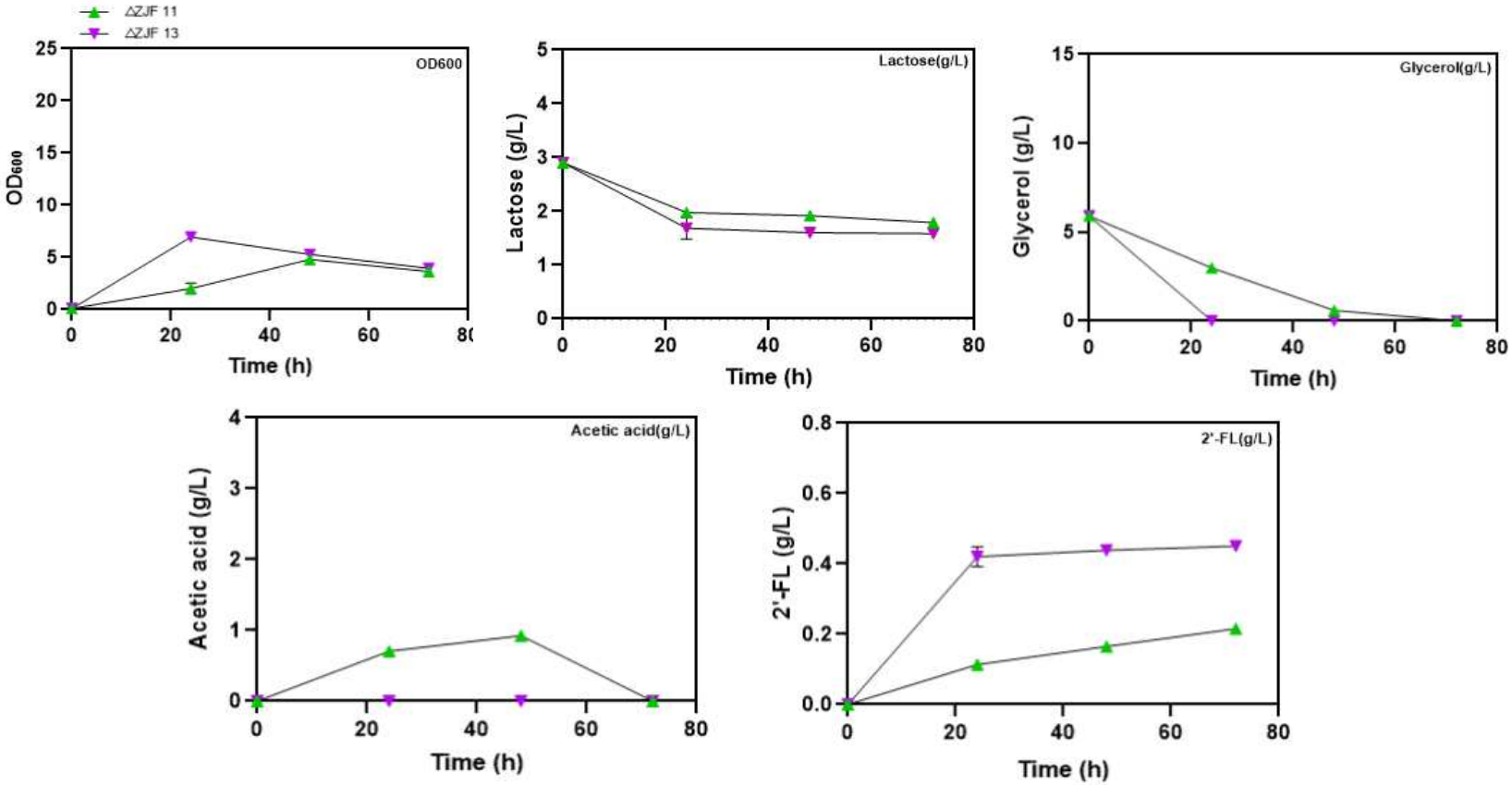


Fig. 11. Batch Fermentation Analysis of *E. coli* △ZJF 11 and △ZJF 13 Strains: Effect of T7 vs. Trc Promoters.

(Supplementary with 2g/L lactose and 5g/L glycerol.) Symbols: △ZJF 11(Green triangle), △ZJF 13(Purple inverted triangle)

3.3.2. Enhancement of 2'-FL Production via Fed-Batch Fermentation

The previous results confirmed that the Trc promoter based expression system enables efficient expression of α-1,2-fucosyltransferase, leading to a significant increase in 2'-FL accumulation. However, during the later stages of batch fermentation, the increase in 2'-FL production plateaued, which is presumed to be due to the limitation of the biosynthetic pathway caused by the depletion of glycerol, the main carbon source in the medium. Accordingly, to compensate for carbon source depletion during cultivation and further enhance 2'-FL productivity, a fed-batch fermentation strategy was implemented. The fermentation was carried out for 124hours, during which glycerol was periodically supplemented at 5g/L whenever depletion was observed, allowing continuous precursor synthesis. This strategy aimed to maintain the performance of the expression system while preventing productivity decline caused by carbon source limitation.

The △ZJF 12 strain produced a final 2'-FL concentration of approximately 1.4g/L under fed-batch conditions, representing a 2-fold increase compared to the batch condition, where the production was around 0.7g/L. Similarly, the △ZJF 13 strain showed a final 2'-FL production of approximately 1.0 g/L under fed-batch conditions, indicating a 2.5-fold increase compared to the batch condition, where the production was around 0.4g/L.(Fig. 12)

These results suggest that not only the regulation of expression

levels but also the design of fermentation process conditions significantly influences precursor accumulation. In particular, the implementation of a fed-batch strategy effectively alleviates metabolic limitations caused by carbon source depletion, thereby establishing essential conditions for enhancing DFL productivity based on a stable supply of 2'-FL.

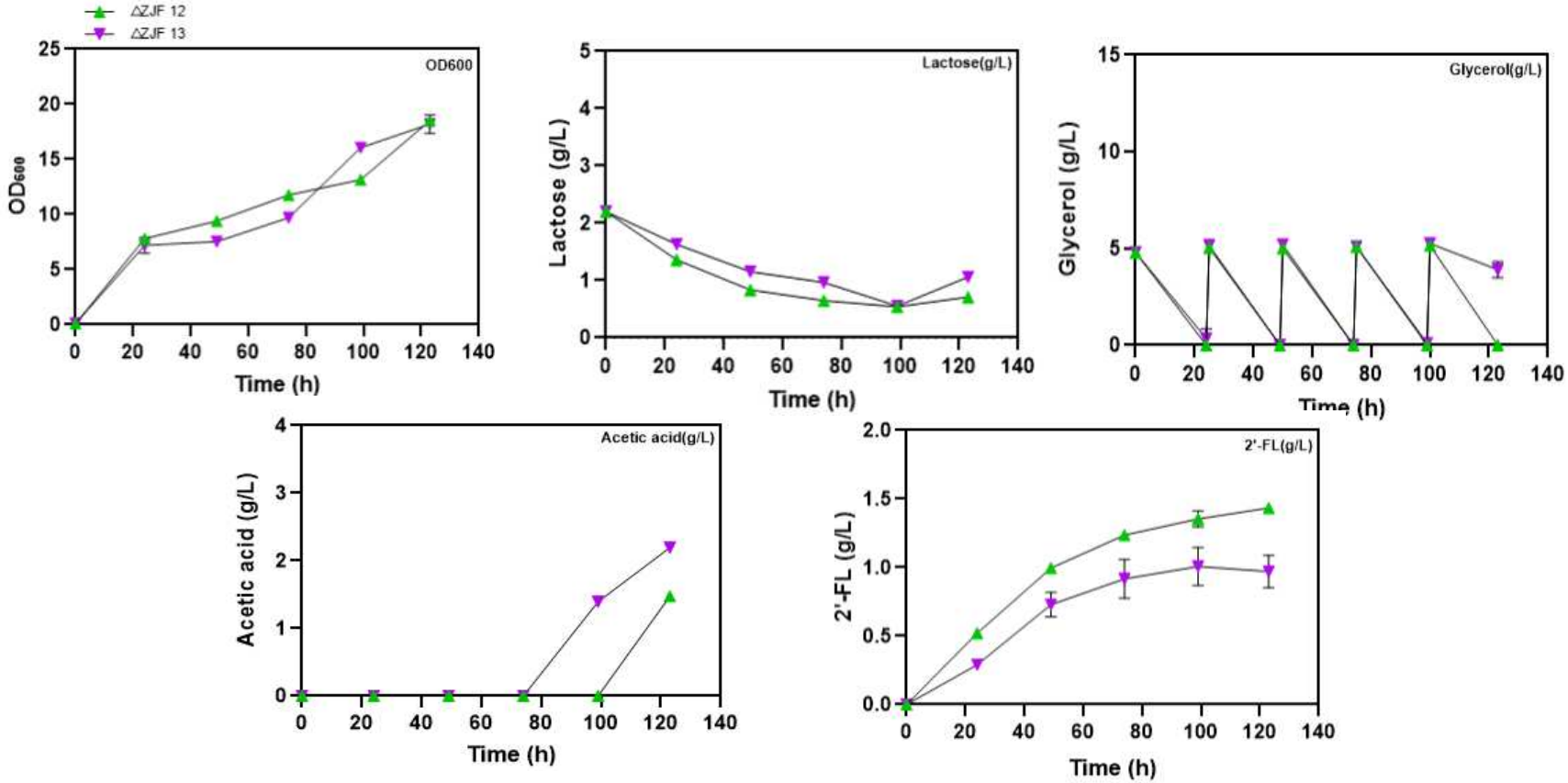


Fig. 12. Fed-batch Fermentation Analysis of *E. coli* △ZJF 12 and △ZJF 13 Strains

(Supplemented with 2g/L lactose and 5g/L glycerol; additional glycerol was supplied every 24hours after depletion.)

Symbols: △ZJF 12(Green triangle), △ZJF 13(Purple inverted triangle)

3.3.3. Enhancement of DFL Production via Introduction of the *Hp3/4FT*(Y131D) Variant

As the third strategy to enhance DFL production, a point mutated variant of *Hp3/4FT*(Y131D) was introduced to improve the catalytic activity of α-1,3/4-fucosyltransferase. Unlike the previous approaches that focused on precursor supply, this strategy aimed to increase the final DFL titer by enhancing the intrinsic enzymatic efficiency of the fucosyltransferase. The Y131D variant involves the substitution of the 131st amino acid residue of *Hp3/4FT*, tyrosine(Y), with aspartic acid(D), thereby inducing a structural modification that can enhance the enzyme's catalytic reactivity. According to previous reports, this variant exhibited approximately 1.2-fold higher DFL production compared to the wild-type *Hp3/4FT* (Zhu et al. 2024).

In this study, the *Hp3/4FT*(Y131D) variant was expressed under fed-batch fermentation conditions to evaluate changes in DFL production compared to the wild-type enzyme. This approach aimed to assess the applicability of an enzyme activity-based strategy for enhancing DFL productivity.

3.3.4. Evaluation of DFL Production under Optimized Conditions

To evaluate the overall impact of the combined strategies—Trc promoter introduction, fed-batch fermentation, and incorporation

of the *Hp3/4FT* variant—two *E. coli* strains were constructed, each expressing either the wild-type *Hp3/4FT* or the Y131D mutant. Both strains were engineered to express *wbgL* under the control of the Trc promoter and were cultivated under identical fed-batch conditions to compare the production levels of 2'-FL and DFL. As shown in Fig. 13, both strains exhibited a marked increase in 2'-FL accumulation compared to the previous strategy, with the Y131D mutant strain producing approximately 1.0g/L and the wild-type strain producing about 0.8g/L of 2'-FL. However, the conversion of 2'-FL to DFL remained limited, resulting in final DFL concentrations of 0.24g/L for the Y131D variant and 0.229g/L for the wild-type strain—indicating only a modest increase in product formation relative to precursor accumulation.

These results suggest that while the combined strategies—Trc promoter replacement, fed-batch fermentation, and enzyme variant introduction—were effective in enhancing 2'-FL accumulation, the in vivo catalytic activity of *Hp3/4FT* remains a major bottleneck in improving overall DFL productivity. In particular, the limited conversion of 2'-FL to DFL despite its sufficient accumulation indicates that further improvements are required in factors such as enzyme expression level, substrate accessibility, and structural stability.

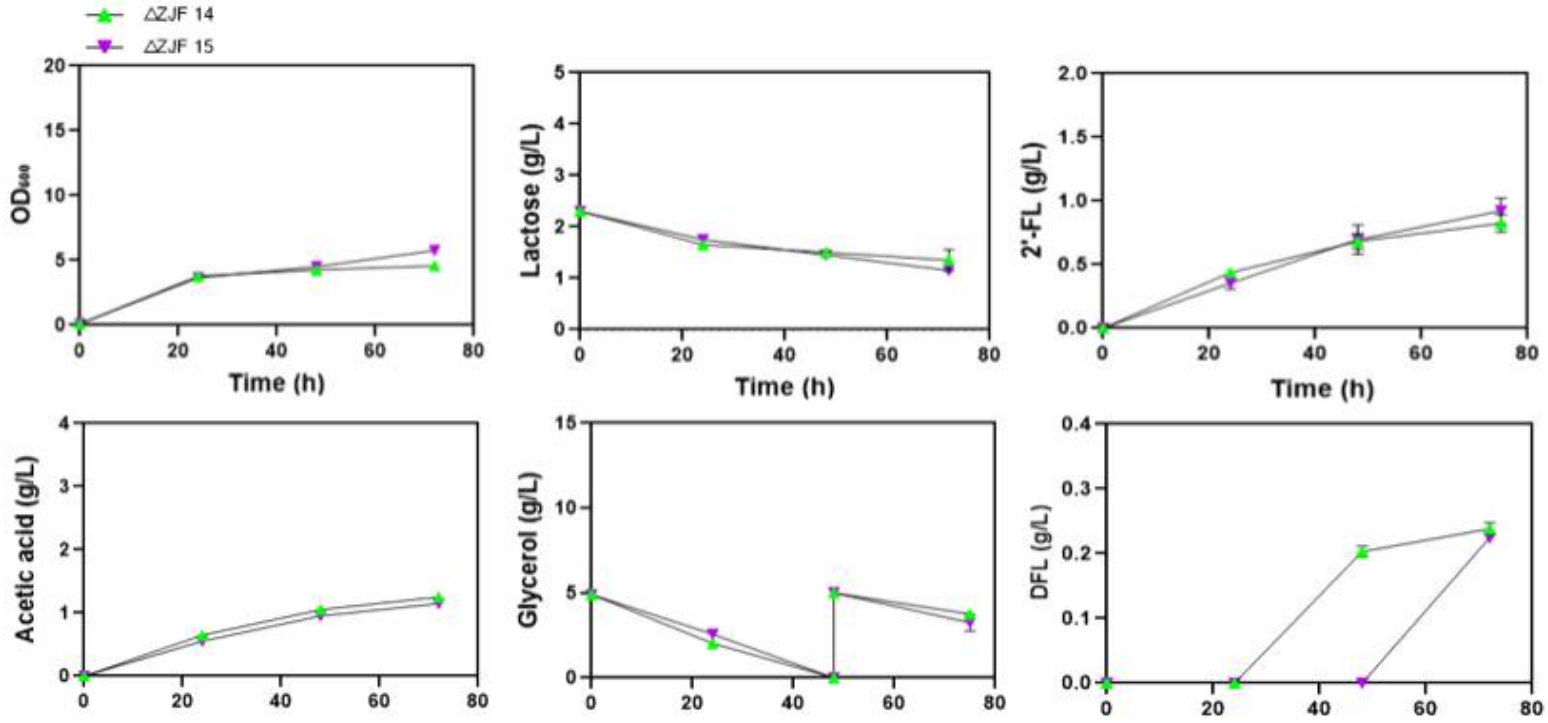


Fig. 13. Final evaluation of DFL production following the integrated optimization strategy.

(Supplemented with 2g/L lactose and 5g/L glycerol; additional glycerol was supplied every 24hours after depletion.)

Symbols: △ZJF 14 (Green triangle, *Hp3/4FT* Y131D*)*, △ZJF 15 (Purple inverted triangle, wild-type *Hp3/4FT*)

4. Discussion

This study was initiated as a strategic approach to enable microbial production of DFL. To this end, a genetic framework for DFL production was established, and the expression of key enzymes was systematically optimized to improve overall productivity. To ensure a stable supply of lactose and to increase the intracellular accumulation of GDP-L-fucose, three metabolic engineering strategies were implemented.

First, *E. coli* JM109(DE3), a β-galactosidase-deficient strain, was chosen as the host to ensure a stable supply of lactose during fermentation. Second, to block pathways that competitively consume GDP-L-fucose, the *waaF* and *wcaJ* genes were individually deleted using the CRISPR-Cas9 system. Third, the *lacZ* gene, responsible for the hydrolysis of lactose into glucose and galactose, was deleted to prevent unnecessary metabolic loss of the precursor.

Subsequently, to optimize the expression efficiency of the α-1,2-fucosyltransferase step, the expression strengths of various constitutive promoters(J23100, J23108, J23117) were evaluated using an EGFP reporter system. Based on these data, the *FucT2* and *wbgL* genes were individually integrated into the strains, and the actual 2'-FL productivity was compared according to the promoter used. In the EGFP assay, the expression strength was observed in the order of promoter J23100, J23108, and J23117.

In terms of actual 2'-FL production, no significant differences were observed among the three promoters. This result suggests that differences at the transcriptional level may not necessarily correlate with consistent differences in metabolite production. Meanwhile, when comparing the same promoter conditions, the *wbgL* expressing strain exhibited generally higher 2'-FL productivity compared to the *FucT2* expressing strain. This difference is likely attributed to variations in enzyme conversion efficiency or expression stability.

Based on these results, this study selected the △ZJF 02 strain as the base strain and attempted DFL production by chromosomally integrating the *Hp3/4FT* in both full-length and truncated forms. However, even in the strain expressing truncated *Hp3/4FT*, the final DFL yield was only 0.15g/L, while no DFL was detected in the strain expressing the full-length form. In particular, in the truncated form, despite the complete depletion of 2'-FL, the DFL yield remained low. In contrast, in the full-length form, only 2'-FL was detected without any conversion to DFL. These results suggest that the bottleneck in DFL biosynthesis may not only be due to the activity of α-1,3/4-fucosyltransferase but also to the insufficient supply of the precursor, 2'-FL.

To address these limitations and enhance DFL production, three key strategies were sequentially implemented in this study. As the first strategy, the expression of α-1,2-fucosyltransferase was regulated using a Trc promoter to enhance 2'-FL production.

Second, a fed-batch cultivation strategy was introduced to overcome productivity stagnation caused by glycerol depletion under batch fermentation conditions. Third, the Y131D variant of the *Hp3/4FT* gene was introduced to improve the catalytic activity of α-1,3/4-fucosyltransferase.

As a result of introducing the Trc promoter, 2'-FL production increased more than twofold compared to the conventional T7-based system. In addition, under fed-batch cultivation conditions, the periodic supplementation of glycerol enabled continuous precursor accumulation, and the *wbgL*(Trc) expressing strain exhibited the highest level of 2'-FL production. Lastly, in the strain expressing the truncated *Hp3/4FT*(Y131D) variant, DFL production showed a slight increase compared to the wild-type, but the final DFL titer remained at a relatively low level.

These findings suggest that the catalytic activity of *Hp3/4FT* remains a major bottleneck in the overall DFL biosynthetic pathway. Therefore, further strategies are required to enhance the enzymatic activity of α-1,3/4-fucosyltransferase. Specifically, fusing solubility-enhancing protein tags such as TrxA, GroS, or NusA to the N-terminus may improve expression levels and solubility, while structure-guided directed mutagenesis could be employed to precisely optimize the active site for improved catalytic performance. In conclusion, this study successfully established a set of effective strategies to enhance 2'-FL precursor production. With further improvements in the catalytic

efficiency of α-1,3/4-fucosyltransferase, the development of a high-yield DFL production system is expected to be increasingly feasible.

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ABSTRACT IN KOREAN

사람 모유 올리고당(Human milk oligosaccharides, HMOs)은 장내 미생물 군 형성, 병원성 세균의 부착 억제, 면역 체계 조절 등 다양한 생리학적 기능을 수행하는 필수 생리활성 성분이다. 이 중 difucosyllactose (DFL)는 두 개의 푸코스 잔기를 포함하는 독특한 구조로 인해 우수한 생리활성을 나타내며, 기능성 식품 성분 및 의약 소재로서의 활용 가능성이 주목받고 있다. 본 연구에서는 *Escherichia coli*를 이용하여 DFL을 생산하기 위한 대사공학 전략을 개발하였다. DFL의 공여체인 GDP-L-fucose의 축적을 향상시키기 위해, 생합성 경로에 관여하는 *waaF* 및 *wcaJ* 유전자를 제거하였다. 또한 수용체인 락토스의 분해를 억제하기 위해 *lacZ* 유전자를 제거하였다. 이후 2'-FL 합성을 위해 α-1,2-fucosyltransferase인 *WbgL* 와 *FucT2*를 도입해주었으며, 효소발현을 최적화 시키기위해 다양한 프로모터 조건 하에서 발현 정도를 체계적으로 비교 평가하였다. 이후 *WbgL*과 함께 작용하여 DFL을 생산할 수 있는 α-1,3/4-fucosyltransferase인 *Hp3/4FT* 를 도입하여 이중 푸코실화 경로를 구축하였으며, 이로써 DFL 생산과 동시에 부산물인 3-FL 생성을 최소화할 수 있었다. 또한 효소 발현성과 촉매 성능 향상 가능성을 평가하기 위해 *Hp3/4FT*의 wild-type과 truncated 변이체의 DFL 생산량을 비교하였다. 구축된 경로를 기반으로 한 초기 발효에서는 낮은 수준의 DFL 생산성이 확인되었으며, 이를 개선하기 위해 2'-FL의 생합성을 프로모터 조절을 통해 향상시켰다. 또한, 배양 조건을 최적화하고, *Hp3/4FT*의 변이체를 도입함으로써 효소 발현성과 전체적인 DFL 생산성의 향상을 도모하였다.

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