INTRODUCTION

The trisaccharide, 2′-fucosyllactose (2′-FL) and 3-fucosyllactose (3-FL), are essential immunologically active ingredients in human milk, with significant physiological functions such as promoting infant brain development, modulating immune responses, suppressing inflammation and resisting pathogenic microbial infections, and are extensively applied in infant formulas, nutraceuticals and food additives (Han et al., 2012).

Particularly, the US and EU food safety regulators have authorized the use of 2′-FL as a nutritional supplement in infant formula (Sprenger et al., 2017). However, biotechnological bottlenecks and high costs of FL preparation have restricted its widespread market application.

In the last decade, various approaches, including breast milk isolation, chemical synthesis, enzymatic conversion and microbial fermentation, have been utilized to produce FL (Agoston et al., 2019; Anderson & Donald, 1981; Baumgärtner et al., 2013). Considering
environment-friendly and sustainable production strategies, the utilization of fast-growing biological expression systems with high substrate consumption rates, such as *Escherichia coli* (Huang et al., 2017), yeast (Hollands et al., 2019) and *Bacillus subtilis* (Deng et al., 2019), can further improve the productivity of FL during fermentation and facilitate the development of HMOs industrial biotechnology. GDP-L-fucose, a precursor of the colanic acid biosynthetic pathway, can be accumulated via de novo pathway or salvage pathway (Figure 1). Noteworthy, the intracellular availability of GDP-L-fucose determines the overall yield of FL (Lee et al., 2011). The salvage pathway is generally considered unsuitable for industrial production of FL due to expensive substrate feedstocks (L-fucose, ATP and GTP) (Jung et al., 2019). Deletion of the wcaJ and lon genes and overexpression of the rcsA gene in the de novo pathway has been confirmed to favour the intracellular accumulation of GDP-L-fucose (Huang et al., 2017). The selection of strong promoters or high-copy plasmids also contributed to enhancing expression levels of critical genes in the GDP-L-fucose pathway (Li, Li, et al., 2021; Li, Zhu, et al., 2021). Furthermore, the carbon fluxes of other critical intermediates (e.g., fru-6-P and GDP-D-mannose) in this pathway also determine the intracellular availability of GDP-L-fucose, which will be an innovative point in our work. Lactose is an essential receptor substrate in FL production, and its intracellular availability is achieved by inactivating the lacZ gene (Chin et al., 2015; Chin et al., 2017). Based on previous studies, there are rate-limiting enzymes in the FL synthesis pathway, particularly α-1,2/3-fucosyltransferase, which are engaged in catalysing the final step of the metabolic reaction (Drouillard et al., 2006). The low catalytic activity and poor soluble expression of these enzymes have become bottlenecks in FL biosynthesis (Chin et al., 2015). The acquisition of high-activity α-1,2/3-fucosyltransferase is not limited to the screening of enzyme sources. Strategies such as modulating transcriptional and translational control elements, improving enzyme properties and protein fusion can also effectively increase the catalytic activity of rate-limiting enzymes and promote the expression of soluble recombinant proteins by reducing the formation of inactive inclusion bodies caused by protein misfolding (Lin et al., 2022). Related studies have rarely been reported in FL production; therefore, in this study, the performance of α-1,2/3-fucosyltransferase was further improved by optimizing ribosome binding sites (RBS), fusion peptides and multi-copy genes.

**FIGURE 1** Engineered pathway for de novo synthesis 2′-FL and 3-FL in *E. coli*. Overexpressed genes are highlighted in purple, deleted genes are annotated in red. manA, manB, phosphomannomutase; manC, α-Dmannose-1-phosphate guanylyltransferase; gmd, GDP-D-mannose-4,6-epimerase; wcaG, GDP-L-fucose synthetase; HpfutC, α-1,2-fucosyltransferase; HPM32, α-1,3-fucosyltransferase; pfkA, ATP-dependent 6-phosphofructokinase 1; pfkB, ATP-dependent 6-phosphofructokinase 2; fsaA, fructose-6-phosphate aldolase 1; fsaB, fructose-6-phosphate aldolase 2; mtd, mannitol-1-phosphate dehydrogenase; nudK, GDP-mannose hydrolase; wcaJ, UDP-glucose lipid carrier transferase; lacZ, β-galactosidase; lon, temperature-sensitive ATP-dependent protease.
E. coli is an important industrial chassis widely used in FL production due to its rapid growth cycle and genetic manipulability (Chen et al., 2020; Choi et al., 2016). Through genetic engineering approaches, E. coli has been developed as one of the 2′-FL producers using substrates including glycerol, glucose, xylose and sucrose (Lee et al., 2020; Li et al., 2020; Li, Zhu, et al., 2021; Parschat et al., 2020). Currently, multiple metabolic engineering strategies, involving modular pathway optimization, cofactor and ATP/GTP supply, modification of chassis microorganisms and lactose-independent pathway construction, have been exploited to increase FL production (Huang et al., 2017; Li, Li, et al., 2021; Sprenger et al., 2017). However, the relatively low yield and high cost are still problems to be solved in FL industrial production. Parschat et al. (2020) developed a 2′-FL-producing strain using sucrose as a substrate, which produced 2′-FL without the addition of lactose, significantly reducing production costs. Currently, 3-FL-producing strains have only been reported in E. coli and in low yields. Ni et al. (2020) improved the availability of cofactors and GTP and produced 35.72 g/L of 3-FL in strains lacking lacZ, wcaJ and nudD. Other 3-FL-producing strains with higher yields have not been reported.

In this work, multi-level combinatorial strategies were utilized to achieve high-level de novo biosynthesis of FL in engineered E. coli using glycerol and lactose. We attempted to establish optimal FL synthesis pathways, screen for α-1,2,3-fucosyltransferase, alleviate metabolic bottlenecks, improve the catalytic performance of rate-limiting enzymes and scale up production in bioreactors. The final engineered strains proved to be effective in producing FL of high titer, yield and productivity, providing a promising platform for practical industrial production.

**EXPERIMENTAL PROCEDURES**

**Materials**

The vectors pRSFDuet-1 and pETDuet-1 were purchased from Novagen. 2′-FL and 3-FL standards were obtained from Sigma-Aldrich. Molecular reagents such as plasmid extraction kits, DNA purification kits and high-fidelity DNA polymerase were purchased from Vazyme and Takara. All other chemicals were of analytical and molecular biology grade and obtained from Sinopharm.

**Strains and plasmids construction**

All strains, plasmids and oligonucleotides used in this study are summarized in Tables S1, S2 and S3, respectively. E. coli DH5α was employed as the host strain for molecular cloning and plasmid construction. The engineered strain BZ (E. coli derivate), constructed in previous studies for 2′-FL synthesis (Li, Li, et al., 2021), was used as the starting strain for metabolic engineering. For cloning and subcloning, the vectors pRSFDuet-1 and pETDuet-1 were selected.

The codon-optimized α-1,2-fucosyltransferase genes, comprising HpfutC (GenBank: WP_026938579.1), TvfutC (GenBank: WP_011056838.1), TsutfC (GenBank: WP_024124365.1), PtutC (GenBank: WP_154905360.1), EcwbgL (GenBank: AAL67559.1) and BfwcfB (GenBank: ADN43847.1), were synthesized by Tianlin (Wuxi, China) and inserted into the vector pETDuet-1, generating the plasmids pET-HpfutC, pET-TvfutC, pET-TsutfC, pET-PtutC, pET-EcwbgL and pET-BfwcfB, respectively. Similarly, the vector pETDuet-1 was employed to construct genes encoding α-1,3-fucosyltransferase, including Hpm32 (PDB: S50Z), HpfutA (GenBank: O30511.1), HpfutB (GenBank: WP_000487428.1) and HpfutD (GenBank: WP_064780098.1), respectively. A combinatorial screen was performed using the previously constructed plasmid pRSF-CBGW with different sources of α-1,2,3-fucosyltransferase.

To boost the expression of the rate-limiting enzyme gene HpfutC and Hpm32, the corresponding plasmids were constructed based on pET-HpfutC and pET-Hpm32 by replacing the RBS with different strengths. Five fusion peptides, including ubiquitin-related small modification protein (SUMO), 3x Flag, haemagglutinin (HA), pacte lyase B of Erwinia carotovora CE (pelB) and prokaryotic translation initiation factor IF2 (InfB), were subsequently fused at the N-terminus of HpfutC and Hpm32 with a (GGGGS)2 linker. Finally, two- and three-copy plasmids containing HpfutC/Hpm32 gene fragments were constructed. All plasmids successfully generated were sequenced and electrotransformed into E. coli-derived strains for FL synthesis.

**Media and culture conditions**

E. coli DH5α was utilized for gene cloning and propagated by aeration at 37°C in Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) or LB agar plates (LB medium with 15 g/L agar). For FL biosynthesis, the engineered E. coli BL21(DE3) and derivative strains were cultivated in a modified-M9CA medium. The modified-M9CA medium contains 30 g/L glycerol, 10 g/L yeast extract, 2 g/L casamino acid, 6.8 g/L Na2HPO4·7H2O, 3.0 g/L KH2PO4, 1.0 g/L NH4Cl, 1.0 g/L NaCl, 1.4 g/L MgSO4·7H2O, 10 mg/mL thiamine and 1 mL/L trace element solution (25 g/L FeCl3·6H2O, 2 g/L CaCl2·2H2O, 2 g/L ZnCl2, 2 g/L Na2MoO4·2H2O, 1.9 g/L CuSO4·5H2O and 0.5 g/L H3BO3), pH 6.8. Appropriate antibiotics, including ampicillin (Amp, 100 μg/mL) and kanamycin (Kan, 50 μg/mL), were utilized.
to screen positive transformants or to maintain plasmid replication.

For shake-flask cultivation, the FL-producing strains were first inoculated into test tubes containing 4 ml of LB medium and incubated for 12 h at 37°C to the late exponential phase. Then, fresh seed solution (0.5 ml) was inoculated into modified-M9CA medium (50 ml) containing Amp (100 μg/ml) and Kan (50 μg/ml) in 250 ml non-baffled flasks, and cultured at 37°C for 3 h with shaking (200 rpm). Once the cell optical density (OD 600) reached approximately 0.6–0.8, 0.3 mM IPTG and 10 g/L lactose were supplemented to induce the expression of pathway enzymes (manC, manB, gmd, wcaG and HpfutC/HpM32) to produce 2'-FL or 3-FL. After induction, the fermentation temperature was lowered to 25°C and incubated for a further 72 h. The biological experiments were conducted in triplicate.

**Gene knockout through CRISPR-Cas9**

To strengthen cellular metabolic flow, the bypass genes encoding wcaJ, nudD, nudK, pfkA, pfkB, mtlD, lon, fsaA, fsaB and fucIK were selected and removed from *E. coli* BL21(DE3) ΔlacZ chromosome through CRISPR-Cas9 gene editing technique (Figure 3A). A dual plasmid system including pCas9 (Addgene plasmid # 62225) and pTargetF (Addgene plasmid # 62226) was used for gene knock-out, with pCas9 containing the genes encoding Cas9 nuclease and λ-Red recombinase (Jiang et al., 2015). The N20 sequence of the deletion site was designed using the CHOPCHOP website. A single-guide RNA (sgRNA) expression cassette carrying the N20 sequence on pTargetF plasmid was used to identify the target site of the gene. The donor DNA repair template was the upstream and downstream homologous arm sequences of deleted genes, which were linked by SOE-PCR (Zerbini et al., 2017). To facilitate homologous recombination, *E. coli* BL21(DE3) ΔlacZ harbouring pCas9 plasmid was incubated and supplemented with arabinose (30 mM) for λ-Red induction. Various targeting plasmids, including pTF-ΔwcaJ, pTF-ΔnudD, pTF-ΔpfkA, pTF-ΔpfkB, pTF-Δlon, pTF-ΔfsaA, pTF-ΔfsaB, pTF-ΔmtlD, pTF-ΔnudK and pTF-ΔfucIK, and corresponding repair templates were individually electrotransformed into competent cells. Afterward, LB medium was rapidly added to resuscitate the cells. Cells were further incubated at 30°C for 90 min, centrifuged, and coated onto LB agar with Kan (50 μg/ml) and Spectinomycin (Spe, 50 μg/ml). The positive transformants were identified by colony PCR and gene sequencing. The successfully identified colonies were induced by IPTG and incubated at 42°C to eliminate the dual plasmid system. Using the aforementioned methods, *E. coli* strains with different combinations of gene defects were constructed.

**Fed-batch fermentation in 3-L bioreactors**

For fed-batch cultivation, the final optimized strains EC49 and EM47 were initially grown overnight in 50 ml of LB medium as seed solution at 37°C with 200 rpm shaking. The seed solution was then transferred to 3-L bioreactor vessels (DasGip, Germany) with 1 L working volume of the modified-M9CA media. The bioreactor system was run at 37°C, 200 rpm as the initial stirring speed and gradually increased to maximally 800 rpm, 36 L/h as the initial airflow and increased to maximally 180 L/h, 21 L/h as the initial oxygen and increased to maximally 100 L/h, pH = 6.8 (maintained by adding 28% [w/v] NH₄OH), and the dissolved oxygen (DO) level was maintained at 20%–30% by adjusting the stirring speed, airflow, oxygen and substrate feeding. For FL production, 0.5 mM IPTG and 15 g/L lactose were injected into the bioreactors when the OD 600 reached 20, and the temperature was adjusted to 28°C after induction. The feeding solution containing glycerol (800 g/L) and MgSO₄·7H₂O (20 g/L) was automatically pumped into the bioreactor in pH-stat control mode to replenish the carbon supply (Chin et al., 2015). During fermentation, 30 ml of lactose (300 g/L) was injected into the 3-L fermenters when the lactose concentration was below 2 g/L.

**Metabolite analysis**

During cultivation, cell optical density was determined periodically by OD 600 analysis using a UV–Vis spectrophotometer (Shimazu, Japan). A conversion equation (1 OD600 = 0.36 g/L) was used to calculate the dry cell weight (DCW) of engineered *E. coli* from OD 600. To assay the concentrations of metabolites, 1 ml of the culture broth was harvested, boiled for 10 min and centrifuged (10,000 × g, 5 min) as the test sample. The components in the supernatant (10 μl) were quantified using a high-performance liquid chromatography (HPLC) system (Waters e2695) equipped with a refractive index (RI) detector and a Rezex ROA-Organic Acid H⁺ column (Phenomenex) as described previously. The column was eluted with 5 mM H₂SO₄ at 0.6 ml/min and 50°C. To confirm the biosynthesis of FL, the standards and samples were analysed by ultra-performance liquid chromatography-quadrupole tandem time-of-flight mass spectrometer (UPLC-Q-TOF-MS) using a SCIEX X500R QTOF mass spectrometer. To verify the biosynthesis of FL, standards and samples were evaluated with an ultra-performance liquid chromatography-quadrupole tandem time-of-flight mass spectrometer.
tandem time-of-flight mass spectrometer (UPLC-Q-TOF-MS) equipped with a SCIEX X500R QTOF mass spectrometer (Yang et al., 2021).

RT-qPCR analysis

The RT-qPCR primers are shown in Table S3. As a leading control, the housekeeping gene (16S rRNA) was utilized. According to the manufacturer's protocol, the total RNA of harvested cells was extracted using TaKaRa MiniBEST Universal RNA Extraction Kit (Takara) and frozen immediately at −80°C. The cDNA was obtained through mRNA reverse transcription using the PrimeScript™ RT reagent Kit (Takara). Using cDNA as the template, RT-qPCR was performed in a 96-well plate on a CFX96 Real-Time System (Bio-Rad, CA, USA) with the Titanium One-Step RT-PCR Kit (Takara). The qPCR cycling procedure consisted of pre-denaturation at 95°C for 30 s, and 40 cycles of 95°C for 3 s and 60°C for 30 s. The relative transcript levels of target genes (including Hp futC and Hp M32) were analysed by the 2−ΔΔCT method (Livak & Schmittgen, 2001).

RESULTS AND DISCUSSION

Construction of FL biosynthesis pathway and screening of α-1,2/3-fucosyltransferase

There is no endogenous synthesis pathway of fucosyl-lactose in E. coli. To construct efficient FL-producing strains, α-1,2-fucosyltransferase (Hp futC) and α-1,3-fucosyltransferase (Hp M32) were introduced, and the GDP-L-fucose pathway was enhanced to facilitate the supply of precursors in E. coli (Figure 2A). Given that wild-type E. coli consumes lactose preferentially for cell growth, the utilization of lactose for FL production is relatively low. The gene encoding β-galactosidase (lacZ, responsible for breaking lactose into galactose and glucose) has been inactivated, yielding E. coli BL21 (DE3) ΔlacZ as the original strain (Li, Li, et al., 2021). Heterologous expression of Hp futC/Hp M32 and homologous expression of GDP-L-fucose-producing enzyme genes including man B, man C, gmd and wcaG were carried out in E. coli BL21(DE3) ΔlacZ for 2′-FL/3-FL production and GDP-L-fucose synthesis enhancement (Figure 2B). A dual plasmid system consisting of pRSFDuet-1 and pETDuet-1 was used for co-expression of pathway genes, resulting engineered strains EC01-04, EM01-04 (Table S1). To increase 2′-FL/3-FL biosynthesis, the intracellular supply of GDP-L-fucose was enhanced by independently expressing man C-man B and gmd-wcaG along with Hp futC/Hp M32. The 2′-FL titers of the two engineered strains (EC02 and EC03) were 0.22 and 0.54 g/L, with a gain of 22% and 183% compared to that of EC01. The 3-FL titers of the two engineered strains (EM02 and EM03) were 0.18 and 0.35 g/L, respectively, with an increase of 83% and 257% compared to that of EM01. For co-expression of the two gene clusters, the titers of 2′-FL and 3-FL were further enhanced to 0.72 and 0.61 g/L, respectively (Figure 2C). Our results, therefore, suggest that these two gene clusters have a combinatorial effect on FL biosynthesis. The biomass levels of several engineered strains did not differ significantly, indicating that the growth of the strains was less influenced by the expression of pathway genes. Additionally, the synthesis of FL was preliminary determined by HPLC and MS analysis. HPLC profiles showed that a compound was detected in the fermentation sample with a retention time consistent with the 2′-FL/3-FL standard at 8.0/7.8 min (Figure 3B). UPLC-Q-TOF-MS further identified the formation of 2′-FL/3-FL (Figure S1).

Several α-1,2/3-fucosyltransferase from prokaryotes were screened (Figure 2D). According to our results, H p futC of H. pylori ATCC 26695 and mutant Hp M32 (S45F/D127N/R128E/H131I/Y199N/E340D/V368A) of H. pylori NCTC 11639 have higher FL production capacity. It has been reported that H. pylori-derived futC has a higher substrate affinity with a lower Km value of 168 μM (Albermann et al., 2001). With regard to H. pylori NCTC 11639-derived α-1, 3-fucosyltransferase, its crystal structure has been resolved and the 3-FL synthesis capacity of mutant M32 was increased by 14-fold [Kcat/Km value from 2.53 L/(mmol·s) to 15.59 L/(mmol·s)] by directed evolution (Tan et al., 2019). Collectively, the pathway gene combinations and the screening for rate-limiting enzymes initially enhanced the yield of FL. In addition, metabolic network optimization to balance the distribution of carbon flow between cell growth and product synthesis is crucial for obtaining high-yielding strains. Recently, Yu et al. (2022) developed a temperature-sensitive multi-module time-sequential bidirectional regulatory system in the model industrial microorganism Bacillus subtilis and applied it to optimize the metabolic network of 2′-FL. Finally, the stability and robustness of the system were verified in a 5-L fermenter, and the yield of 2′-FL reached 28.2 g/L.

Elimination of competing pathways to increase FL production

Based on metabolic flux analysis, the branching metabolism of three critical intermediates (fru-6-P, GDP-D-mannose and GDP-L-fucose) may hinder the biosynthesis and accumulation of FL. To maximize the flow of carbon flux to the FL synthesis pathways, the CRISPR/Cas9 system was utilized to weaken the three branching pathways of the intermediate fru-6-P: fru-6-P to fru-1,6-P by two isoforms of ATP-dependent phosphofructokinase (encoded by pfkA and pfkB),
fru-6-P to glyceraldehyde-3-P by fructose-6-phosphate aldolase (encoded by *fsaA* and *fsaB*) and fru-6-P conversion to mannitol-1-P by mannitol-1-phosphate dehydrogenase (encoded by *mtlD*). In this work, the above five genes associated with fru-6-P were deleted separately from BZ strain, generating the new strains BZPA, BZPB, BZFA, BZFB and BZMD (Table S1). The results showed that the 2′-FL and 3-FL titers were enhanced to varying degrees, with a maximum increase to 2.62 and 0.91 g/L, an increase of 263% and 49% compared to EC04 and EM04 strains (Figure 3C,E). In a previous study, Ni et al. (2020) obtained several beneficial 2′-FL production strains by reducing the sensitivity of mannosyl hydrolase (encoded by *nudD*) and GDP-mannose hydrolase (encoded by *nudK*) to inhibit GDP-D-mannose consumed in the regulation of cell wall biosynthesis. To validate this strategy in fucosyllactose production, *nudD* and *nudK* were also disrupted in BZ strain, obtaining BZND and BZNK strains, respectively. As shown in Figure 3C,E, deletion of *nudD* was more productive compared to deletion of *nudK*, which was consistent with previous reports. Deletion of *nudD* (EC10 and EM10 strains) resulted in a 216% and 96% increase in 2′-FL and 3-FL titers, reaching 2.28 and 1.20 g/L, respectively. Noteworthy, the intracellular availability of GDP-L-fucose determines the overall yield of FL (Wan et al., 2021). GDP-L-fucose is a precursor of the colanic acid biosynthesis pathway. To weaken the competitive pathway and reduce the undesirable consumption of GDP-L-fucose, UDP-glucose-lipid carrier transferase (encoded by *wcaJ*) was also targeted for deletion. Simultaneously, *RcsA*, as a positive transcriptional regulator of the GDP-L-fucose synthesis genes in *E. coli*, has been reported to be susceptible to rapid degradation via the temperature-sensitive ATP-dependent protease (encoded by *lon*), and thus
Protease deficiency was thought to facilitate the efficient accumulation of FL (Huang et al., 2017). Here, the effects of the absence of wcaJ and lon on FL production also were evaluated. We found that deletion of the lon gene was more predominant in E. coli BZ strain, leading to the enhanced production of 2′-FL and 3-FL to 2.50 g/L (247% increased) and 1.34 g/L (119% increased), respectively.

The mutant strains of the above-selected deletion genes (pfkA, pfkB, mtlD, fsaA, fsaB, nudD, nudK and lon) all exhibited significant FL production performance compared to the wcaJ deletion strain. On the basis of these results, another round of superimposed knockout was performed in the BZW strain, obtaining the new strains including BZWFA, BZWFB, BZWL, BZWMD, BZWND, BZWNK, BZWPA and BZWPB. As shown in Figure 3D,F, there was no significant difference in biomass of all defective strains, indicating that the strains exhibited excellent growth in M9CA medium containing glycerol and yeast extract. In light of titers, the deletion of pfkA in E. coli BZW was the best combination for FL production, and the attained strain BZWPA produced 4.62 g/L of 2′-FL and 2.02 g/L of 3-FL. Consistent with previous reports, Zhang et al. (2018) demonstrated that deletion of pfkA blocked the metabolic flux from fru-6-P to fru-1,6-P, which effectively increased the production of fructosylated chondroitin. Moreover, the strains containing pfkA, nudD and lon deletions presented superior production performance among all mutant strains, as shown by the results of single and double knockout experiments. We speculate that deletion of the above three genes may result in a substantial increase in carbon flux of the FL biosynthetic pathway.

To verify this hypothesis, the knockout was carried out on the basis of the BZWND strain, individually or in tandem, producing strains BZWNDL, BZWNPB and BZWNDPAL. The absence of lon in E. coli BL21 ΔlacZΔwcaJΔnudD (BZWND) resulted in a 13.8% and 43.5% increase in the formation of 2′-FL and 3-FL (Figure 4). In addition, the BZWNDPA strain with pfkA removed led to a further enhancement in FL production, with the concentration of 2′-FL and 3-FL rising to 5.67 and 3.32 g/L, respectively. Remarkably, 6.24 g/L 2′-FL and 3.56 g/L 3-FL were produced in the BZWNDPAL strain, respectively, indicating that the simultaneous elimination of pfkA and lon was accompanied by a higher FL level. Here, 6.5 and 3.8 g/L lactose were consumed by EC30 and EM28 from BZWNDPAL, yielding 0.66 mol 2′-FL/mol lactose and 0.68 mol 3-FL/mol lactose. However, 3-FL-producing strains generally exhibited slightly lower biomass compared to 2-FL-producing strains, probably due to the heterologous expression of

![Figure 3](image-url)
downstream α-1,3-fucosyltransferase limiting the carbon flux for cell growth (Ni et al., 2021).

Optimization of rate-limiting enzyme expression through combinatorial expression element engineering

α-1,2-Fucosyltransferase and α-1,3-fucosyltransferase are involved in the final step of 2'-FL and 3-FL biosynthesis, catalysing the transfer of the fucose group in the GDP-L-fucose and linking the lactose-terminated galactosyl group via the α-1,2/α-1,3 bond (Li, Li, et al., 2021; Li, Zhu, et al., 2021). Based on previous reports, the poor solubility and low catalytic activity of fucosyltransferase have become bottlenecks in the preparation of fucosyllactose by microbial methods (Jung et al., 2019; Zhu et al., 2022). Thus, multi-level combinatorial strategies (including RBS, fusion peptides and multi-copy gene screening) were designed to improve the technical bottleneck for fucosyltransferase application.

The ribosome binding site (RBS) is an efficient regulatory element for translation initiation and protein expression in bacteria. In most cases, translation initiation is the rate-limiting step (Sun et al., 2020). To further strengthen the expression level of the rate-limiting enzymes (HpfutC and HpM32), RBS engineering was utilized as a viable approach, which has been extensively applied in synthetic biology research (Santos-Navarro et al., 2021). Here, 13 selected RBS sequences were substituted between the −35 and −10 regions of the original RBS from pET-HpfutC and pET-HpM32, respectively, to construct 26 artificial RBS, forming plasmids as shown in supplementary materials. The translation strength of the selected RBS is available from the MIT Registry of Biological Standard Parts (Figure 5A). EC30 and EM28 strains with pRSF-CBGW and pET-HpfutC/HpM32 were used as the starting strains, and the HpfutC/HpM32 expression cassettes were ligated with different strengths of RBS to construct 26 strains to determine the optimal expression.

The production performance of FL containing different RBS strains was investigated. Notably, the strain containing RBS (BBa_B0034) was the most effective after feeding 8 g/L lactose to the medium, producing 7.87 g/L for 2'-FL and 5.26 g/L for 3-FL, which were 25.3% and 47.7% higher than the control, respectively (Figure 5B). Additionally, RBS (BBa_B0030), RBS (BBa_B0032) and RBS (BBa_B0035) also facilitated the FL production, with increases of 15.2%, 9.7% and 18.3% for 2'-FL, 34.2%, 4.7% and 32.8% for 3-FL, respectively. Nevertheless, the 2'-FL and 3-FL titers decreased...
slightly when the remaining RBS was introduced into the BZWNDPAL strain. Analysis shows that the medium expression intensity of RBS (BBa_B0034) is more suitable for the translation level and protein expression of HpfutC and HpM32. Specifically, the mRNA levels of HpfutC and HpM32 were 3.48 and 4.54-fold higher than those in control (Figure S2). To verify this result, the secondary structure of RBS (BBa_B0034) was predicted using the mFold Web Server. The secondary structure formed in the 5′ end of by RBS (BBa_B0034) is more likely to expose the binding region to a greater extent (Figure 5C). It has been shown that the Gibbs free energy (ΔG) is negative when there is an attractive interaction between ribosome and mRNA (Salis et al., 2009). RBS (BBa_B0034) had the lowest ΔG of all selected RBS (ΔG = −8.60), indicating that the ribosome and mRNA had the strongest binding capacity, thus producing a superior FL production. RBS optimization of rate-limiting enzymes can also be applied to enhance the biosynthesis of other high-value-added chemicals. Li et al. (2019) further improved isoprene production in E. coli by reasonable optimization of RBS and screening of critical enzymes. For lacto-N-triose II (LNT II) production, Zhu et al. (2021) used different strengths of RBS to regulate the translation rate of critical enzymes, and the RBS-optimized engineered strains were more favourable for soluble expression of lgtA and biosynthesis of LNT II.

Following the validation of the positive effect of RBS optimization on HpfutC and HpM32 expression, we considered further modifications by fusion peptides to improve the soluble expression of fucosyltransferase. The fusion peptides SUMO, 3x FLAG, HA, pleB and lnfB were fused to the N-terminus of HpfutC and HpM32. Specifically, the mRNA levels of HpfutC and HpM32 were 3.48 and 4.54-fold higher than those in control (Figure S2). To verify this result, the secondary structure of RBS (BBa_B0034) was predicted using the mFold Web Server. The secondary structure formed in the 5′ end of by RBS (BBa_B0034) is more likely to expose the binding region to a greater extent (Figure 5C). It has been shown that the Gibbs free energy (ΔG) is negative when there is an attractive interaction between ribosome and mRNA (Salis et al., 2009). RBS (BBa_B0034) had the lowest ΔG of all selected RBS (ΔG = −8.60), indicating that the ribosome and mRNA had the strongest binding capacity, thus producing a superior FL production. RBS optimization of rate-limiting enzymes can also be applied to enhance the biosynthesis of other high-value-added chemicals. Li et al. (2019) further improved isoprene production in E. coli by reasonable optimization of RBS and screening of critical enzymes. For lacto-N-triose II (LNT II) production, Zhu et al. (2021) used different strengths of RBS to regulate the translation rate of critical enzymes, and the RBS-optimized engineered strains were more favourable for soluble expression of lgtA and biosynthesis of LNT II.

Following the validation of the positive effect of RBS optimization on HpfutC and HpM32 expression, we considered further modifications by fusion peptides to improve the soluble expression of fucosyltransferase. The fusion peptides SUMO, 3x FLAG, HA, pleB and lnfB were fused to the N-terminus of HpfutC and HpM32 to obtain fusion recombinant protein for FL production (Figure 5D). The strains EC36 and EM34 containing plasmid pET(BBa_B0034)-pelB-HpfutC/HpM32 obtained 8.57 g/L of 2′-FL and 5.78 g/L of 3-FL, an increase of 8.8% for 2′-FL and 12% for 3-FL compared to the control (strains EC30 and EM28). In a previous study, an optimal 21 nucleotide lnfB (1-21 bp) sequence fused to a GFP reporter gene exhibited strongly increased expression levels. In this work, strains containing the fusion peptide lnfB also exhibited slightly higher FL production capacity. However, the biomass and yield of the strains containing the remaining fusion peptides (SUMO, 3x FLAG and HA) were significantly reduced, probably because the incorrect protein folding impacted the enzymatic activity of HpfutC and HpM32 (Lin et al., 2022). It has been reported that fusion tags such as the three-aspartate tag and the SUMOstar tag contribute to enhancing the soluble expression of futC (Chin et al., 2015; Hollands et al., 2019). With regard to α-1,3-fucosyltransferase, its soluble expression can also be substantially enhanced by codon optimization and truncation or extension of the C terminus (Yu et al., 2018).

To further enhance the production potential of FL synthesis strains, multi-copy plasmid construction was achieved by tandem rate-limiting enzymes,
subsequently generating strains EC49 and EM47 (two-copy) and strains EC50 and EM48 (three-copy) on the basis of strains EC47 and EM45. As shown in Figure 5E, the highest 2′-FL concentration was obtained for the two-copy strain EC49 with a yield of 9.36 g/L, an increase of 9.2% compared to the single-copy strain EC47. In our previous study, lacZ-wcaJ-deleted E. coli produced 2.24 g/L of 2′-FL in a glycerol-defined medium, and in this work, the titer of 2′-FL was 4.17-fold higher than that in the previous study (Li, Li, et al., 2021). Correspondingly, the two-copy strain EM47 also contributed to an increase in 3-FL production, with a yield of 6.28 g/L, an increase of 8.6% compared to the single-copy strain EM45. However, the biomass of the strain decreased as the copy number increased, probably because the increased plasmid loop length affected the growth and metabolic rate of the strains. On the other hand, the expression content of the rate-limiting enzyme at different copy numbers was examined. The results showed that the best expression content of the rate-limiting enzyme was obtained under two-copy strains (Figure 5F), which is consistent with the results described above.

Production of FL in 3L Bioreactors

To evaluate the performance of strains EC49 and EM47 under more industrial production conditions, we performed fed-batch fermentations in 3L bioreactors using modified M9CA medium (Figure 6). To initiate FL production, IPTG and lactose were supplemented to the bioreactor at a final concentration of 0.5 mM and 12 g/L when the OD₆₀₀ reached 20. To balance the growth and metabolism of the strain, glycerol was continuously fed after induction by pH-stat control mode. The entire control process maintains the glycerol concentration at a lower level. The consumption of lactose is accompanied by the production of FL. The bioreactor was fed lactose solution intermittently to maintain a concentration between 2 and 12 g/L. The strains started to accumulate 2′-FL or 3-FL at 8 h, and finally produced 64.62 g/L for 2′-FL and 40.68 g/L for 3-FL at 100 h, which were 6.9 and 6.4-fold of that in shake-flask cultivation. The yield and productivity of 2′-FL were measured to be 0.65 mol 2′-FL/mol lactose and 0.65 g/L/h, respectively. The yield and productivity of 3-FL reached 0.67 mol 3-FL/mol lactose and 0.41 g/L/h. The DCW of the two strains at the end of fermentation reached 73.4 and 64.3 g/L, which

![Figure 6](image-url)
were 5.5 and 5.8 times more than those in shake flasks. The superior cell growth and considerable FL titers in the FL-producing strains may be attributed to: cell factory modification increasing the carbon flux for FL synthesis; efficiently regulation of correct folding and functional expression of the rate-limiting enzyme by regulating the translation initiation rate, binding the fusion peptide as well as gene copies; and the higher biomass and FL productivity of the strains in the optimized M9CA medium.

CONCLUSIONS

In this work, a multi-level metabolic engineering approach was employed to achieve efficient production of FL from low-cost glycerol and lactose. Our work identified several critical intermediates involved in GDP-L-fucose synthesis and deleted potential competing target genes via gene editing. More specifically, the absence of wcaJ, nudD, pfkA and lon maximized the flow of carbon flux towards the GDP-L-fucose biosynthesis pathway, significantly enhancing the level of FL production. Our results also suggest that, although the screened H. pylori-derived α-1,2/3-fucosyltransferase helps to alleviate bottlenecks in FL production, the inherent negative properties of the enzyme (e.g., low catalytic activity and poor soluble expression) still need to be improved. Therefore, fine-tuning the translation control element RBS and screening for different fusion peptides and gene copy numbers could improve the intrinsic properties of rate-limiting enzymes while further increasing FL production. Both batch and fed-batch cultivation results of strains EC49 and EM47 confirmed that the engineered E. coli made a significant leap in FL titers at the gram-litre level. Particularly, in 3L fed-batch cultivation, the maximal titers of 2′-FL and 3-FL reached 64.62 g/L and 40.68 g/L, indicating that the engineered strain is capable of adapting to high-density cultivation and has potential for industrial application. Overall, we demonstrated the effectiveness of multi-level metabolic engineering strategies based on synthetic biology tools in addressing metabolic bottlenecks. The strategies adopted in this work will be equally applicable to the construction of platform strains for other high-value-added compounds in microbial cell factories. Future efforts will focus on developing antibiotic-free and inducer-free strains of high-yielding FL using cheaper biomass feedstocks. In addition, the directed evolution of α-1,2,3-fucosyltransferase will be carried out through multiple enzyme molecular modification strategies.

ACKNOWLEDGEMENTS

This research was financially supported by the Major Science and Technology Innovation Project of Shandong Province (2020CXGC010601) and Kitty Hawk Project of Zhejiang Provincial Administration for Market Regulation (CY2022004).

FUNDING INFORMATION

This research was financially supported by the Major Science and Technology Innovation Project of Shandong Province (2020CXGC010601) and Kitty Hawk Project of Zhejiang Provincial Administration for Market Regulation (CY2022004).

CONFLICT OF INTEREST

The authors declare no competing financial interest.

ORCID

Tao Zhang https://orcid.org/0000-0003-3194-2028

REFERENCES

Agoston, K., Hederos, M.J., Bajza, I. & Dekany, G. (2019) Kilogram scale chemical synthesis of 2′-fucosyllactose. Carbohydrate Research, 476, 71–77.

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

Anderson, A. & Donald, A.S. (1981) Improved method for the isolation of 2′-fucosyllactose from human milk. Journal of Chromatography, 211, 170–174.

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

Chen, Q., Wu, H., Ji, M., Xie, Y., Li, S., Li, Y. et al. (2020) Engineering L- fucose synthesis and deleted potential competing FL from low-cost glycerol and lactose. Our work identified metabolic pathway enhancement.

Drouillard, S., Driguez, H. & Samain, E. (2006) Large-scale synthesis of 2′-fucosyllactose in engineered Escherichia coli by expressing putative α-1,2-fucosyltransferase, WcfB from Bacteroides fragilis. Journal of Biotechnology, 257, 192–198.

Chin, Y.W., Kim, J.Y., Lee, W.H. & Seo, J.H. (2012) Enhanced production of 2′-fucosyllactose in engineered Escherichia coli for manufacturing 2′-fucosyllactose. Process Biochemistry, 94, 79–85.

Chin, Y.W., Kim, J.Y., Jung, S.M. & Seo, J.H. (2017) Improved synthesis of the milk oligosaccharide 2′-fucosyllactose in engineered Escherichia coli by expressing putative α-1,2-fucosyltransferase, WcfB from Bacteroides fragilis. Journal of Biotechnology, 257, 192–198.

Chin, Y.W., Kim, J.Y., Lee, W.H. & Seo, J.H. (2015) Improved production of 2′-fucosyllactose in engineered Escherichia coli BL21star(DE3) by modulation of lactose metabolism and fusosyltransferase. Journal of Biotechnology, 210, 107–115.

Choi, S.Y., Park, S.J., Kim, W.J., Yang, J.E., Lee, H., Shin, J. et al. (2016) One-step fermentative production of poly (lactate-syltransferase). Nature Biotechnology, 34, 435–440.

Deng, J.Y., Gu, L.Y., Chen, T.C., Huang, H., Yin, X.Q., Lv, X.Q. et al. (2019) Engineering the substrate transport and cofactor regeneration systems for enhancing 2′-fucosyllactose synthesis in Bacillus subtilis. ACS Synthetic Biology, 8, 2418–2427.

Drouillard, S., Driguez, H. & Samain, E. (2006) Large-scale synthesis of H-antigen oligosaccharides by expressing Helicobacter pylori alpha1,2-fucosyltransferase in metabolically engineered Escherichia coli cells. Angewandte Chemie (International Ed. in English), 45, 1778–1780.

Han, N.S., Kim, T.J., Park, Y.C., Kim, J. & Seo, J.H. (2012) Biotechnological production of human milk oligosaccharides. Biotechnology Advances, 30, 1268–1278.

Hollands, K., Baron, C.M., Gibson, K.J., Kelly, K.J., Krasley, E.A., Laffend, L.A. et al. (2019) Engineering two species of yeast as cell factories for 2′-fucosyllactose. Metabolic Engineering, 52, 232–242.

Huang, D., Yang, K.X., Liu, J., Xu, Y.Y., Wang, Y.Y., Wang, R. et al. (2017) Metabolic engineering of Escherichia coli for the production of 2′-fucosyllactose and 3-fucosyllactose through modular pathway enhancement. Metabolic Engineering, 41, 23–38.

Jiang, Y., Chen, B., Duan, C.L., Sun, B.B., Yang, J.J. & Yang, S. (2015) Multigene editing in the Escherichia coli genome.
via the CRISPR-Cas9 system. *Applied and Environmental Microbiology*, 81, 2506–2514.

Jung, S.M., Chin, Y.W., Lee, Y.G. & Seo, J.H. (2019) Enhanced production of 2′-fucosyllactose from fucose by elimination of rhamnose isomerase and arabinose isomerase in engineered *Escherichia coli*. *Biotechnology and Bioengineering*, 116, 2412–2417.

Lee, J.W., Kwak, S., Liu, J.J., Yu, S., Yun, E.J., Kim, D.H. et al. (2020) Enhanced 2′-fucosyllactose production by engineered *Saccharomyces cerevisiae* using xylose as a co-substrate. *Metabolic Engineering*, 62, 322–329.

Lee, W.H., Chin, Y.W., Han, N.S., Kim, M.D. & Seo, J.H. (2011) Enhanced production of GDP-1-fucose by overexpression of NADPH regenerator in recombinant *Escherichia coli*. *Applied Microbiology and Biotechnology*, 91, 967–976.

Li, C., Wu, M., Gao, X., Zhu, Z., Li, Y., Lu, F. et al. (2020) Efficient biosynthesis of 2′-fucosyllactose using an in vitro multienzyme cascade. *Journal of Agricultural and Food Chemistry*, 68, 10763–10761.

Li, M.J., Chen, H.L., Liu, C.Q., Guo, J., Xu, X., Zhang, H.B. et al. (2019) Improvement of isoprene production in *Escherichia coli* by rational optimization of RBSs and key enzymes screening. *Microbial Cell Factories*, 18, 4.

Li, M.L., Li, C.C., Hu, M.M. & Zhang, T. (2021) Metabolic engineering strategies of *de novo* pathway for enhancing 2′-fucosyllactose synthesis in *Escherichia coli*. *Microbial Biotechnology*, 15, 1561–1573.

Li, W., Zhu, Y.Y., Wan, L., Guang, C.E. & Mu, W.M. (2021) Pathway optimization of 2′-fucosyllactose production in engineered *Escherichia coli*. *Journal of Agricultural and Food Chemistry*, 69, 1567–1577.

Lin, L., Gong, M., Liu, Y., Li, J., Lv, X., Du, G. et al. (2022) Combinatorial metabolic engineering of *Escherichia coli* for de novo production of 2′-fucosyllactose. *Bioresource Technology*, 351, 126949.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^−\Delta\Delta CT method. *Methods*, 25, 402–408.

Ni, Z.J., Li, Z.K., Wu, J.Y., Ge, Y.F., Liao, Y.X., Yuan, L.X. et al. (2020) Multi-path optimization for efficient production of 2′-fucosyllactose in an engineered *Escherichia coli C41 (DE3)* derivative. *Frontiers in Bioengineering and Biotechnology*, 8, 1–13.

Ni, Z.J., Wu, J.Y., Li, Z.K., Yuan, L.X., Wang, Y., Chen, X.S. et al. (2021) Enhanced bioproduction of fucosylated oligosaccharide 3-fucosyllactose in engineered *Escherichia coli* with an improved de novo pathway. *Bioscience, Biotechnology, and Biochemistry*, 85, 1772–1781.

Parschat, K., Schreiber, S., Wartenberg, D., Engels, B. & Jennewein, S. (2020) High-tier de novo biosynthesis of the predominant human milk oligosaccharide 2′-fucosyllactose from sucrose in *Escherichia coli*. *ACS Synthetic Biology*, 9, 2784–2796.

Salis, H.M., Mirsky, E.A. & Voigt, C.A. (2009) Automated design of synthetic ribosome binding sites to control protein expression. *Nature Biotechnology*, 27, 946–950.

Santos-Navarro, F.N., Vignoni, A., Boada, Y. & Pico, J. (2021) RBS and promoter strengths determine the cell-growth-dependent protein mass fractions and their optimal synthesis rates. *ACS Synthetic Biology*, 10, 3290–3303.

Sprenger, G.A., Baumgärtner, F. & Albermann, C. (2017) Production of human milk oligosaccharides by enzymatic and whole-cell microbial biotransformations. *Journal of Biotechnology*, 258, 79–91.

Sun, H., Yang, J. & Song, H. (2020) Engineering mycobacteria artificial promoters and ribosomal binding sites for enhanced sterol production. *Biochemical Engineering Journal*, 162, 107739.

Tan, Y., Zhang, Y., Han, Y., Liu, H., Chen, H., Ma, F. et al. (2019) Directed evolution of an α1,3-fucosyltransferase using a single-cell ultrahigh-throughput screening method. *Science Advances*, 5, eaaw8451.

Wan, L., Zhu, Y.Y., Chen, G., Luo, G.C., Zhang, W.L. & Mu, W.M. (2021) Efficient production of 2′-fucosyllactose from L-fucose via self-assembling multienzyme complexes in engineered *Escherichia coli*. *ACS Synthetic Biology*, 10, 2488–2498.

Yang, L., Fan, W. & Xu, Y. (2021) GC x GC-TOF/MS and UPLC-Q-TOF/MS based untargeted metabolomics coupled with physicochemical properties to reveal the characteristics of different type daqus for making soy sauce aroma and flavor type baijiu. *LWT*, 146, 111416.

Yu, J., Shin, J., Park, M., Seydametova, E., Jung, S.M., Seo, J.-H. et al. (2018) Engineering of α-1,3-fucosyltransferases for production of 3-fucosyllactose in *Escherichia coli*. *Metabolic Engineering*, 48, 269–278.

Yu, W.W., Jin, K., Wu, Y.K., Zhang, Q.W., Liu, Y.F., Li, J.H. et al. (2022) A pathway independent multi-modular ordered control system based on thermosensors and CRISPR improves bioproduction in *Bacillus subtilis*. *Nucleic Acids Research*, 50, 6587–6600.

Zerbini, F., Zanella, I., Fraccascia, D., König, E., Irene, C., Frattini, L.F. et al. (2017) Large scale validation of an efficient CRISPR/Cas-based multi gene editing protocol in *Escherichia coli*. *Microbial Cell Factories*, 16, 68.

Zhang, Q., Yao, R., Chen, X.L., Liu, L.M., Xu, S.Q., Chen, J.H. et al. (2018) Enhancing fructosylated chondroitin production in *Escherichia coli* k4 by balancing the UDP-precursors. *Metabolic Engineering*, 47, 314–322.

Zhu, Y.Y., Wan, L., Li, W., Ni, D.W., Zhang, W.L., Yan, X. et al. (2022) Recent advances on 2′-fucosyllactose: physiological properties, applications, and production approaches. *Critical Reviews in Food Science and Nutrition*, 62, 2083–2092.

Zhu, Y.Y., Wan, L., Meng, J.W., Luo, G.C., Chen, G., Wu, H. et al. (2021) Metabolic engineering of *Escherichia coli* for lactono-α-tirso II production with high productivity. *Journal of Agricultural and Food Chemistry*, 69, 3702–3711.

**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**How to cite this article:** Li, M., Li, C., Luo, Y., Hu, M., Liu, Z. & Zhang, T. (2022) Multi-level metabolic engineering of *Escherichia coli* for high-tier biosynthesis of 2′-fucosyllactose and 3-fucosyllactose. *Microbial Biotechnology*, 15, 2970–2981. Available from: [https://doi.org/10.1111/1751-7915.14152](https://doi.org/10.1111/1751-7915.14152)