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Metabolic engineering strategies of *de novo* pathway for enhancing 2′-fucosyllactose synthesis in *Escherichia coli*

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Summary

2′-Fucosyllactose (2′-FL), one of the most abundant human milk oligosaccharides (HMOs), is used as a promising infant formula ingredient owing to its multiple health benefits for newborns. However, limited availability and high-cost preparation have restricted its extensive use and intensive research on its potential functions. In this work, a powerful *Escherichia coli* cell factory was developed to ulteriorly increase 2′-FL production. Initially, a modular pathway engineering was strengthened to balance the synthesis pathway through different plasmid combinations with a resulting maximum 2′-FL titre of 1.45 g l^{-1}. To further facilitate the metabolic flux from GDP-L-fucose towards 2′-FL, the CRISPR-Cas9 system was utilized to inactivate the genes including *lacZ* and *wcaJ*, increasing the titre by 6.59-fold. Notably, the co-introduction of NADPH and GTP regeneration pathways was confirmed to be more conducive to 2′-FL formation, achieving a 2′-FL titre of 2.24 g l^{-1}. Moreover, comparisons of various exogenous α1,2-fucosyltransferase candidates revealed that *futC* from *Helicobacter pylori* generated the highest titre of 2′-FL. Finally, the viability of scaled-up production of 2′-FL was evidenced in a 3 l bioreactor with a maximum titre of 22.3 g l^{-1} 2′-FL and a yield of 0.53 mole 2′-FL $mole^{-1}$ lactose.

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Introduction

Human milk oligosaccharides (HMOs), the third largest solid component of breast milk (5–15 g l^{-1}) after fat and lactose, play an irreplaceable role in the growth and development of neonatal (Gonia *et al*., 2015). Increasing animal experiments and clinical trials have confirmed the beneficial properties of HMOs, such as maintaining intestinal homeostasis as probiotics, resisting the adhesion of pathogenic bacteria, modulating immune responses and promoting the development and repair of the nervous system (Weichert *et al*., 2013; Bych *et al*., 2019). Notably, 2′-fucosyllactose (2′-FL) has been proved to be the most abundant oligosaccharide in HMOs (Castanys-Muñoz *et al*., 2013). 2′-FL is approved for addition to infant formulas, dietary supplements and medical foods by U.S. Food and Drug Administration (FDA) and the European Food Safety Authority (Vandenplas *et al*., 2018). Studies have shown that infants fed formula with 2′-FL have the same variety of intestinal flora as breastfed infants (Marriage *et al*., 2015; Goehring *et al*., 2016). Therefore, 2′-FL has received great attention as a functional food ingredient for nutritional health and medicinal purposes.

Generally, 2′-FL is produced by isolation from breast milk or chemical synthesis (Fernandez-Mayoralas and Martin-Lomas, 1986). However, the efficient production of 2′-FL could not be achieved due to the limited supply of breast milk and the chemical synthesis process requiring precise side-chain protection and deprotection (Bode *et al*., 2016; Petschacher and Nidetzky, 2016). The enzymatic synthesis of 2′-FL allows the screening of appropriate enzymes based on the configuration of the receptor and glycosyl donor (Ye *et al*., 2019; Li *et al*., 2020). Unfortunately, the nucleoside donor, GDP-L-fucose, is expensive and the catalytic activity of α1,2-fucosyltransferase is low, making it impossible to achieve large-scale industrial production (Lee *et al*., 2012; Baumgärtner *et al*., 2013). Synthetic biology and metabolic engineering provide the most efficient method for the direct synthesis of 2′-FL from microorganisms (Chin *et al*., 2015, 2017). The biosynthetic pathway of

2′-FL in *E. coli* is illustrated in Fig. 1. The *de novo* pathway is the main pathway for microbial synthesis of GDP-L-fucose, which transforms mannose-6-phosphate into GDP-L-fucose via four enzymes, phosphomannomutase (*manB*, E.C. 5.4.2.8), mannose-1-phosphate guanyltransferase (*manC*, E.C. 2.7.7.22), GDP-D-mannose-4,6-ehydratase (*gmd*, E.C. 4.2.1.47) and GDP-L-fucose synthetase (*wcaG*, E.C. 1.1.1.271) (Zhai *et al.*, 2015; Huang *et al.*, 2017).

Currently, the biosynthetic pathway constructing for 2′-FL in host bacteria such as *E. coli* (Parschat *et al.*, 2020), *Bacillus subtilis* (Deng *et al.*, 2019), *Saccharomyces cerevisiae* and *Yarrowia lipolytica* (Hollands *et al.*, 2019) has become a research hotspot. Most researches were conducted on *E. coli* due to the simplicity of the culture conditions and the availability of genetic tools. As an example, a batch fermentation of *E. coli* JM109 (DE3) expressing *H. pylori* α1,2-fucosyltransferase *resulted in a 3.4-fold increase in 2′-FL production compared with the original (FucT2)* produced 1.23 g l^{-1} 2′-FL, while the addition of a triple-Asp-tag to the N-terminus bacteria (Chin *et al.*, 2015). WbgL, the gene encoding *FucT2* in *E. coli* O126, has high expression activity and catalytic specificity for lactose in *E. coli*, allowing most of the GDP-L-fucose in the reaction system to be used in the catalytic process, with a titre of 20.1 g l^{-1} of 2′-FL (Engels and Elling, 2014). Baumgärtner *et al.* (2013) integrated two GDP-L-fucose metabolic pathways into the genome of *E. coli* JM109, leading to 20.3 g l^{-1} 2′-FL in fed-batch fermentation.

Cofactors (NADPH and GTP) play an essential role in achieving high productivity and high titres of metabolites (Chemler *et al.*, 2010). In microbial cells, the pentose

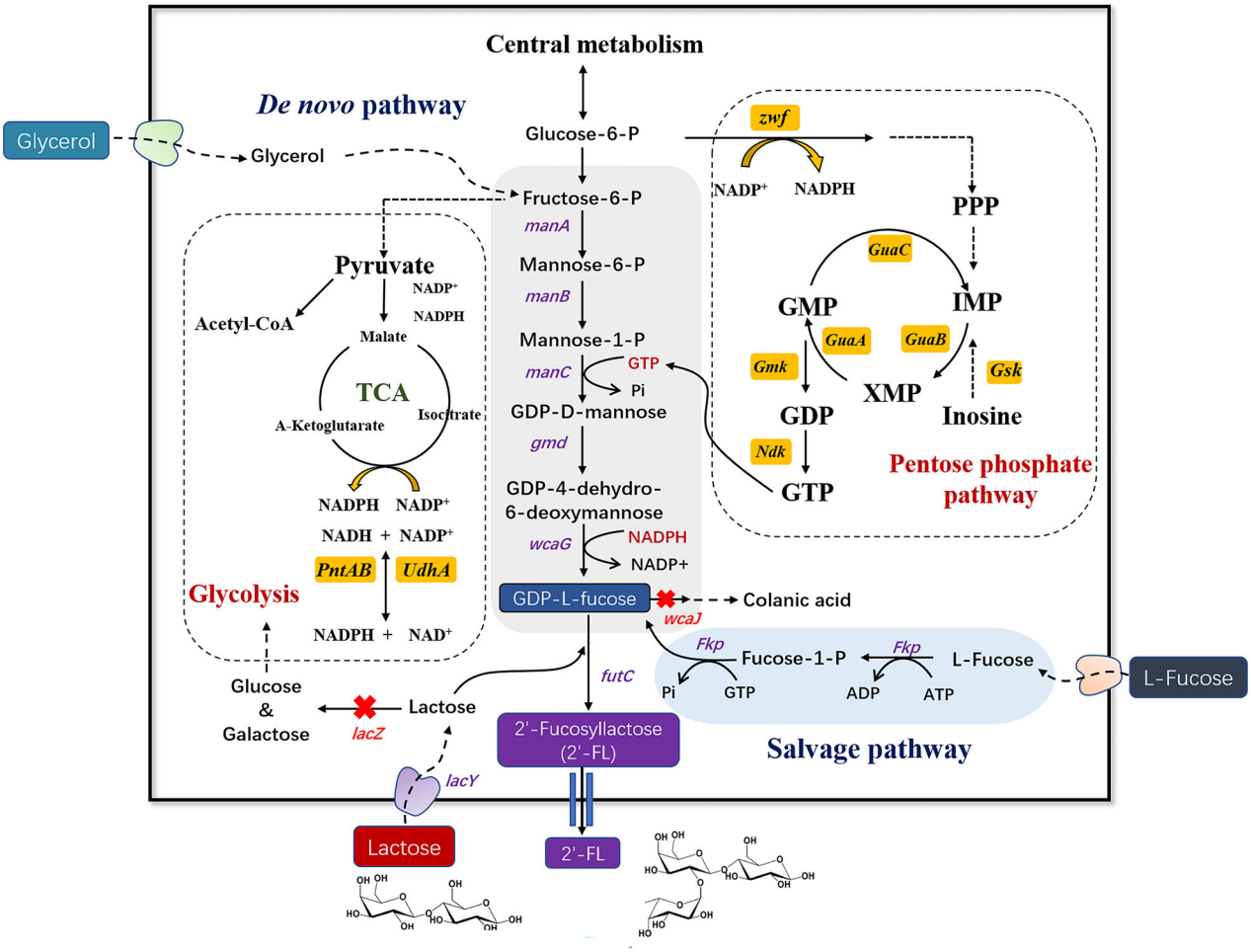

Fig. 1. 2′-FL biosynthesis model (*de novo* or salvage pathway) in engineered *E. coli*. The genes in purple denote overexpressed enzymes, and the genes in red denote the inactivated enzymes. The abbreviations of the enzymes are as follows: *manA*, mannose-6-phosphate isomerase; *manB*, phosphomannomutase; *manC*, α-D-mannose-1-phosphate guanyltransferase; gmd, GDP-D-mannose-4,6-ehydratase; *wcaG*, GDP-L-fucose synthetase; *wcaJ*, UDP-glucose lipid carrier transferase; *lacZ*, β-galactosidase; *zwf*, glucose-6-phosphate dehydrogenase; *Gsk*, guanosine-inosine kinase; *GuaB*, IMP dehydrogenase; *GuaA*, GMP synthetase; *GuaC*, GMP reductase; *Gmk*, guanylate kinase; *Ndk*, nucleotide diphosphate kinase; *PntAB*, membrane-bound transhydrogenase and *UdhA*, soluble transhydrogenase.

phosphate pathway (PPP) and the tricarboxylic acid (TCA) cycle are the dominant sources of NADPH and GTP (Sauer *et al*., 2004). During 2′-FL synthesis, NADPH and GTP are involved in the formation of GDP-L-fucose. The researchers constructed a *de novo* pathway of GDP-fucose in *E. coli* (*wcaJ*$^{-}$) and further expressed *zwf* and *gsk* genes to enhance intracellular NADPH and GTP expression, and the production of GDP-L-fucose could be accumulated to a maximum titre of 106 mg l^{-1} (Wan *et al*., 2020). Thus, improving the intracellular redox state can effectively improve the performance of the host bacteria and increase the yield of the target.

In this work, a modular metabolic engineering modification strategy was introduced in engineered *E. coli*, which regulates the metabolic flux between modules through different plasmid combinations based on the *de novo* pathway. To weaken branch bypass, 2′-FL synthesis was promoted by inactivating the β-galactosidase (*lacZ*) and UDP-glucose lipid carrier transferase (*wcaJ*) genes using the CRISPR-Cas9 system. The NADPH and GTP regeneration pathways were engineered as embedded modules to significantly improve the yield of 2′-FL. The best choices among several exogenous α1,2-fucosyltransferase (*futC*) for 2′-FL production performance were screened. Finally, a fed-batch fermentation was performed to further explore the production potential of the optimal strain BZW-24.

Results and discussion

Plasmid combinatorial optimization for 2′-FL production

To optimize the 2′-FL synthetic pathway, a modular metabolic engineering modification strategy was introduced in *E. coli*. The metabolic flux of the different modules was regulated by changing the transcriptional control factor (plasmid copy number) of each module to balance the synthetic pathway. Given that GDP-L-fucose is an essential precursor substance for the 2′-FL synthesis pathway, however, the yield of GDP-L-fucose is extremely low, thus requiring a fine modification of this synthetic pathway (Wan *et al*., 2020). The critical enzymes relation to the GDP-L-fucose pathway (*manB*, *manC*, *gmd* and *wcaG*) were used as module I responsible for the conversion of mannose-1-P to GDP-L-fucose. It has been reported that *futC* converting lactose and GDP-L-fucose into 2′-FL was a rate-limiting enzyme in the metabolic pathway due to its low activity (Drouillard *et al*., 2006). However, overexpression of *lacY* can effectively strengthen the efficiency of lactose transport. Therefore, *futC* and *lacY* were divided into module II to investigate the methods to alleviate this bottleneck (Fig. 2A).

The module expression intensity was determined by promoter strengths and gene copy numbers (Tolia and Joshua-Tor, 2006; Wu *et al*., 2014). In this study, five different copies of plasmids under the control of T7 promoter were used including pRSFDuet-1 (RSF ori), pETDuet-1 (pBR322 ori), pCDFDuet-1 (CDF ori), pACYCDuet-1 (p15A ori) and pCOLADuet-1 (ColA ori). Under different plasmid combinations, eighteen engineered host strains (BZW1–18) were constructed to achieve the *de novo* synthesis of 2′-FL (Fig. 2B). The fermentation products were detected by HPLC, and 2′-FL was validated by ESI-MS. The results of shake flask fermentation indicated that under the fixed expression of module II, the accumulation of products showed an upward tendency with increasing expression of module I. For example, the combination of downstream module II (pET-CY) and upstream module I with different copy numbers produced recombinant strains BZW-1, BZW-9, BZW-13 and BZW-16 and remarkably improved the titres of 2′-FL to 1.45, 1.02, 0.86 and 0.82 g l^{-1}, respectively (Fig. 2B). This suggested that the upstream module I at a higher expression level favours the accumulation of intracellular GDP-L-fucose. Similarly, the same trend was observed for fixation of the other modules. Interestingly, fixing the expression of module I, module II showed the highest titre at an expression slightly lower than that of module I. For example, the strains BZW-5, BZW-10 and BZW-15 had 8.4%, 5.1% and 6.0% higher 2′-FL production compared with the strains BZW-4, BZW-9 and BZW-14 respectively. Combinatorial optimization revealed that BZW-1 (carrying plasmids pRSF-CBGW and pET-CY) was the best fermenting strain with a 2′-FL concentration of 1.45 g l^{-1}. A plausible explanation was that the high-intensity expression of module II resulted in an inhibition of *futC* conversion, which may trigger metabolic burden and energy inefficiency in the host strain (Lee and Kim, 2015).

In order to compare the mRNA levels of 2′-FL biosynthetic enzymes (*manB*, *manC*, *gmd*, *wcaG*, *futC* and *lacY*) in different strains, we divided the eighteen engineered strains into five groups according to module I. The strains with high expression levels (BZW-1, BZW-5, BZW-10 and BZW-15) and low expression levels (BZW-4, BZW-8, BZW-12 and BZW-16) were selected for RT-qPCR assays (Fig. 5A). As expected, BZW-1 exhibited higher transcription levels in the above strains. In particular, the mRNA levels of *manB* and *wcaG* were 11.8- and 12-fold higher than those in BZW-16. This indicated that the increase of plasmid copy number in module I resulted in an increased expression level of the gene for 2′-FL synthesis in strain BZW-1, which effectively boosted the synthesis of the precursor metabolite GDP-L-fucose. For the mRNA levels of *futC* and *lacY* in module II, the expression of *futC* and *lacY* in the other strains was increased to varying degrees compared with strain BZW-16; however, the increase was not significant.

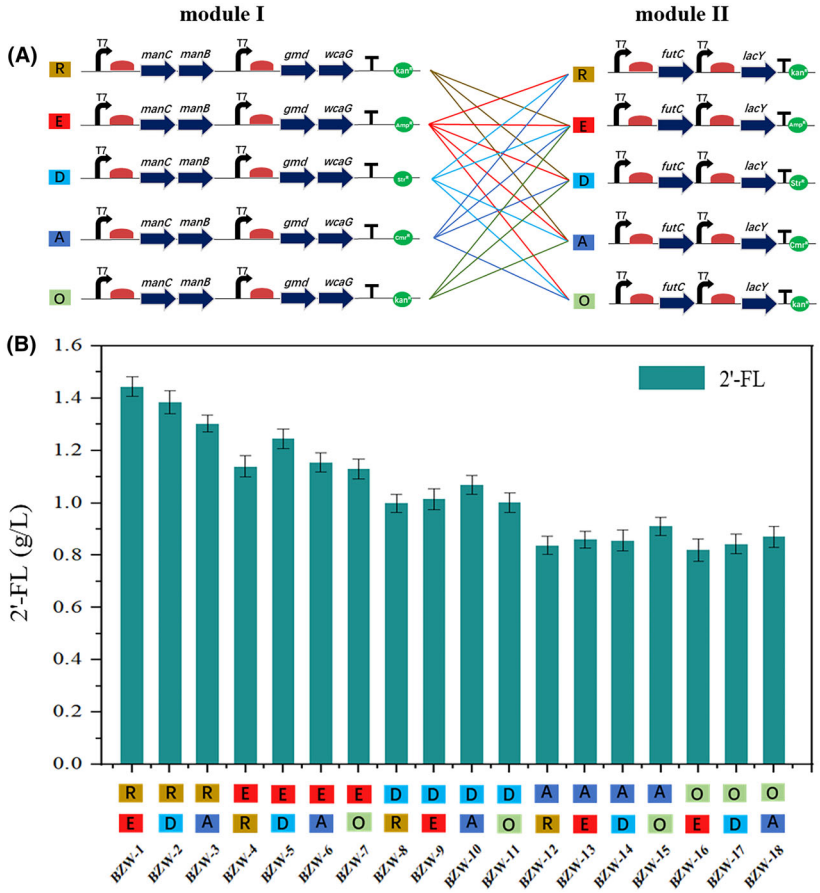

Fig. 2. Plasmid combinatorial optimization for 2′-FL production. A. Schematic design of different plasmid combinations. Module I includes *manB*, *manC*, *gmd* and *wcaG*. Module II contains *futC* and *lacY*. Both modules were expressed under the control of extremely strong (pRSFDuet-1, denoted by R), relatively strong (pETDuet-1, denoted by E), moderate (pCDFDuet-1, denoted by D), weak (pACYCDuet-1, denoted by A) or very weak (pCOLADuet-1, denoted by O) plasmids. B. Influences of modular engineering of 2′-FL. Triplicate experiments were conducted, and error bars denote the standard deviation.

Overall, lower downstream gene dosage and higher upstream gene dosage resulted in higher 2′-FL yield, and it is possible that this combination increased the accumulation of intermediates and promoted efficient conversion of *futC*.

Effects of strengthening the cofactors NADPH and GTP regeneration pathways on 2′-FL production

NADPH and GTP are essential cofactors involved in bioenergy and carbon metabolism, which help maintain intracellular redox balance (Sauer *et al*., 2004). Currently, enhancing endogenous cofactor pathways associated with metabolites has become an effective approach to improve the yield of industrial microbial chemicals. For this reason, we coupled cofactor and metabolic engineering to construct a cofactor regeneration system based on strain BWZ1.

Previous studies have shown that the overexpression of *zwf* in PPP to enhance NADPH regeneration and the overexpression of *Gsk* in the guanosine nucleotide biosynthesis pathway to facilitate GTP regeneration were both effective in increasing the production of the key precursor GDP-L-fucose (Wan *et al*., 2020). Here, the cumulative effect of cofactor engineering on 2′-FL was assessed by overexpression of multiple genes.

The *zwf*, *pntAB*, *UdhA*, *Gsk*, *GuaA*, *GuaB*, *GuaC*, *Gmk* and *Ndk* were selected as candidate genes, of which the mechanisms for energy regeneration are shown in Fig. 3A. Single or combined genes were cloned into the vector pCDFDuet-1 and introduced into strain BWZ1. The performance of the resulting strain was explored to further improve its redox status.

The single gene overexpression experiments enhanced the yield of 2′-FL to varying degrees. The strains overexpressing *zwf* (BZW-19) and *pntAB* (BZW-20) achieved better performance in four expression systems including *zwf* (BZW-19), *pntAB* (BZW-20), *UdhA* (BZW-21) and *Gsk* (BZW-22). The increased NADPH availability contributed to the accumulation of maximum

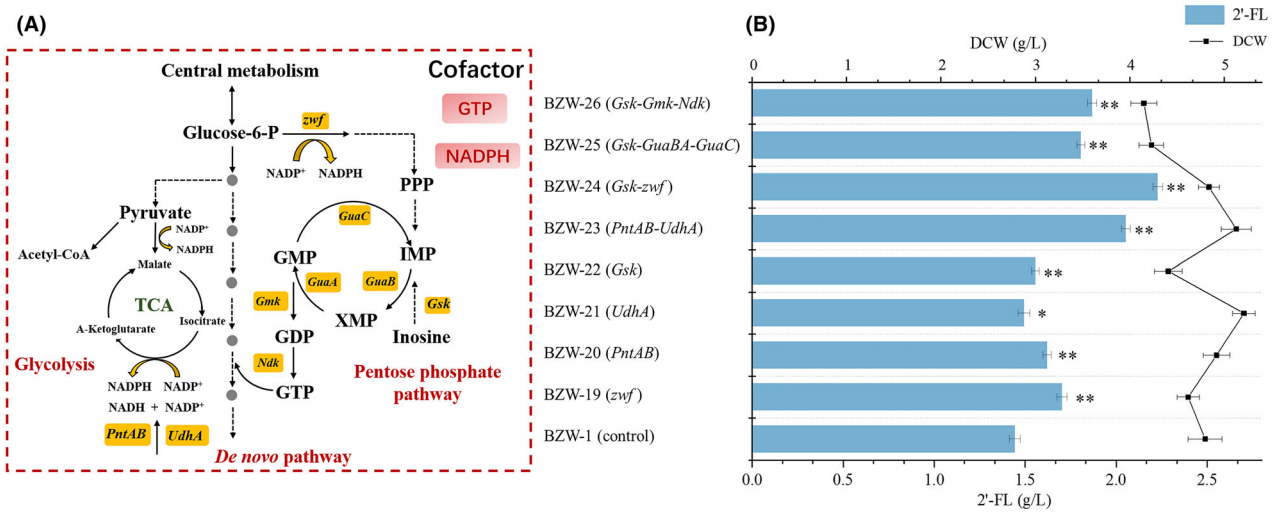

Fig. 3. Effect of increased cofactor NADPH and GTP availability on 2′-FL production. A. Cofactor regeneration pathways associated with the *de novo* pathway. The genes involved in the cofactor engineering include *zwf*, *pntAB*, *UdhA*, *Gsk*, *GuaA*, *GuaB*, *GuaC*, *Gmk* and *Ndk*. B. Shake flask fermentation results of different engineered strains with single and multiple gene overexpression involving cofactor regeneration. Triplicate experiments were conducted, and error bars denote the standard deviation. * and ** mean $P < 0.05$ and $P < 0.01$ respectively.

titres of 1.71 (BZW-19) and 1.62 g l^{-1} (BZW-20) of 2′-FL, respectively, which were 17.9% and 11.7% higher than the titres of the control strain (Fig. 3B). These results revealed that overexpression of the genes *zwf* and *pntAB* intensified the NADPH regeneration pathway, as they directed the strain to a highly reduced state to overproduce 2′-FL. Remarkably, the 2′-FL yield was significantly improved over that of the single gene in the *E. coli* strains co-expressing *PntAB-UdhA*, *Gsk-zwf*, *Gsk-GuaBA-GuaC* and *Gsk-Gmk-Ndk*. The 2′-FL titres of strains BZW-23, BZW-24, BZW-25 and BZW-26 were 2.06, 2.24, 1.81 and 1.87 g l^{-1}, which were 42.1%, 53.8%, 24.8% and 29.0% higher than that of the control respectively. This demonstrates that NADPH and GTP regeneration has indeed effectively increased the yield of 2′-FL. Particularly, the optimal performance of the BZW-24 strain might be attributed to the fact that the increased carbon flux through the overexpression of *zwf* was directed to adequate NADPH supply and that the overexpression of *Gsk* enhanced the GTP biosynthetic pathway, promoting the biotransformation of inosine and guanosine to IMP and GMP (Ledesma-Amaro *et al.*, 2013). Furthermore, *manC* requires GTP for the bioconversion of GDP-D-mannose, while *wcaG* requires NADPH for GDP-L-fucose biosynthesis in the 2′-FL *de novo* synthetic pathway. Efficient feeding of cofactors NADPH and GTP is essential for yield improvement. Previous studies have attempted to improve NADPH regeneration or enhance guanosine nucleotide biosynthesis through overexpression or evolution of genes corresponding to the rate-limiting steps in the target cofactor regeneration pathway (Wan *et al.*, 2020).

Effect of the deletion of lacZ and wcaJ on 2′-FL production

To further maximize the flow of metabolites to the target pathway, the CRISPR-Cas9 gene editing system was implemented to knockout *lacZ* and *wcaJ* in *E. coli* BL21 (DE3). Deletion of the UDP-glucose lipid carrier transferase (*wcaJ*) blocks the metabolic flux from GDP-L-fucose to colanic acid, favouring the accumulation of GDP-L-fucose (Huang *et al.*, 2017). β-Galactosidase (*lacZ*) cleaves lactose to galactose and glucose, catalysing the first step of lactose metabolism (Chin *et al.*, 2015). The intracellular availability of lactose is essential in the efficient biosynthesis of 2′-FL, thus inactivating the *lacZ* gene in engineered *E. coli*.

As shown in Fig. 4B, the deletion of *wcaJ* gene in *E. coli* strains BWS and BWZS resulted in an increase of 98.5% and 44.1% in intracellular GDP-L-fucose, compared with the original strain. The DCW and growth conditions of the knockout strain were not noticeably affected, which was consistent with previous findings (Wan *et al.*, 2020). Furthermore, weakening of the *lacZ* branch bypass further facilitated the accumulation of 2′-FL. The titres of 2′-FL for strains BWD, BZD and BWZ-1 were 0.392, 1.09 and 1.45 g l^{-1}, illustrating a 1.78-, 4.95- and 6.59-fold increase compared with BD (0.22 g l^{-1} at 60 h). Among them, strains BZD and BWZ-1 achieved yields of 0.59 and 0.61 mole 2′-FL $mole^{-1}$ lactose, which were 8.4 and 8.8 times higher than that of strain BD (Fig. 4C). To validate the effect of *wcaJ* and *lacZ* deletion on metabolic pathways, several relevant genes (*manC*, *manB*, *gmd*, *wcaG*, *lacY*, *lacZ*

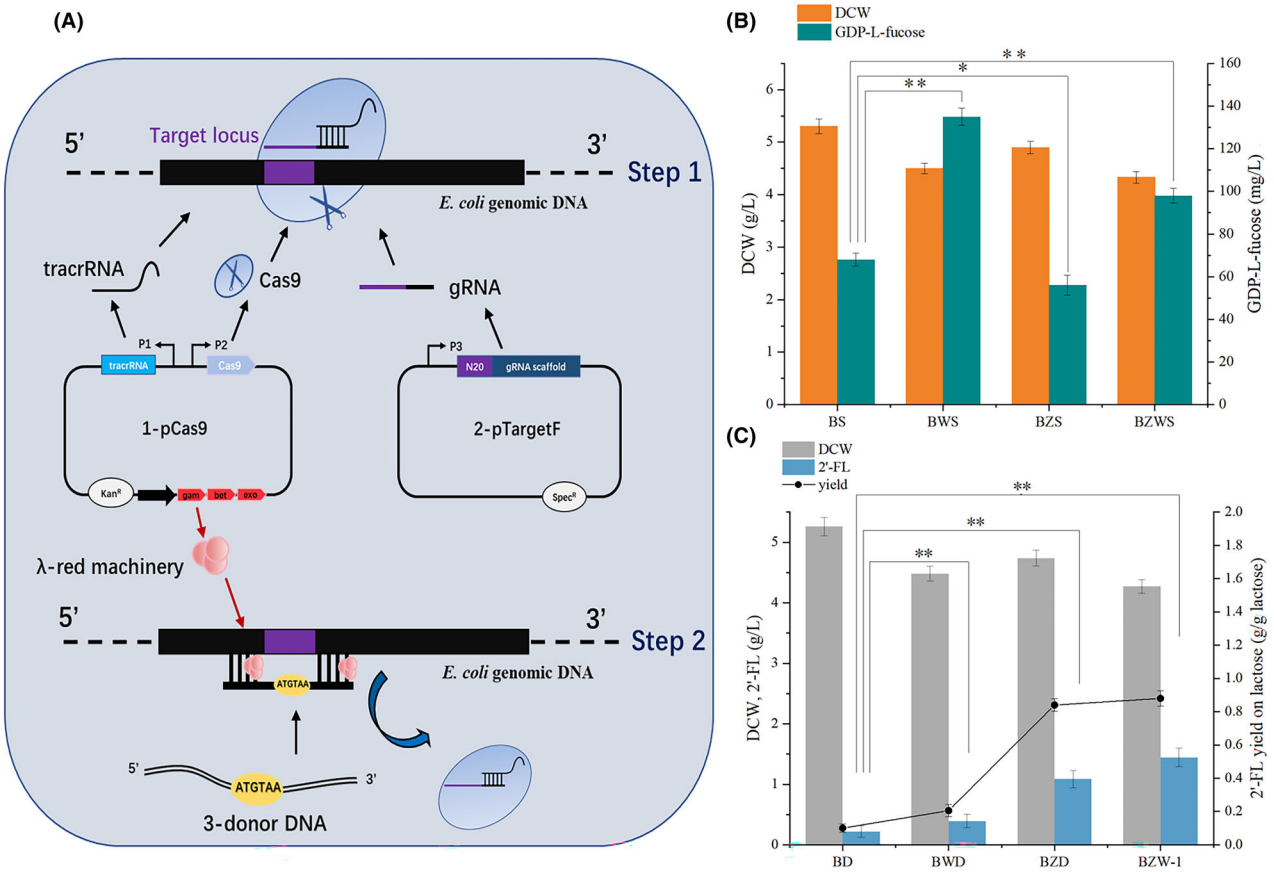

Fig. 4. Effect of gene knockout on 2′-FL biosynthesis. A. Overview of CRISPR/Cas9 genome editing strategies in *E. coli*. Genome editing involves three main components: (1) a pCas9 plasmid expressing the λ Red machinery and Cas9 endonuclease; (2) a pTargetF plasmid carrying the sgRNA gene; (3) a homologous repair template, donor-DNA. B. GDP-L-fucose titre and DCW of BWS, BZS, BWZS and the control host BS. C. 2′-FL titre, DCW and 2′-FL yield on lactose in BD, BWD, BZD and BWZ-1. Triplicate experiments were performed, and the error bars indicate the standard deviation. * and ** mean $P < 0.05$ and $P < 0.01$ respectively.

and *wcaJ*) in the metabolic pathway were selected. The mRNA levels of the above genes (*manC*, *manB*, *gmd*, *wcaG* and *lacY*) were significantly increased in the defective strains (BL21(DE3)Δ*wcaJ*, BL21(DE3)Δ*lacZ* and BL21(DE3)Δ*lacZ*Δ*wcaJ*) compared with those in the original BL21(DE3) strain (Fig. 5B). Obviously, the knockout of *wcaJ* and *lacZ* genes greatly reduced their transcription levels. These results indicated that deletion of *wcaJ* and *lacZ* effectively enhanced the titres of GDP-L-fucose and 2′-FL and significantly improved the accumulation of the target pathway.

Improving 2′-FL production by comparing various α1,2-fucosyltransferases

α1,2-Fucosyltransferase allows fucosylation modification of the substrate to achieve enzymatic conversion from the donor GDP-L-fucose and the acceptor lactose to the product 2′-FL. Herein, four α1,2-fucosyltransferases from prokaryotes were selected, namely HpFutC1 (protein ID: KY499613), HpFutC2 (protein ID: AFV41312.1), BfwcfB (protein ID: WP_005817145.1) and EcwbgL (protein ID: ADN43847.1). To evaluate the contribution of different α1,2-fucosyltransferases to 2′-FL, batch cultivation of four strains (BZW-24, BZW-27, BZW-28 and BZW-29) was employed using a metabolic engineering strategy (Fig. 6). Overexpression of different sources of α1,2-fucosyltransferases may result in different levels of 2′-FL production. The BZW-24 strain (containing HpFutC1) achieved an optimal 2′-FL concentration of 2.24 g l^{-1} from 20 g l^{-1} glycerol and 14 g l^{-1} lactose at 60 h, with the yield of 0.60 mole 2′-FL $mole^{-1}$ lactose. (Fig. 6A). Compared with BZW-24, the remaining three strains exhibited slightly lower 2′-FL titres, with BZW-27, BZW-28 and BZW-29 exhibiting 2′-FL titres of 1.80, 1.69 and 1.95 g l^{-1}, respectively (Fig. 6B–D). The higher 2′-FL concentration obtainable with HpFutC1 might be attributed to the higher affinity of HpFutC1 on lactose. Albermann *et al*. (2001) found that the *K*m value of HpfutC (168 μM) was lower than that of other sources of α1,2-fucosyltransferases, thus giving *futC* a higher affinity for the substrate. It should be noted that

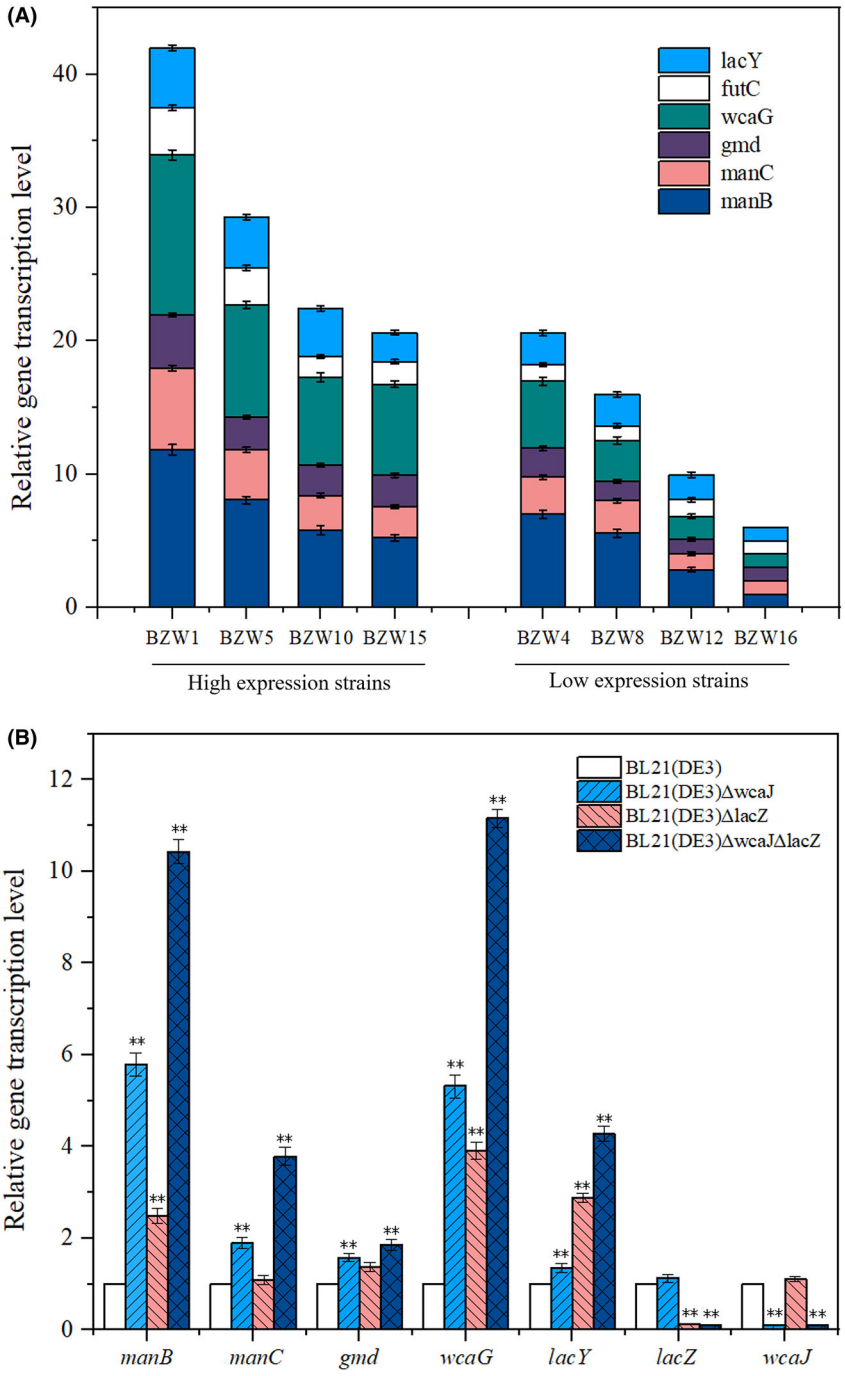

Fig. 5. Relative expression levels of genes within 2′-FL biosynthetic pathway in the engineered strains. A. Expression profiles of the genes (*manB*, *manC*, *gmd*, *wcaG*, *futC* and *lacY*) in engineered *E. coli* strains (BZW-1, BZW-5, BZW-10, BZW-15, BZW-4, BZW-8, BZW-12 and BZW-16). B. Effect of gene knockdown on the expression of related genes (*manC*, *manB*, *gmd*, *wcaG*, *lacY*, *lacZ* and *wcaJ*) in the 2′-FL biosynthetic pathway. Statistical significance was assessed by Tukey's HSD test. * and ** mean $P < 0.05$ and $P < 0.01$ respectively.

α1,2-fucosyltransferase itself exhibits weak enzymatic activity and low soluble expression, and future work will be directed towards the evolution of *futC* to improve the productivity of 2′-FL in the *de novo* synthetic pathway.

Production of 2′-FL in 3 l bioreactors

To achieve high titres of 2′-FL for the engineered strain under certain conditions, glycerol fed-batch fermentation

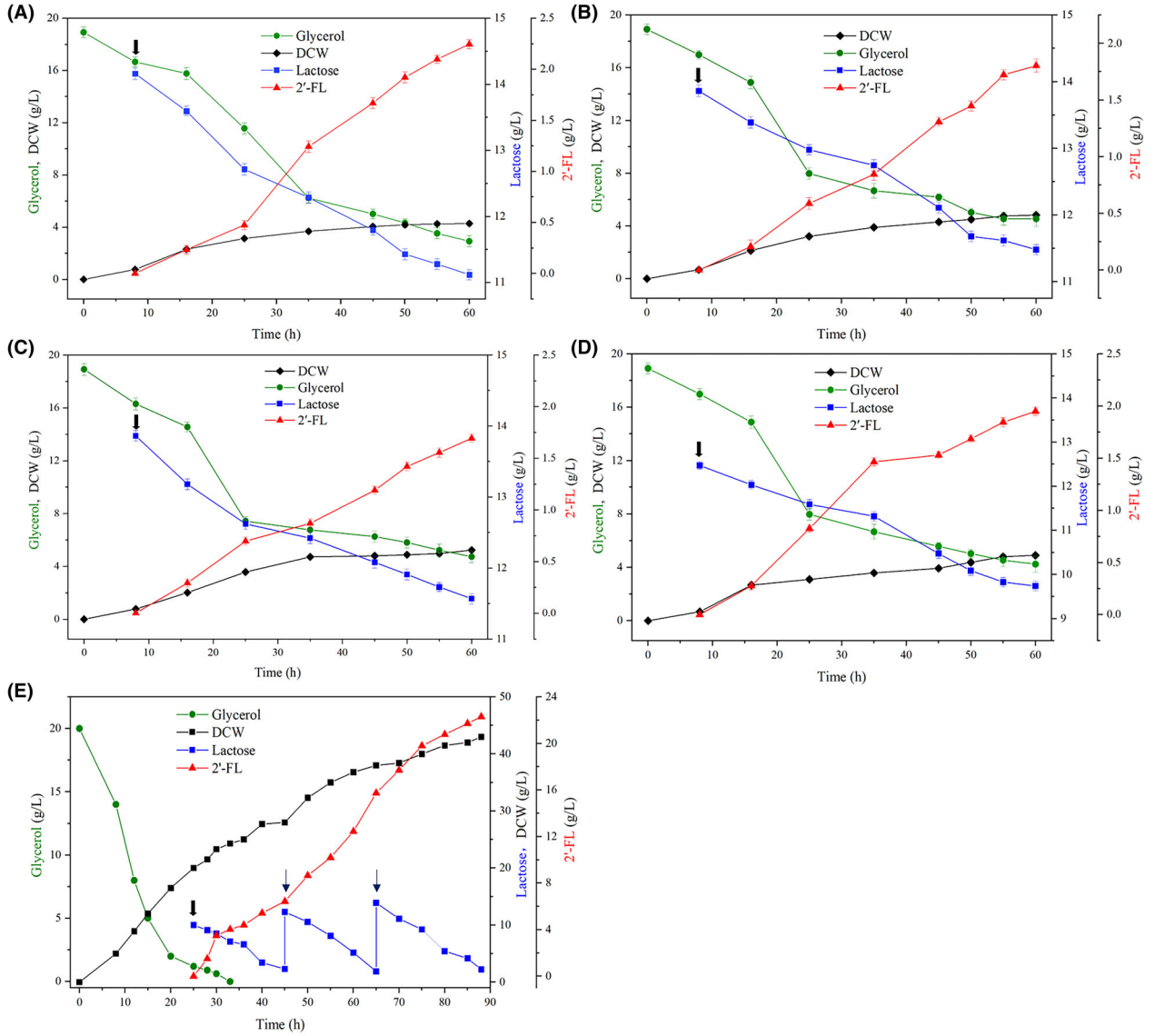

Fig. 6. Fig. Biosynthesis of 2′-FL by batch and fed-batch fermentation. Production of 2′-FL from glycerol with batch fermentations using several α1,2-fucosyltransferase including HpFutC1 (A), HpFutC2 (B), Bfwcfb (C) and EcwbgL (D). (E) Production of 2′-FL by strain BWZ24 with HpFutC1 in a 3 l bioreactor. Thick arrow, IPTG induction and initial addition of lactose; thin arrows, intermittent addition of lactose.

of engineered *E. coli* BZW-24 was carried out with intermittent lactose addition. Here, glycerol was employed as a carbon source for cell growth and lactose was added as a fucose receptor during the fermentation process. The reason for this is that both glycerol and lactose are transported into the cell via the non-PTS system (Yazdani and Gonzalez, 2007). The growth of the strain is not inhibited, and the substrates are low cost and easily available for large-scale production. After the initial addition of glycerol was completely depleted, glycerol was fed in a pH-constant mode.

Fermentation results revealed that the product started to accumulate gradually with the addition of IPTG (0.2 mM) at OD_{600} of 20 (Fig. 6E). After the initial lactose was exhausted from the medium, lactose was supplemented to the culture broth to sustain 2′-FL biosynthesis. As a result, 22.3 g l^{-1} of 2′-FL was obtained at the end of fed-batch fermentation, which was 9.96 times of that in the shake flask. As shown in Table S3, the strain was assimilated by glycerol to maintain cell growth to a final cell dry weight of 43 g l^{-1}. As calculated that the 2′-FL yield was 0.53 mole 2′-FL $mole^{-1}$ lactose or 0.52 g 2′-FL g^{-1} DCW, which is basically consistent with the yield of the batch fermentations. These results demonstrated that continuous supplementation of carbon sources, efficient NADPH and

GTP regeneration and metabolic flux to the desired biosynthetic pathway significantly facilitated the biosynthesis of 2′-FL. Here, *E. coli* BL21 (DE3) rather than JM109 (DE3) was utilized as a cell factory to produce 2′-FL. This was attributed to the fact that the biofilms formed by JM109 cause serious problems during fermentation, whereas BL21 (DE3) readily exhibits active sugar metabolism and tolerance against metabolic pressure, making it relatively easier for BL21 (DE3) to grow to high cell densities (Lee *et al*., 2012; Teodósio *et al*., 2012).

Conclusions

In this work, a biosynthetic pathway for the efficient production of 2′-FL was constructed using low-cost glycerol as a carbon source and lactose as a precursor. The intracellular titre of 2′-FL in engineered *E. coli* was effectively increased by modular optimization of cellular metabolic pathways. Gene knockout of metabolic bypass, regeneration of cofactors (NADPH and GTP) and screening of different α1,2-fucosyltransferase significantly increased the yield of 2′-FL. The best engineered strain BZW-24 had a 2′-FL titre of 22.3 g l^{-1} and a yield of 0.53 mole 2′-FL $mole^{-1}$ lactose. The work reported here indicates that this engineered strain is a promising cell factory for efficient production of 2-FL. It should be noted that the activity and soluble expression levels of α1,2-fucosyltransferase are still lower. More efforts should be made in the future, such as establishing a high-throughput screening method for α1,2-fucosyltransferase and performing directed evolution studies on the enzyme.

Experimental procedures

Bacterial strains, plasmids and medium

All strains and plasmids used in this study are summarized in Tables 1 and S1 respectively. *E. coli* BL21 (DE3) was used as a host strain, and *E. coli* DH5a was employed for amplification and construction of plasmids. The vectors with different copy numbers, including pRSFDuet-1, pETDuet-1, pCDFDuet-1, pACYCDuet-1 and pCOLADuet-1, were selected for cloning and subcloning. All molecular reagents such as high-fidelity DNA polymerases, plasmid extraction kits, DNA purification kits and restriction enzymes were purchased from Vazyme Biotech (Nanjing, China).

The engineered strains are routinely grown on Luria–Bertani (LB) medium (5 g l^{-1} yeast extract, 10 g l^{-1} tryptone and 10 g l^{-1} NaCl) or LB agar plates (LB medium with 15 g l^{-1} agar), containing appropriate antibiotics for plasmid screening at 37°C. Batch and fed-batch cultivations were performed in glycerol-defined medium [13.5 g l^{-1} KH_2PO_4, 1.7 g l^{-1} citric acid, 4.0 g l^{-1} $(NH_4)_2HPO_4$, 1.4 g l^{-1} $MgSO_4{\cdot}7H_2O$ and 10 ml l^{-1} trace element solution (10 g l^{-1} Fe(III) citrate, 2.25 g l^{-1} $ZnSO_4{\cdot}7H_2O$, 1.0 g l^{-1} $CuSO_4{\cdot}5H_2O$, 0.35 g l^{-1} $MnSO_4{\cdot}H_2O$, 0.23 g l^{-1} $Na_2B_4O_7{\cdot}10H_2O$, 0.11 g l^{-1} $(NH_4)_6Mo_7O_{24}$ and 2.0 g l^{-1} $CaCl_2$), pH 6.8] with 20 g l^{-1} glycerol as a carbon source.

Gene cloning and plasmid construction

The linear fragments were assembled for plasmid construction using Clone Express II one-step cloning kit (Vazyme, Nanjing, China). The gene templates for *manC-manB*, *gmd-wcaG* and *lacY* were derived from the *E. coli* K12 MG1655, and the primers are shown in Table S2. The coding sequence of α1,2-fucosyltransferases has been codon optimized and synthesized by Genewiz (Suzhou, China). The vector linear backbone fragments were amplified by reverse PCR. First, the amplified *manC-manB* gene clusters fragment was inserted into pRSFDuet-1, pETDuet-1, pCDFDuet-1, pACYCDuet-1 and pCOLADuet-1 at the *Nco* I site, generating the plasmids pRSF-CB, pET-CB, pCDF-CB, pCDYC-CB and pCOLA-CB respectively. Subsequently, the gmd-wcaG cluster was further inserted into the aforementioned plasmids containing *manC-manB* cluster at the *Nde* I site to construct pRSF-CBGW, pET-CBGW, pCDF-CBGW, pACYC-CBGW and pCOLA-CBGW. Similarly, the *futC* and *lacY* fragments and vectors with different copy number were ligated to obtain recombinant plasmids pRSF-CY, pET-CY, pCDF-CY, pACYC-CY and pCOLA-CY. Finally, recombinant expression vectors with different plasmid combinations (Fig. 2A) were transformed into *E. coli* competent cells to obtain the recombinant strains.

The overexpressed genes involved in NADPH regulation and the cofactors GTP and ATP regeneration (Fig. 3A) in this study were *zwf*, *pntAB*, *UdhA*, *Gsk*, *GuaA*, *GuaB*, *GuaC*, *Gmk* and *Ndk*. The templates for genes cloning were derived from the *E. coli* K12 MG1655 genome. As constructed above, the vector and target fragments were ligated to obtain the plasmids pCDF-*zwf*, pCDF-*pntAB*, pCDF-*UdhA*, pCDF-*Gsk*, pCDF-*pntAB-UdhA*, pCDF-*Gsk-zwf*, pCDF-*Gsk-GuaBA-GuaC* and pCDF-*Gsk-Gmk-Ndk*.

Gene knockout through CRISPR-Cas9

The genes encoding *lacZ* and *wcaJ* were targeted for deletion from *E. coli* chromosome via CRISPR-Cas9 system (Fig. 4A). The two-plasmid-based CRISPR-Cas9 system was used for genome editing, in which the pCas9 plasmid encodes the Cas9 nuclease and the pTargetF plasmid carries single-guide RNA (sgRNA) with a certain N20 sequence (Jiang *et al*., 2015). pCas9 and pTargetF were deposited in Addgene, numbered 62225

Table 1. List of strains used in this study.

Strains	Relevant genotype or relevant properties	Reference
E. coli DH5a	F^- *endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG* Φ80*dlacZ*ΔM15 Δ*(lacZYA-argF)* U169, *hsdR17 (rK^- mK^+)*, λ^-	Novagen
E. coli BL21 (DE3)	*fhuA2 (DeLong et al.) ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int: (lacI::PlacUV5::T7 gene1) i21 Δnin5*	Novagen
BW	*E. coli* BL21 (DE3): Δ*wcaJ*	This study
BZ	*E. coli* BL21 (DE3): Δ*lacZ*	This study
BZW	*E. coli* BL21 (DE3): Δ*lacZ*Δ*wcaJ*	This study
BS	BL21 (DE3) harboring plasmids pRSF-CBGW	This study
BD	BL21 (DE3) harboring plasmids pRSF-CBGW and pET-CY	This study
BWS	BW harboring plasmids pRSF-CBGW	This study
BWD	BW harboring plasmids pRSF-CBGW and pET-CY	This study
BZS	BZ harboring plasmids pRSF-CBGW	This study
BZD	BZ harboring plasmids pRSF-CBGW and pET-CY	This study
BZWS	BZW harboring plasmids pRSF-CBGW	This study
BZW-1	BZW harboring plasmids pRSF-CBGW and pET-CY	This study
BZW-2	BZW harboring plasmids pRSF-CBGW and pCDF-CY	This study
BZW-3	BZW harboring plasmids pRSF-CBGW and pACYC-CY	This study
BZW-4	BZW harboring plasmids pET-CBGW and pRSF-CY	This study
BZW-5	BZW harboring plasmids pET-CBGW and pCDF-CY	This study
BZW-6	BZW harboring plasmids pET-CBGW and pAYC-CY	This study
BZW-7	BZW harboring plasmids pET-CBGW and pCOLA-CY	This study
BZW-8	BZW harboring plasmids pCDF-CBGW and pRSF-CY	This study
BZW-9	BZW harboring plasmids pCDF-CBGW and pET-CY	This study
BZW-10	BZW harboring plasmids pCDF-CBGW and pACYC-CY	This study
BZW-11	BZW harboring plasmids pCDF-CBGW and pCOLA-CY	This study
BZW-12	BZW harboring plasmids pAYC-CBGW and pRSF-CY	This study
BZW-13	BZW harboring plasmids pAYC-CBGW and pET-CY	This study
BZW-14	BZW harboring plasmids pAYC-CBGW and pCDF-CY	This study
BZW-15	BZW harboring plasmids pAYC-CBGW and pCOLA-CY	This study
BZW-16	BZW harboring plasmids pCOLA-CBGW and pET-CY	This study
BZW-17	BZW harboring plasmids pCOLA-CBGW and pCDF-CY	This study
BZW-18	BZW harboring plasmids pCOLA-CBGW and pACYC-CY	This study
BZW-19	BZW-1 harboring plasmids pCDF-*zwf*	This study
BZW-20	BZW-1 harboring plasmids pCDF-*pntAB*	This study
BZW-21	BZW-1 harboring plasmids pCDF-*UdhA*	This study
BZW-22	BZW-1 harboring plasmids pCDF-*Gsk*	This study
BZW-23	BZW-1 harboring plasmids pCDF-*PntAB-UdhA*	This study
BZW-24	BZW-1 harboring plasmids pCDF-*Gsk-zwf*	This study
BZW-25	BZW-1 harboring plasmids pCDF-*Gsk-GuaBA-GuaC*	This study
BZW-26	BZW-1 harboring plasmids pCDF-*Gsk-Gmk-Ndk*	This study
BZW-27	BZW harboring plasmids pRSF-CBGW, pET-TY and pCDF-*Gsk-zwf*	This study
BZW-28	BZW harboring plasmids pRSF-CBGW, pET-FY and pCDF-*Gsk-zwf*	This study
BZW-29	BZW harboring plasmids pRSF-CBGW, pET-GY and pCDF-*Gsk-zwf*	This study

and 62226 respectively. Knockout gene targets were designed using online software at http://www.regenome.net/cas-offinder/. The sgRNA expression cassette with the N20 sequence was constructed using the pTargetF plasmid as a template. The successfully constructed knockout plasmids were named pTF-Δ*lacZ* and pTF-Δ*wcaJ* respectively. *E. coli* BL21 (DE3) competent cells harbouring pCas9 were prepared as described previously (Sharan *et al*., 2009). The repair templates targeting anti-selection marker genes were designed to remove sequences between the start codon and stop codon of the target gene. Long repair templates (500–1000 bp arms) were constructed by linking homology arms upstream and downstream of target genes (Zerbini *et al*., 2017). Arabinose (final concentration 10 mM) was supplemented to the culture for λ-red induction to facilitate homologous recombination. The knockout plasmid pTF-Δ*lacZ* or pTF-Δ*wcaJ* and the homologous repair template were electrotransformed into competent cells. Positive transformants were screened by colony PCR and DNA sequencing on LB solid plates containing kanamycin (50 μg ml^{-1}) and spectinomycin (50 μg ml^{-1}). After that, the double-plasmid system in the defective bacteria was eliminated by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for induction and overnight incubation of positive transformants at 42°C.

Batch fermentation for 2′-FL production

For batch fermentation, the engineered strains were inoculated into 3 ml of LB medium and cultured at 37°C

and 200 rpm. Subsequently, 0.5 ml of seed solution was transferred into 50 ml of defined medium with 20 g l^{-1} glycerol to expand growth in 250 ml shake flasks. IPTG and lactose were supplemented at the final concentration of 0.2 mM and 10 g l^{-1} until the cells' optical density (OD_{600}) was approximately 0.6–0.8. After the IPTG induction, the cells were incubated at 25°C for efficient expression of proteins. If necessary, ampicillin (100 μg ml^{-1}), chloramphenicol (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}) and streptomycin (50 μg ml^{-1}) were added to the medium.

Fed-Batch fermentation in a 3 l bioreactor

Fed-batch cultivations were performed in a 3 l bioreactor with 1 l working volume of the defined medium and 5% of inoculation. Main culture was employed at 37°C, and when OD_{600} reached 20, IPTG and lactose were added to a final concentration of 0.2 mM and 20 g l^{-1}. During induction, the temperature was adjusted to 25°C and the pH of the medium was maintained at 6.8 by addition of 28% NH_4OH. The feeding solution comprising 600 g l^{-1} of glycerol and 20 g l^{-1} of $MgSO_4{\cdot}7H_2O$ was streamed into the fermentation broth to replenish the carbon source by pH-stat mode (Chin *et al*., 2015). The fermentation process was controlled by a cascade system that regulated the speed, aeration and oxygen to achieve 20% dissolved oxygen in the fermenter.

Analytical methods

During cultivation, the culture broth was sampled at intervals and growth conditions were determined by OD_{600} analysis using a spectrophotometer (P7 Double Beam UV-VIS Spectrophotometer, Mapada, China). Dry cell weight (g l^{-1}) was calculated from OD_{600} using conversion factor of 0.36 (Chin *et al*., 2013).

The concentrations of glycerol, lactose and 2′-FL in the fermentation broth were quantified with an HPLC system (Waters e2695) equipped with a refractive index (RI) detector and a Rezex ROA-Organic Acid H^+ column (Phenomenex, Torrance, CA, USA). The column was eluted with 0.01 M H_2SO_4 at a flow rate of 0.6 ml min^{-1} at 50°C (Chin *et al*., 2016). To assay 2′-FL, culture broth samples (1 ml) were boiled for 10 min to break the cells completely and centrifuged at 12 000 *g* for 10 min. The supernatant was used to estimate the total oligosaccharide content. To identify the product of 2′-FL, 2′-FL standards and samples were analysed by electro-spray ionization-mass spectroscopy (ESI-MS) in negative ion mode under standard conditions on a Waters MALDI SYNAPT Q-TOF MS (Milford, MA, USA). The content of GDP-L-fucose in fermentation broth was determined according to Huang *et al*. (2017).

Real-time quantitative PCR (RT-qPCR)

To evaluate the mRNA levels of intracellular 2′-FL biosynthetic enzymes in engineered *E. coli*, RT-qPCR was employed for analysis. Total RNA of the strains was extracted during the mid-exponential phase using FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme-Nanjing, China) following the manufacturer's protocol. The RNA was reverse transcribed to cDNA using the HiScript III RT SuperMix for qPCR (+gDNA wiper) kit (Vazyme). The RT-qPCR was performed using the CFX96 Real-Time system (Bio-Rad, Pleasanton, CA, USA) and ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China). The housekeeping gene (16S rRNA) was used as a leading control. The relative transcript levels of target genes were analysed from three biological replicates by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primer sequences for RT-qPCR are shown in Table S2.

Statistical analysis

All numerical results are the average of at least three independent replicates. The mean differences were determined by Tukey's HSD test ($P < 0.05$) using analysis of variance (one-way ANOVA) in OriginPro 9.0 software (Origin Lab, Northampton, MA, USA).

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Conflict of interest

The authors declare no competing financial interest.

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Supporting information

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

Table S1. List of plasmids used in this study.

Table S2. Primers used in this study.

Table S3. Results on the batch and fed-batch fermentations of engineered *E. coli* strains for 2′-FL production.

Fig. S1. Identification of 2′-FL by HPLC and MS. (a) The overlaid HPLC spectra of fermentation sample, 2′-FL standard and control. (b) Analysis of 2′-FL standard by TOF-MS. (c) Analysis of 2′-FL from fermentation broth by TOF.

