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Original Research Article

Construction of an engineered *Escherichia coli* for effective synthesis of 2′-fucosyllactose *via* the *salvage* pathway

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ABSTRACT

2′-Fucosyllactose (2′-FL) is one of the important functional oligosaccharides in breast milk. So far, few attempts on biosynthesis of 2′-FL by the *salvage* pathway have been reported. Herein, the *salvage* pathway enzyme genes were introduced into the *E. coli* BL21star(DE3) for synthesis of 2′-FL. The 2′-FL titer increased from 1.56 to 2.13 g/L by deleting several endogenous genes on competitive pathways. The α-1,2-fucosyltransferase (WbgL) was selected, and improved the 2′-FL titer to 2.88 g/L. Additionally, the expression level of pathway enzyme genes was tuned through optimizing the plasmid copy number. Furthermore, the spatial distribution of WbgL was enhanced by fusing with the MinD C-tag. After optimizing the fermentation conditions, the 2′-FL titer reached to 7.13 g/L. The final strain produced 59.22 g/L of 2′-FL with 95% molar conversion rate of lactose and 92% molar conversion rate of fucose in a 5 L fermenter. These findings will contribute to construct a highly efficient microbial cell factory to produce 2′-FL or other HMOs.

1. Introduction

2′-Fucosyllactose (2′-FL) is the most abundant oligosaccharide in human milk oligosaccharides (HMOs) [1]. 2′-FL has been found to have many functions, such as preventing pathogen adhesion [2], modulating the gut intestinal barrier and immune system [3,4], and promoting the growth of probiotics [5]. Based on health benefits and safety of 2′-FL, 2′-FL as novel food ingredient has been permitted in Europe, the United States, etc. [6].

2′-FL can be produced by three methods, viz. chemical/enzymatic synthesis, and whole cell biosynthesis [7]. Compared with the other two methods, the whole cell biosynthesis method is the most economical and mainstream method to synthesize 2′-FL [8]. In the biosynthesis pathway, the heterologous expressed α-1,2-fucosyltransferases transfers the fucosyl residue from guanosine 5′-diphosphate (GDP)-L-fucose to lactose to synthesize 2′-FL. GDP-L-fucose is the important donor, and it can be synthesized through the *salvage* pathway or the *de novo* pathway *in vivo* [9,10]. In the *salvage* pathway, GDP-L-fucose is produced from adenosine/guanosine triphosphate and fucose under the catalysis of fucokinase/GDP-L-fucose pyrophosphorylase (Fkp) [11]. The *de novo* pathway exists in the wide-type *E. coli*, transforming mannose-6-phosphate into GDP-L-fucose through the following four enzymes, phosphate mannosidase ManB, mannose 1-phosphate guanylyltransferase ManC, GDP-D-mannose-4,6-dehydratase Gmd and GDP-L-fucose synthase Fcl or wcaG [12]. Compared with the *salvage* pathway, the *de novo* pathway is longer, requiring more enzymatic reactions and cofactors for the 2′-FL synthesis. Therefore, the *salvage* pathway is a good approach to synthesize 2′-FL.

So far, many strategies for synthesis of 2′-FL through the *de novo* pathway have been investigated [13–19]. However, few attempts on biosynthesis of 2′-FL by the *salvage* pathway have been reported. Chin et al. have constructed the *salvage* pathway in *E. coli*, and eliminated the β-galactosidase (LacZ), fucose isomerase (FucI) and fuculose kinase (FucK) to reduce lactose and fucose degradation, leading to produce 23.1 g/L of 2′-FL [20]. The authors further eliminated another two enzymes responsible for fucose degradation, D-arabinose isomerase (AraA) and L-rhamnose isomerase (RhaA), to improve the titer of 2′-FL in engineered strain. The engineered strain finally produced 47 g/L of 2′-FL with 52% molar conversion rate of fucose [21]. In another study, a multi-enzyme cascade reaction system was designed to efficiently regenerate cofactors in the process of 2′-FL production *in vitro*, resulting in the titer of 2′-FL up to 25.88 g/L [22]. With the aim to increase the stability of pathway enzymes and prevent the intermediate diffusion, a

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Abbreviations

2′-FL	2′-Fucosyllactose
HMOs	Human milk oligosaccharides
GDP	Guanosine 5′-diphosphate
IPTG	Isopropyl β-D-thiogalactopyranoside
LB	Luria Bertani
DCW	Dry cell weights
MS	Mass spectra
HPLC	High performance liquid chromatography

Table 1
Strains used in this study.

Strains	Characteristics	Source
E. coli DH5α	F^-, *φ*80d, *lacZ*Δ*M15* Δ*(lacZYA-argF)*U169, *endA1*, *recA1*, *hsdR17*(rK^- mK^+), *supE44*, *thi-1*, *gyrA96*, *relA1*	Invitrogen (CA, USA)
E. coli BL21star (DE3)	F^-, *ompT*, *hsdSB* (r_B^- m_B^-), *gal*, *dcm*, *rne131* (DE3)	Invitrogen (CA, USA)
BLS-L	*E. coli* BL21star(DE3) Δ*lacZ*	This study
BLS-LW	BLS-L Δ*wcaJ*	This study
BLS-LWF	BLS-LW Δ*fucIK*	This study
BLS-LWFA	BLS-LWF Δ*araA*	This study
BLS-LWFR	BLS-LWF Δ*rhaA*	This study
BLS-LWFAR	BLS-LWFA Δ*rhaA*	This study
BLS01	BLS-L harboring plasmid pCOLA-PC	This study
BLS02	BLS-LW harboring plasmid pCOLA-PC	This study
BLS03	BLS-LWF harboring plasmid pCOLA-PC	This study
BLS04	BLS-LWFA harboring plasmid pCOLA-PC	This study
BLS05	BLS-LWFR harboring plasmid pCOLA-PC	This study
BLS06	BLS-LWFAR harboring plasmid pCOLA-PC	This study
BLS07	BLS-LWFAR harboring plasmid pCOLA-PL	This study
BLS08	BLS-LWFAR harboring plasmid pCOLA-PB	This study
BLS09	BLS-LWFAR harboring plasmid pCOLA-PT	This study
BLS10	BLS-LWFAR harboring plasmid pCDF-PL	This study
BLS11	BLS-LWFAR harboring plasmids pCDF-L and pET-P	This study
BLS12	BLS-LWFAR harboring plasmids pCDF-L and pRSF-P	This study
BLS13	BLS-LWFAR harboring plasmid pET-PL	This study
BLS14	BLS-LWFAR harboring plasmids pET-L and pCDF-P	This study
BLS15	BLS-LWFAR harboring plasmids pET-L and pRSF-P	This study
BLS16	BLS-LWFAR harboring plasmid pRSF-PL	This study
BLS17	BLS-LWFAR harboring plasmids pRSF-L and pCDF-P	This study
BLS18	BLS-LWFAR harboring plasmids pRSF-L and pET-P	This study
BLS19	BLS-LWFAR harboring plasmids pRSF-LD and pCDF-P	This study
BLS20	BLS-LWFAR harboring plasmids pRSF-L and pCDF-PD	This study
BLS21	BLS-LWFAR harboring plasmids pRSF-LD and pCDF-PD	This study

self-assembling multienzyme system has been designed to enhance the 2′-FL biosynthesis, and the 2′-FL titer was up to 30.5 g/L with the 66% molar conversion rate of fucose [23].

In this study, multiple combinations of synthetic biology strategies were designed to increase the titer of 2′-FL in *E. coli* BL21star(DE3) *via* the *salvage* pathway. The two genes *fkp* and *futC* were introduced into β-galactosidase-deleted *E. coli*. A series of endogenous genes related to the degradation of substrates were deleted. Different α-1,2-fucosyltransferases were also selected to improve the fucosylation of lactose. The transcriptional level of *wbgL* and *fkp* were then tuned through varying the plasmid copy number. Moreover, the spatial distribution of WbgL and Fkp was optimized through fusing with MinD C-tag. The engineered strain finally produced 59.22 g/L of 2′-FL with 95% molar conversion rate of lactose and 92% molar conversion rate of fucose in a 5 L fermenter.

2. Materials and methods

2.1. Reagents

2′-FL standard and fucose were obtained from Aladdin (Shanghai, China). Lactose was purchased from Beijing AOBOX Biotechnology Co., Ltd (Beijing, China). Molecular reagents were obtained from Vazyme (Nanjing, China). Kanamycin, ampicillin and spectinomycin were purchased from Inalco Pharmaceuticals (CA, USA). Other chemical reagents were obtained from Beijing BioDee Biotechnology Co., Ltd (Beijing, China).

2.2. Gene knock-out through CRISPR-Cas9 system

The CRISPR/Cas9 system was used to delete a series of endogenous genes, including *lacZ*, *wcaJ*, *fucI*, *fucK*, *araA*, and *rhaA* [24]. Specifically, N20 sequences, which targets the gene for editing, were designed using the online tool (https://www.benchling.com/). Plasmid pTargetF and corresponding donor DNA fragment were together electrotransformed into competent cells harboring pCas9 plasmid. After the gene was successfully deleted, the colonies were induced by isopropyl β-D-thiogalactopyranoside (IPTG) to eliminate the pTargetF plasmid and cultivated at 37 °C to eliminate the pCas plasmid. Primers used for gene editing are given in Table S1.

2.3. Plasmids and strains

The plasmids from Duet series and wild type *E. coli* BL21star(DE3) were obtained from Novagen (WI, USA). *E. coli* DH5α, used for plasmids construction, was purchased from Weidi Biotechnology Co., Ltd. (Shanghai, China). All strains and plasmids are presented in Table 1 and S2, respectively. All primers are given in Table S3.

Construction of the *salvage* pathway: Two genes *fkp* (*Bacteroides fragilis* NCTC9343; GenBank: WP_010993080.1) and *futC* (*Helicobacter pylori*; GenBank: KY499613.1) fragments were amplified by the primers *fkp*-F/R and *futC*-F/R, respectively. The plasmid fragments were amplified from pCOLADuet-1 using pCOLA-1-F/R and pCOLA-2-F/R. Four fragments were assembled using the Clone kit (Vazyme, Nanjing, China) to generate the plasmid pCOLA-PC.

Selection of different α-1,2-fucosyltransferases: To replace the *futC* in pCOLA-PC with other α-1,2-fucosyltransferase genes from different species, the gene *wbgL* (*E. coli* O126; GenBank: ADN43847.1) was amplified by the primers wbgL-F/R, and the plasmid fragment was amplified from pCOLA-PC using pCOLA-W-F/R. The recombinant plasmid pCOLA-PL was then constructed. The plasmids pCOLA-PB and pCOLA-PT were constructed by the similar way.

Optimization of the plasmid copy number: To construct the plasmid pCDF-PL, the gene fragment including *wbgL* (*E. coli* O126; GenBank: ADN43847.1) and *fkp* (*Bacteroides fragilis* NCTC9343; GenBank: WP_010993080.1) was amplified with primers PL-F/R, and ligated into the vector pCDFDuet-1, which was amplified *via* primers pCDF-1-F/R. The similar way was used to generate the following plasmids: pCDF-P, pCDF-L, pET-PL, pET-P, pET-L, pRSF-PL, pRSF-P, and pRSF-L.

Spatial distribution of WbgL and Fkp: The Mind-C tag fragment (ACTAGTGGTTCGGGATCGCCGTTGCAAGTCTTGGAGGAACAAAA-CAAAGGGATGATGGCTAAAATCAAATCATTTTTCGGTGTACGTTCC-TAACCTAGGTTTGGATCCGGCTGCTAA-CAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGC) was amplified with primers PGM-F/R, and ligated into the vector pCDFDuet-1-*fkp* (plasmid pCDF-PD). The similar way was used to generate the plasmids pRSF-LD.

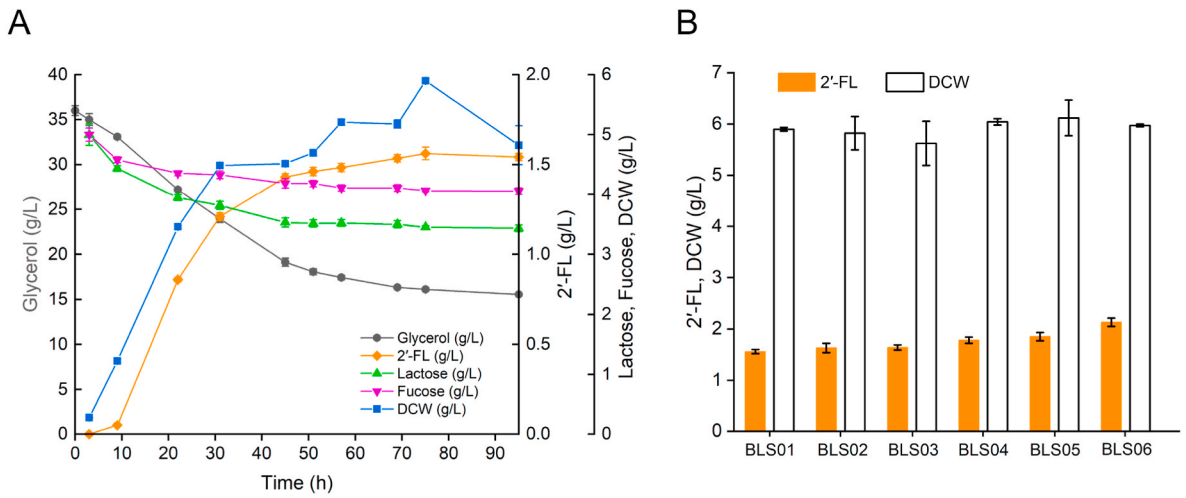

Fig. 1. Construction of engineered *E. coli* strains for 2′-FL production *via* the *salvage* pathway. (A) Substrates consumption, cell growth, and 2′-FL biosynthesis of engineered *E. coli* strain BLS01. (B) 2′-FL accumulation and cell growth of engineered *E. coli* strains BLS01 ~ 06.

2.4. Culture conditions

For construction of plasmids, *E. coli* DH5α containing corresponding plasmids were grown in Luria Bertani (LB) medium (Kanamycin, 50 μg/L; Ampicillin, 100 μg/L; Spectinomycin, 50 μg/L). All recombinant strains were incubated in LB medium for approximately 12 h at the conditions of 37 °C and 220 rpm (Shanghai Zhichu Instrument Co., Ltd. Shanghai, China). Next, 1% of seed liquid was transferred to 50 mL of M9 medium as described by the previous study [25]. When the OD_{600} value of engineered strains increased to 0.6, IPTG, lactose and fucose were added at the final concentrations of 0.2 mM, 5 g/L and 5 g/L, respectively. Then, the temperature was adjusted to 25 °C for 2′-FL production. All experiments were performed in triplicate.

Fed-batch fermentation experiment was carried on in a 5 L fermenter (BXBIO, Shanghai, China) according to the literature with minor adjustment [21]. Briefly, 150 mL of seed solution was added to 1.35 L of the defined medium. When OD_{600} value reached 15–20, lactose, fucose and IPTG were added at the final concentrations of 15 g/L, 15 g/L and 0.5 mM, respectively. The temperature was adjusted from 37 °C to 28 °C for 2′-FL production. pH-stat mode was used to add the feeding solution, composing 600 g/L glycerol. The concentrations of lactose and fucose were maintained 5–15 g/L throughout the fed-batch fermentation process. The pH value was controlled at pH 6.8 with addition of aqueous ammonia.

2.5. Analytical methods

The growth condition of engineered strains was characterized by dry cell weights (DCW) with the conversion equation ($1.0OD_{600} = 0.35$ g/L). The OD_{600} value of engineered strains was measured using an ultraviolet spectrophotometer (TU-1900, Beijing Parsee General Instrument Co. Ltd., Beijing, China). The concentrations of glycerol, 2′-FL, lactose and fucose were determined according to the previous study [23]. To confirm the production of 2′-FL, the 2′-FL standard and culture broth from the strain BLS01 were analyzed and identified by Mass spectra (MS). MS were obtained in a Bruker UltrafleXtreme MALDI-TOF instrument equipped with a 2 KHz smartbeam-II™ laser and FlashDetector™ detector at a resolution of 40,000 [26].

3. Results and discussion

3.1. Construction of the salvage pathway in E. coli

The vector pCOLA-PC (pCOLADuet-1 with *fkp* and *futC* genes) was introduced into BLS-L, generating strain BLS01 (*E. coli* BL21star(DE3)Δ*lacZ* harboring plasmid pCOLA-PC). As shown in Fig. 1A, the strain BLS01 produced 2′-FL as expected. To confirm the 2′-FL production by engineered *E. coli* strain BLS01, the 2′-FL standard and the culture broth of the strain BLS01 were performed through high performance liquid chromatography (HPLC) and MS. The standard 2′-FL was eluted with a peak at 7.847 min. Similarly, the culture broth of the strain BLS01 also had a peak at 7.847 min (Fig. S1A). The MS of the 2′-FL standard and the culture broth of the strain BLS01 were determined by positive-ion ESI MS with a peak of $[M + Na]^+$ ions at *m/z* 511.10 (Fig. S1B). The HPLC and MS results indicated that the strain BLS01 could synthesize 2′-FL. 2′-FL and DCW rapidly increased after fermentation of 9 h, and the maximal 2′-FL titer and DCW reached to 1.56 g/L and 5.91 g/L after 72 h fermentation in shake flask (Fig. 1A). Subsequently, a series of endogenous genes relate with the metabolism of fucose and GDP-L-fucose, including *wcaJ*, *fucIK*, *araA,* and *rhaA* were deleted to generate strains BLS02 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ* harboring plasmid pCOLA-PC), BLS03 (BLS02Δ*fucIK*), BLS04 (BLS03Δ*araA*), BLS05 (BLS03Δ*rhaA*) and BLS06 (BLS04Δ*rhaA*). The 2′-FL titers of BLS02 and BLS03 was almost the same, with 1.63 g/L and 1.65 g/L, respectively (Fig. 1B). The 2′-FL titers of BLS04 and BLS05 increased to 1.78 g/L and 1.85 g/L, respectively. Finally, BLS06 yielded 2.13 g/L of 2′-FL, which was significantly improved. Furthermore, the growth of these gene knockout strains was not obviously affected (Fig. 1B).

Lactose, GDP-L-fucose and fucose are the three key substrates to produce 2′-FL by the *salvage* pathway [27]. Due to the β-galactosidase (LacZ) in wild-type *E. coli* consumes lactose for growth rather than for 2′-FL production, the *lacZ* gene was deleted to increase the synthesis of 2′-FL. GDP-L-fucose can further transform into colanic acid [28]. The *wcaJ* gene involved in this process was also deleted, and the titer of 2′-FL increased 0.07 g/L. Fucose can be metabolized through the FucI and FucK in *E. coli* [29]. Thus, the *fucI* and *fucK* genes were further deleted, resulting in the slightly increased of 2′-FL (Fig. 1B). In addition to metabolizing through FucI and FucK, fucose can be isomerized by D-arabinose isomerase (AraA) and L-rhamnose isomerase (RhaA) [30,31]. Moreover, inactivation of *araA* gene and *rhaA* gene in *E. coli* can block the metabolism of fucose and increase 2′-FL production [21,23]. Therefore, after the elimination of these endogenous genes on competitive pathways, the titer of 2′-FL had been significantly improved by 37% in this study.

3.2. Effect of different α-1,2-fucosyltransferases on 2′-FL biosynthesis

Three α-1,2-fucosyltransferases from different sources (WbgL,

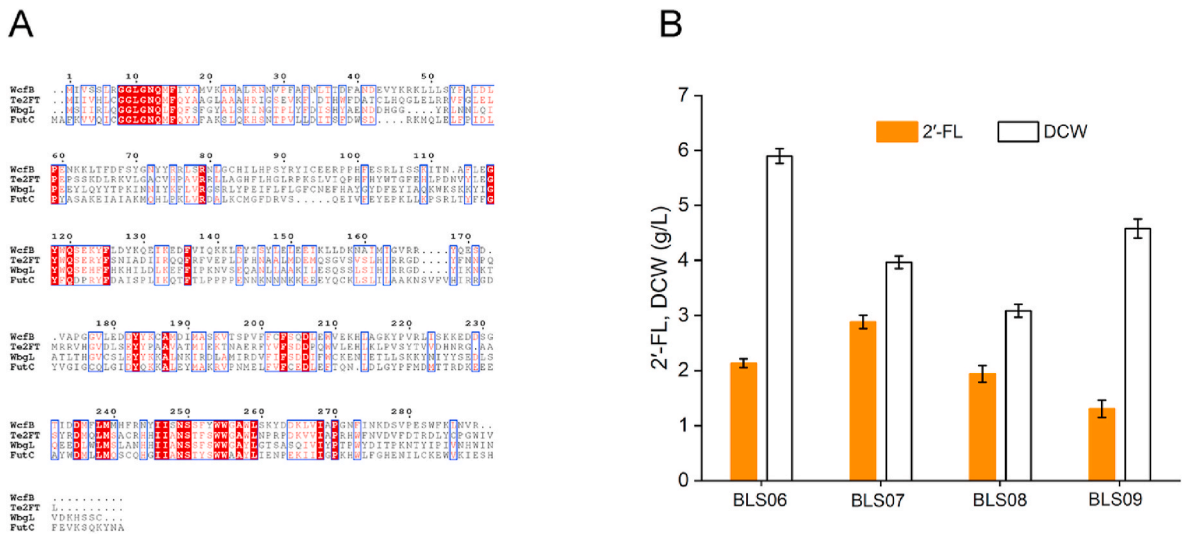

Fig. 2. Effect of different α-1,2-fucosyltransferases on the 2′-FL titer and cell growth in engineered *E. coli* strains. (A) Multiple sequence alignments of four α-1,2-fucosyltransferases from different microorganisms. FutC: α-1,2-fucosyltransferase from *Helicobacter pylori* (GenBank: KY499613); WbgL: α-1,2-fucosyltransferase from *E. coli* O126 (GenBank: ADN43847.1); WcfB: α-1,2-fucosyltransferase from *Bacteroides fragilis* NCTC9343 (GenBank: WP_005817145.1); Te2FT: α-1,2-fucosyltransferase from *Thermosynechococcus vestitus* (GenBank: WP_011056838.1). Strictly conserved residues are indicated by red background, and highly conserved residues are indicated by blue box background. (B) 2′-FL accumulation and cell growth of engineered *E. coli* strains BLS06 ~ 09.

GenBank: ADN43847.1; WcfB, GenBank: WP_005817145.1; Te2FT, GenBank: WP_011056838.1) were selected to compare the 2′-FL biosynthesis ability, WbgL, WcfB and Te2FT exhibited 27.4%, 29.0%, and 30.3% of amino acid sequence identity with FutC, respectively. WbgL shared 27.7% and 29.5% of amino acid sequence identity with WcfB and Te2FT, respectively. WcfB displayed 33.6% sequence identity with Te2FT (Fig. 2A and Table S4). Compared with BLS06 (harboring FutC), BLS07 (harboring WbgL) produced the higher 2′-FL titer, up to 2.88 g/L. The other two strains, BLS08 (harboring WcfB) and BLS09 (harboring Te2FT) produced the lower 2′-FL titers of 1.94 and 1.31 g/L, respectively (Fig. 2B).

α-1,2-Fucosyltransferase, the key enzyme, is responsible for the formation of 2′-FL. A suitable α-1,2-fucosyltransferase is very important to improve 2′-FL yield [14]. Huang et al. identified the best candidate FutC among twelve α-1,2-fucosyltransferases that achieved the maximal yield of 2′-FL in *E. coli* [14]. Seydametova et al. reported that the Te2FT from *Thermosynechococcus elongatus* was selected through ten α-1,2-fucosyltransferase candidates could produce 0.49 g/L 2′-FL in engineered *E. coli*, which was comparable to the yields previously reported for α-1, 2-fucosyltransferases FutC and WcfB [32]. The α-1,2-fucosyltransferase FutBc was successfully expressed in *Saccharomyces cerevisiae,* and the 2′-FL titer reached to 2.54 g/L, which is a 1.8-fold improvement compared with the frequently-used FutC [33]. Another α-1,2-fucosyltransferase WbgL showed a preference for β4-linked galactose substrates, producing high titer of 2′-FL in *E. coli* [15,34]. In this study, WbgL displayed a relatively high fucosylation of lactose (Fig. 2B). It has been reported that WbgL has a higher specific activity than that of FutC [34]. Thus, the BLS07 strain was used for further optimization.

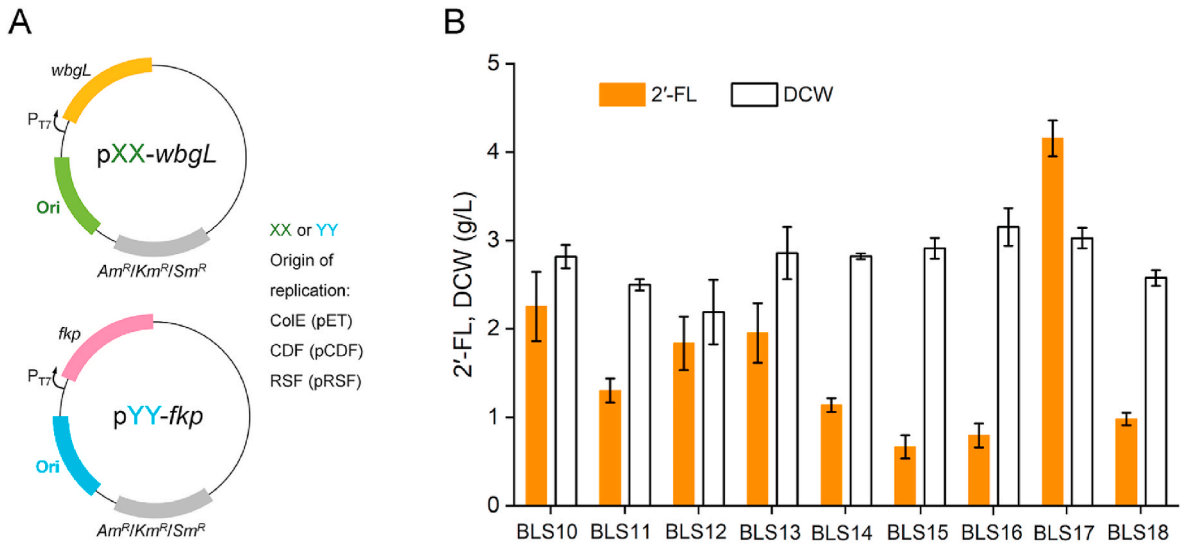

Fig. 3. Effect of optimizing the plasmid copy number on the 2′-FL accumulations and cell growth in engineered *E. coli* strains. (A) Strategies of fine tuning of 2′-FL synthesis by optimizing the plasmid copy number. The expression of *wbgL* or *fkp* was by the three Duet series plasmids with dual T7 promoters. P_{T7}, T7 promoter; Ori, origin of replication; Am^R, apramycin-resistance gene; Km^R, kanamycin-resistance gene; Sm^R, streptomycin/spectinomycin-resistance gene. (B) 2′-FL accumulation and cell growth of engineered *E. coli* strains BLS10 ~ 18.

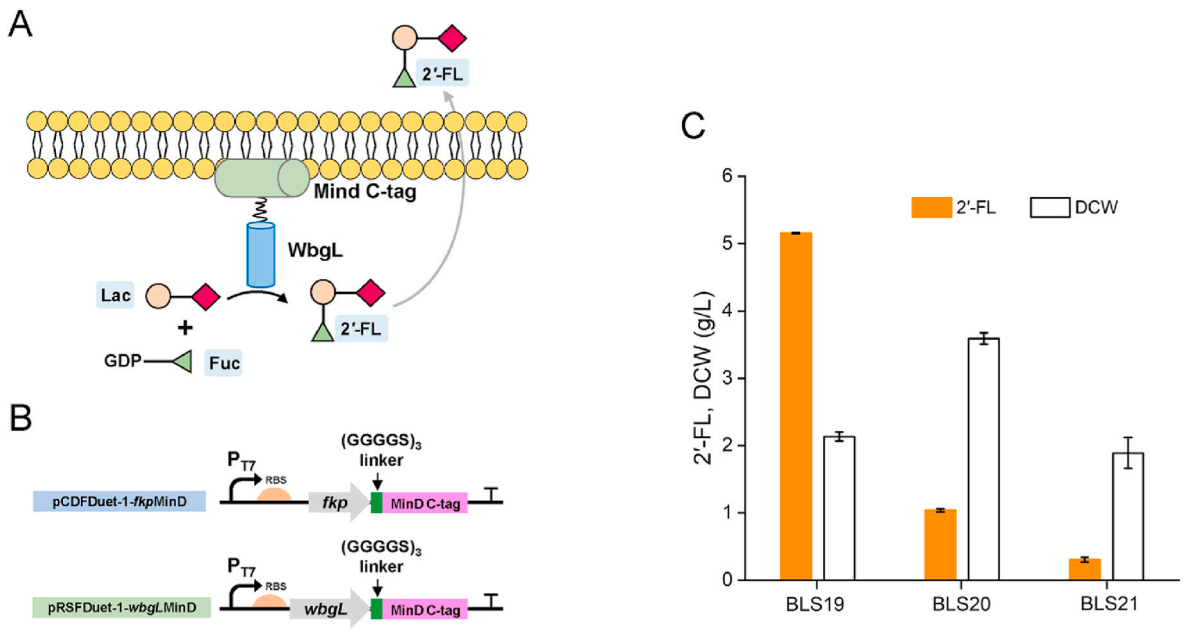

Fig. 4. Effect of spatial distribution of pathway enzymes on the 2′-FL synthesis and cell growth in engineered *E. coli* strains. (A) Schematic of fusing MinD C-tag to Fkp or WbgL. (B) Schematic of plasmids containing *wbgL* and *fkp* genes fused with MinD C-tag. (C) 2′-FL synthesis and cell growth of engineered *E. coli* strains BLS19 ~ 21.

3.3. Tuning of 2′-FL biosynthesis by optimizing the copy number of plasmid

Three different copy number of plasmids (pRSFDuet-1, high copy number; pETDuet-1, medium copy number; pCDFDuet-1, low copy number) were used to tune the metabolic flux in engineered strains. The *wbgL* gene and *fkp* gene were comminated in different plasmids, resulting in nine *E. coli* strains, BLS10 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmid pCDF-PL), BLS11 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmids pCDF-L and pET-P), BLS12 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmids pCDF-L and pRSF-P), BLS13 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmid pET-PL), BLS14 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmids pET-L and pCDF-P), BLS15 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmids pET-L and pRSF-P), BLS16 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmid pRSF-PL), BLS17 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmids pRSF-L and pCDF-P) and BLS18 (*E. coli* BL21star(DE3)Δ*lacZ*Δ*wcaJ*Δ*fucIK*Δ*araA*Δ*rhaA* harboring plasmids pRSF-L and pET-P) (Fig. 3A). BLS17 yielded the maximal 2′-FL titer among the nine strains, up to 4.16 g/L. The lowest 2′-FL titer was observed for BLS15, only 0.66 g/L. The 2′-FL titer of other seven strains ranged from 0.80 g/L to 2.25 g/L. BLS16 containing pRSFDuet-1 plasmid to express two genes *wbgl* and *fkp*, produced 0.80 g/L of 2′-FL. While pETDuet-1 and pRSFDuet-1 were used to express *fkp* and *wbgL*, the 2′-FL titer increased to 0.98 g/L for BLS18. When the expression level of *fkp* using pCDFDuet-1 plasmid was further reduced, BLS17 produced 4.16 g/L 2′-FL, which was 5.2 times that of BLS16 (Fig. 3B).

In order to synthesize the target products at high levels, the genes encoding the corresponding enzymes should be expressed at appropriately balanced levels to avoid the accumulation of toxic intermediate products that result in growth inhibition or low target product yields [35]. Variation of the plasmid copy number is a common approach to achieve this target, and successfully applies to improving fatty acids, hydroxytyrosol and 2′-FL production [36–38]. In this study, the titer of 2′-FL was significantly improved by 44% through varying the plasmid copy number. In the optimal plasmid combination, pCDFDuet-1 and pRSFDuet-1 were used to express *fkp* and *wbgL*, respectively (Fig. 3B). The enzyme activity of α-1,2-fucosyltransferases, including WbgL, is not very high, and hinders the production of 2′-FL [32,39]. Using high copy number plasmid to express α-1,2-fucosyltransferase gene is an effective way to resolve this problem. Previous study showed that the α-1, 2-fucosyltransferase *fucT2* expressed at a relatively high level generating the highest titer of 2′-FL [37]. In summary, *fkp* expressed at a lower level and *wbgl* expressed at a higher level favored the 2′-FL synthesis.

3.4. Spatial distribution of the α-1,2-fucosyltransferase WbgL

Spatial distribution of enzymes in the synthesis pathway is an effective strategy to improve the target production [40]. In this section, we used MinD C-tag method to optimize the spatial distribution of pathway enzymes and further improved 2′-FL synthesis. The effect of fusing MinD C-tag to Fkp or WbgL on 2′-FL production was evaluated (Fig. 4A). MinD C-tag was fused to the C-terminus of WbgL and Fkp, respectively, to generate plasmids pRSF-LD and pCDF-PD (Fig. 4B). Then pCDF-P/pRSF-LD, pCDF-PD/pRSF-L, and pCDF-PD/pRSF-LD were transformed into BLS-LWFAR to construct strains BLS19, BLS20 and BLS21. BLS19 produced 5.14 g/L of 2′-FL, which was approximately 24% higher than that of BLS17. In addition, the cell growth of BLS19 and BLS21 were low compared with BLS17 and BLS20 (Fig. 4C).

MinD is a protein tag, which locates in the cell membrane by 8–12 residue C-terminal amino acids [40,41]. Fusing MinD C-tag with other proteins can promote the secretion of target product by mitigating the intracellular toxicity as well as promoting the catalytic process [41]. Wan et al. had designed a self-assembling multienzyme system based on peptide pairs RIDD/RIAD to improve the 2′-FL biosynthesis [23]. RIAD was separately fused with Fkp, which did not increase obviously the production of 2′-FL. When RIDD was fused with FutC, the titer of 2′-FL significantly increased. The major reason was that the RIDD facilitated the stability of FutC [23]. More recently, Chen et al. reported that combination of two self-assembly methods, peptide pairs RIDD/RIAD and protein tag pairs Spycatcher/SpyTag or PDZ/PDZlig, to fulfil spatial organization of the 2′-FL *de novo* synthesis pathway enzymes [42]. As a result, the 2′-FL production showed 2.1 times increase. In this study, only MinD C-tag fused to the C-terminus of WbgL could enhance the titer of 2′-FL (Fig. 4C). In this situation, the stability of WbgL might be promoted

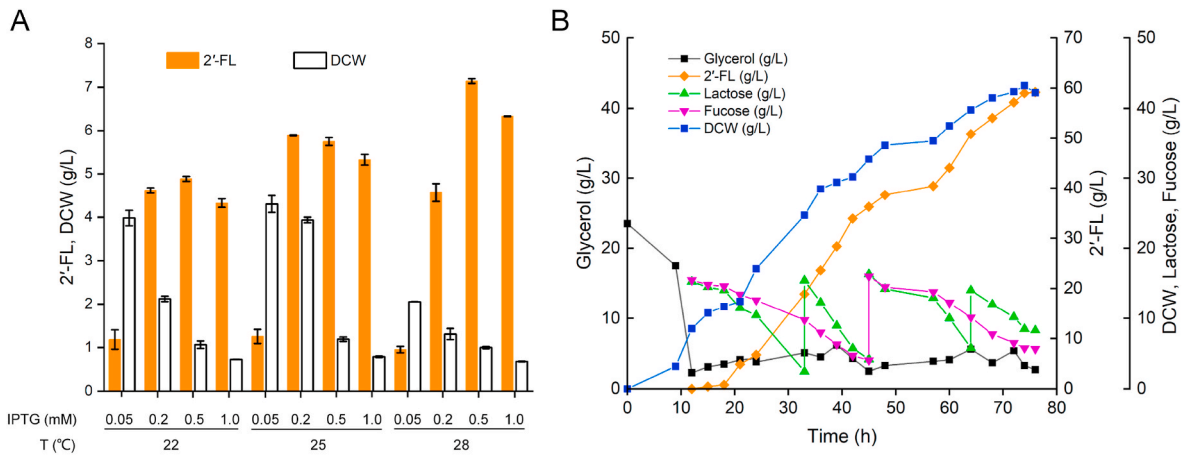

Fig. 5. Effective production of 2′-FL by the engineered *E. coli* strain BLS19. (A) Effect of various culture conditions on 2′-FL synthesis by BLS19. (B) The 2′-FL synthesis of the strain BLS19 in a 5 L bioreactor.

Table 2
Comparison of 2′-FL synthesis capacity by BLS19 with other studies.

Strain	Pathway	2′-FL (g/L)	Productivity (g/L/h)	Molar conversion rate (%)	References
E. coli BL21star(DE3)	*Salvage*	59.22 (Fed-batch)	0.78	92[a]; 95[b]	This study
E. coli BL21star(DE3)	*Salvage*	23.1 (Fed-batch)	0.39	36[a]; 37[b]	[20]
E. coli BL21star(DE3)	*Salvage*	47 (Fed-batch)	0.60	52[a]	[21]
E. coli BL21(DE3)	*Salvage*	30.5 (Fed-batch)	0.48	66[a]; 49[b]	[23]

Note.
[a] Molar conversion rate of 2′-FL to consumed fucose.
[b] Molar conversion rate of 2′-FL to consumed lactose.

by fusing to MinD C-tag and further improved the 2′-FL synthesis. Our finding suggested that fusing MinD C-tag at C-terminus of the α-1, 2-fucosyltransferase WbgL could improve the 2′-FL synthesis.

3.5. Effective production of 2′-FL in a 5 L bioreactor

The fermentation conditions including induction temperature and IPTG concentration were optimized to further improve the 2′-FL biosynthesis by the strain BLS19 (Fig. 5A). The fermentation conditions were optimized to be 28 °C and 0.5 mM, respectively. Under the optimal fermentation conditions, 2′-FL titer reached to 7.13 g/L and DCW was 1.01 g/L (Fig. 5A). To scale-up production of 2′-FL, fed-batch cultivation experiment of strain BLS19 was conducted in a 5 L fermenter. As shown in Fig. 5B, IPTG, fucose and lactose were added when the OD_{600} value reached 15–20 after 12 h of growth. The 2′-FL titer rapidly increased after fermentation for 18 h. The highest DCW was up to 43.22 g/L after fermentation for 74 h. The maximal 2′-FL titer reached 59.22 g/L after fermentation for 76 h, which was 8.3 times more than that in shake flasks. The productivity of 2′-FL was determined to be 0.78 g/L/h. Lactose and fucose conversion yields of 2′-FL were calculated to be 95% and 92%, respectively.

E. coli is the most used strain for 2′-FL production owing to its clear genetic background and mature gene editing tools [43]. The 2′-FL yield capacity of engineered *E. coli via* the *salvage* pathway during past years is summarized in Table 2. The titer of 2′-FL ranged from 23.1 g/L to 47 g/L with the productivity ranged from 0.39 g/L/h to 0.6 g/L/h. Jung et al. reported that overexpression of genes *futC* and *fkp*, and deletion of genes *lacZ*, *fucIK*, *araA* and *rhaA* allowed engineered *E. coli* to produce 47 g/L of 2′-FL [21]. This study significantly improved 2′-FL titer through selecting a suitable α-1,2-fucosyltransferase, fine tuning genes expression level of *wbgL* and *fkp* and enhancing spatial distribution of WbgL, and the final 2′-FL titer was up to 59.22 g/L in fed-batch fermentation (Table 2), which is significantly higher than other reported above. Moreover, the conversions of fucose and lactose were remarkably improved. In this study, all known genes capable of degrading fucose were knocked out. In addition, fucokinase/GDP-fucose pyrophosphorylase (Fkp) and α-1,2-fucosyltransferase (WbgL) were expressed in the appropriate plasmid copy numbers. These strategies significantly increased the fucose conversion in the process of 2′-FL synthesis. The final strain showed the highest titer, productivity, lactose and fucose conversion yields of 2′-FL *via* the *salvage* pathway among the reported engineered *E. coli* strains.

4. Conclusions

In summary, an engineered *E. coli* strain for utilization of lactose and fucose to synthesize 2′-FL was established using multi combinatorial metabolic engineering strategies. It was demonstrated that spatial organization of pathway enzymes was an effective strategy to improve the production of 2′-FL. In addition, the production of 2′-FL and the conversion efficiency of substrates were significantly increased by blocking the bypass metabolism, selecting the suitable α-1,2-fucosyltransferase, and tuning the expression level of pathway enzyme genes. The engineered *E. coli* strain BLS19 produced 59.22 g/L 2′-FL with 95% molar conversion rate of lactose and 92% molar conversion rate of fucose in a 5 L bioreactor. This study will contribute to improving the target product titer and the conversion of substrates in the process of producing 2′-FL or other HMOs.

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Data availability

Data will be made available on request

CRediT authorship contribution statement

Shanquan Liang: Experimental design, Experimental operation, Data collection, Writing - original manuscripts. **Zi He:** Formal analysis, Data Formal analysis. **Dan Liu:** Methodology, Data curation. **Shaoqing Yang:** Writing – review, Project management. **Qiaojuan Yan:** Writing – review, Project management. **Zhengqiang Jiang:** Experimental design, Writing – review & editing, Supervision, Validation.

Declaration of competing interest

All of the authors mutually agree for submitting this manuscript to Synthetic and Systems Biotechnology and declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.01.001.

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