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Engineered *Bacillus subtilis* for the de novo production of 2'-fucosyllactose

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Abstract

Background: The most abundant human milk oligosaccharide in breast milk, 2'-fucosyllactose (2'-FL), has been approved as an additive to infant formula due to its multifarious nutraceutical and pharmaceutical functions in promoting neonate health. However, the low efficiency of de novo synthesis limits the cost-efficient bioproduction of 2'-FL.

Results: This study achieved 2'-FL de novo synthesis in a generally recognized as safe (GRAS) strain *Bacillus subtilis*. First, a de novo biosynthetic pathway for 2'-FL was introduced by expressing the *manB*, *manC*, *gmd*, *wcaG*, and *futC* genes from *Escherichia coli* and *Helicobacter pylori* in *B. subtilis*, resulting in 2'-FL production of 1.12 g/L. Subsequently, a 2'-FL titer of 2.57 g/L was obtained by reducing the competitive lactose consumption, increasing the regeneration of the cofactor guanosine-5'-triphosphate (GTP), and enhancing the supply of the precursor mannose-6-phosphate (M6P). By replacing the native promoter of endogenous *manA* gene (encoding M6P isomerase) with a constitutive promoter P7, the 2'-FL titer in shake flask reached 18.27 g/L. The finally engineered strain BS21 could produce 88.3 g/L 2'-FL with a yield of 0.61 g/g lactose in a 3-L bioreactor, without the addition of antibiotics and chemical inducers.

Conclusions: The efficient de novo synthesis of 2'-FL can be achieved by the engineered *B. subtilis*, paving the way for the large-scale bioproduction of 2'-FL titer in the future.

Keywords: *Bacillus subtilis*, De novo pathway, 2'-fucosyllactose, GDP-L-fucose

Introduction

Studies on the intestinal microbiome in recent years have found that the composition of intestinal microorganisms impacted on human health [1–3]. Breast milk is an important food for infants; its special ingredients, such as oligosaccharides, antibodies, and vitamins, greatly influence the composition of the infant's gut microbiome, thus affecting the infant's health. Human milk oligosaccharides (HMOs) are the third most abundant solid substance in breast milk, following lactose and fat [4]. 2'-Fucosyllactose (2'-FL) is the most abundant

HMOs, accounting for ~30% of the total HMOs [5, 6], and is composed of L-fucose, D-galactose, and D-glucose units. 2'-FL has gained much attention in recent years given its bioactive nature [5], which offers important health and economic benefits. Although indigestible to human infants [7], it plays a crucial role in developing the immune system, regulating the intestinal flora, and suppressing pathogenic infections [8–12]. These known bioactivities and their potential value make HMOs, especially 2'-FL, an attractive research target for preventing or treating diseases in infants and adults. To date, 2'-FL has been approved as a prebiotic for use in infant formula by the U.S. Food and Drug Administration, the European Food Safety Authority, and the Australian Therapeutic Goods Administration.

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Currently, two promising routes are available to produce 2'-FL: chemical synthesis and biosynthesis [13, 14]. The chemical synthesis of 2'-FL is accomplished through multistep protection and deprotection reactions. This method is inefficient and uses toxic chemicals, rendering the chemical method unfit for common use [14]. The reaction steps for 2'-FL biosynthesis are simpler and more environmentally friendly than its chemical synthesis. The two known 2'-FL biosynthetic pathways are the salvage and de novo synthesis pathway. Both pathways are involved in lactose fucosylation (α -1,2-fucosyltransferase; FutC), the difference being in the synthesis pathway of GDP-L-fucose. The salvage pathway was first discovered in *Bacteroides fragilis*. It catalyzes GDP-L-fucose production from the substrate fucose by the fucokinase/GDP-L-fucose phosphorylase [15]. The de novo pathway is part of mannose metabolism, which starts from mannose-6-phosphate (M6P) and is catalyzed by four enzymes, namely, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose-4,6-dehydratase, and GDP-L-fucose synthase, to generate GDP-L-fucose (Fig. 1). The de novo pathway utilizes a low-cost carbon source, such as glucose and sucrose, instead of fucose; it is more economical and industrially applicable. *Escherichia coli*, *Saccharomyces cerevisiae*, and *Corynebacterium glutamicum* have been engineered to produce 2'-FL using the de novo pathway [16–18]. Besides the abovementioned microorganisms, *Bacillus subtilis* has also been selected as a host for producing

functional nutraceuticals due to its generally recognized as safe (GRAS) status [19]. In previous studies, we have achieved the biosynthesis of 2'-FL in the engineered *B. subtilis* by using the salvage pathway [20]. However, there are few reports about the de novo biosynthesis of 2'-FL in the engineered *B. subtilis* strains.

In this study, we achieved 2'-FL de novo synthesis by introducing the de novo pathway of GDP-fucose and FutC in *B. subtilis*. First, *B. subtilis* was tested for 2'-FL tolerance to determine whether this host has the potential to produce high concentrations of 2'-FL. Then, the heterologous 2'-FL de novo synthesis pathway derived from *E. coli* and *Helicobacter pylori* was introduced in *B. subtilis*, and a 2'-FL titer of 1.12 g/L was obtained. Next, the supply of the precursors lactose and M6P and the cofactor (guanosine-5'-triphosphate, GTP) was increased, and the titer was increased by 129%. By optimizing the promoter of M6P isomerase, the 2'-FL titer of 18.27 g/L was obtained in the shake flask using sucrose and lactose as carbon sources. Finally, in a 3-L bioreactor, the 2'-FL titer of the finally engineered strain BS21 reached 88.3 g/L, the highest 2'-FL titer reported so far.

Methods

Plasmids, strains, and culture conditions

The strains used in this study are listed in Table 1. All plasmid constructions were performed using *E. coli* DH5 α . The engineered BS strain (*B. subtilis*-P_{xyLA}-comK::comK) was used as a host strain for the heterologous synthesis

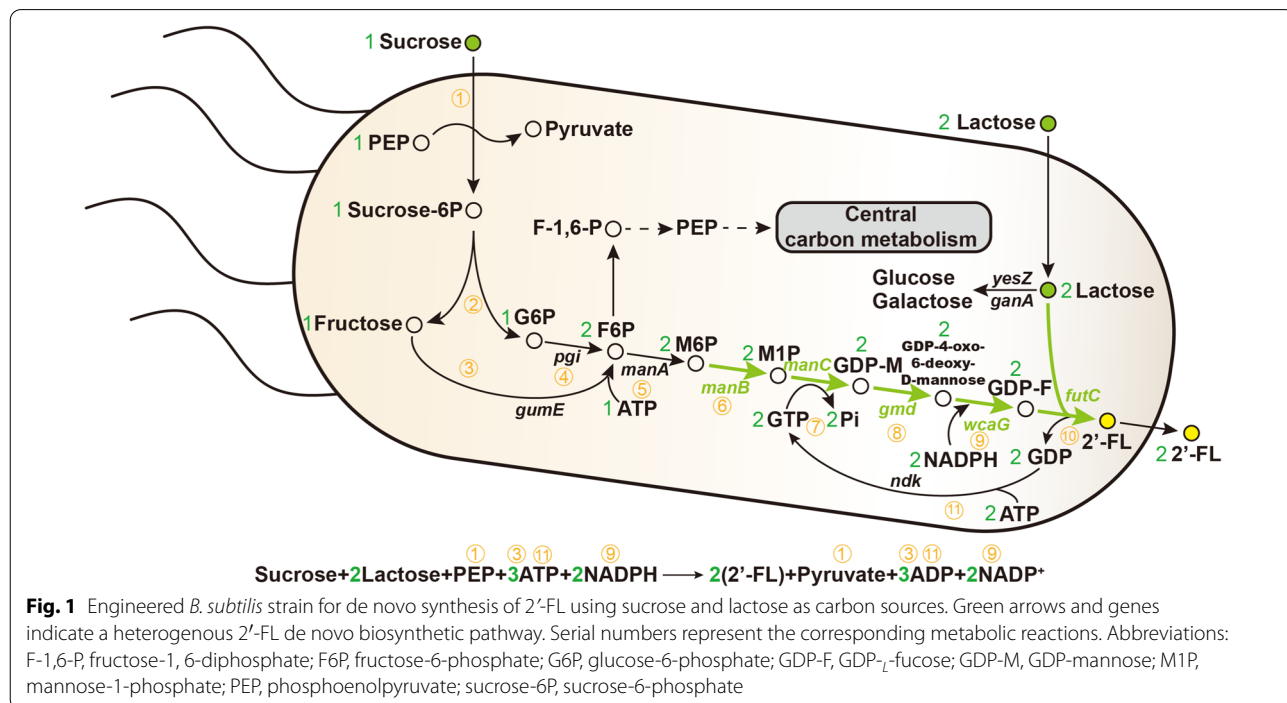


Table 1 Strains used in this study

Strains	Characterization	Source
<i>B. subtilis</i> 168	Standard strain, starting strain	lab stock
<i>E. coli</i> DH5α	Cloning host	lab stock
<i>E. coli</i> BL21 (DE3)	Standard strain	lab stock
BS	<i>B. subtilis</i> 168, P _{xyIA} - <i>comK</i> :: <i>comK</i>	lab stock
BS0	BS, P ₄₃ - <i>manB</i> , P ₄₃ - <i>manC</i> , P ₄₃ - <i>gmd</i> , P ₄₃ - <i>wcaG</i> , P ₄₃ - <i>futC</i>	this work
BS-MB	BS0-pP43NMK- <i>manB</i> -His	this work
BS-MC	BS0-pP43NMK- <i>manC</i> -His	this work
BS-GD	BS0-pP43NMK- <i>gmd</i> -His	this work
BS-WG	BS0-pP43NMK- <i>wcaG</i> -His	this work
BS1	BS0, P ₄₃ - <i>manB</i> , P ₄₃ - <i>gmd</i> , P ₄₃ - <i>wcaG</i> , P ₄₃ - <i>futC</i>	this work
BS2	BS1, P _{xyIA} - <i>manC</i>	this work
BS3	BS2, Δ <i>yesZ</i>	this work
BS4	BS2, Δ <i>ganA</i>	this work
BS5	BS2, Δ <i>yesZ</i> , Δ <i>ganA</i>	this work
BS6	BS5, P _{native} - <i>ndk</i>	this work
BS7	BS5, P _{xyIA} - <i>ndk</i>	this work
BS8	BS5, P _{hbs} - <i>ndk</i>	this work
BS9	BS5, P ₄₃ - <i>ndk</i>	this work
BS10	BS8, P _{native} - <i>yvyI</i>	this work
BS11	BS8, P ₄₃ - <i>yvyI</i>	this work
BS12	BS8, P _{native} - <i>manA</i>	this work
BS13	BS8, P ₄₃ - <i>manA</i>	this work
BS14	BS12, P ₁ - <i>manC</i> :: P _{xyIA} - <i>manC</i>	this work
BS15	BS12, P ₂ - <i>manC</i> :: P _{xyIA} - <i>manC</i>	this work
BS16	BS12, P ₃ - <i>manC</i> :: P _{xyIA} - <i>manC</i>	this work
BS17	BS12, P ₄ - <i>manC</i> :: P _{xyIA} - <i>manC</i>	this work
BS18	BS12, P ₅ - <i>manC</i> :: P _{xyIA} - <i>manC</i>	this work
BS19	BS12, P ₆ - <i>manC</i> :: P _{xyIA} - <i>manC</i>	this work
BS20	BS12, P ₇ - <i>manC</i> :: P _{xyIA} - <i>manC</i>	this work
BS21	BS20, P ₇ - <i>manA</i> :: P _{native} - <i>manA</i>	this work

of 2'-FL. Luria–Bertani (LB) medium (10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) was used to culture *E. coli* DH5α and the engineered *B. subtilis*. The minimal medium with the following composition was used: 17.1 g/L Na₂HPO₄·12H₂O, 3 g/L KH₂PO₄, 1 g/L NH₄Cl, 0.5 g/L NaCl, 0.0001 M CaCl₂, 0.001 M MgSO₄, 0.005 mM FeCl₃, 1 mg/L MnSO₄·4H₂O, 1.7 mg/L ZnCl₂, 0.43 mg/L CuCl₂·2H₂O, 0.6 mg/L CoCl₂·6H₂O, 0.6 mg/L Na₂MoO₄·2H₂O. When needed, antibiotics were added as follows: kanamycin 50 μg/mL, chloramphenicol 5 μg/mL, ampicillin 100 μg/mL. To cultivate *B. subtilis* or *E. coli* strains, a single colony was inoculated in a shake tube with LB medium, as described earlier, at 37 °C and 220 rpm for 8–10 h. A Competent Cell Preparation Kit (Takara Biomedical Technology, Beijing, China) was used to prepare *E. coli* DH5α competent cells. A previously reported method was followed for the preparation

of *B. subtilis* competent cells [20]. To examine the tolerance of *B. subtilis* to 2'-FL, *B. subtilis* was cultured in 200 μL of LB media containing different concentrations of 2'-FL (0 g/L, 10 g/L, 20 g/L and 30 g/L) in 96-well plates (Corning 3603) at 37 °C and 750 rpm. The optical density (OD₆₀₀) was determined at 12-h intervals.

Plasmid construction and DNA manipulation

All plasmids are listed in Additional file 1: Table S1, and the primers and promoters are listed in Additional file 1: Table S2. A CRISPR/Cpf1 genome editing approach was used to perform gene knockout or overexpression in *B. subtilis*, as reported previously [21]. First, the pcrF19NM plasmid was digested using *Bsa*I. T4 DNA ligase was added to connect the crRNA fragment with linearized pcrF19NM plasmid to construct a gene-editing plasmid. In addition, the plasmid with gene-editing DNA

fragments was constructed using the Seamless Cloning Kit (Beyotime Biotechnology, Shanghai, China) according to the manufacturer's instructions. The plasmids of pHT-XCR6 and pcrF19NM-XXX (XXX is the name of the corresponding gene, such as *manB*, *manC*, *gmd*, *wcaG*, and *futC*) were sequentially transformed into *B. subtilis*, and DNA modification was performed. The validated positive colony was inoculated into the LB medium containing 0.005% sodium dodecyl sulfate (SDS) for plasmid curing. Finally, plasmid-free strains were obtained through antibiotic screening. The specific genome integration sites are as follows: *manB* gene was integrated between the *yqiG* and *spo0A* genes, and the *manC* gene was integrated between the *yxkC* and *galE*; the *gmd* gene was integrated between the *ybcI* and *ybzH*; the *wcaG* gene was integrated between the *ybbU* and *alkA*; and the *futC* gene was integrated between the *rpsD* and *tyrS* genes.

Analytical methods

The 2'-FL and xylose concentrations were analyzed using high-performance liquid chromatography (HPLC) system (Agilent Technologies 1260 Series) equipped with a Rezex ROA Organic Acid H+ (8%) Column (Phenomenex, Torrance, CA, USA). The column and the refractive index detector temperature were set at 50 °C, and the mobile phase was 10 mM H₂SO₄ at a flow rate of 0.5 mL/min at 40 °C. The sucrose and lactose concentrations were analyzed using the HPLC system (Agilent Technologies 1260 Series) equipped with an XBridge BEH Amide Column (Waters, Milford, MA, USA). The column and the refractive index detector temperature were set at 35 °C, and the mobile phase was 75% acetonitrile at a flow rate of 1.0 mL/min at 35 °C. The liquid chromatography/mass spectrometry (LC/MS) matrix-assisted laser desorption/ionization time-of-flight system (Waters) was used to identify 2'-FL according to a previous study [22], and the mass range was 100–700 m/z. The relative transcription levels of the regulated genes were determined by quantitative real-time polymerase chain reaction (qRT-PCR) as described previously [23], and the *rpsJ* gene was used as the internal standard [24]. All experiments were independently carried out at least thrice.

Batch fermentation in shake flasks

During shake flask fermentation, the fermentation medium with the following composition was used: 6 g/L tryptone, 12 g/L yeast extract, 12.5 g/L K₂HPO₄·3H₂O, 2.5 g/L KH₂PO₄, and 10 mL/L trace metal solution (composition: 4 g/L FeSO₄·7H₂O, 4 g/L CaCl₂, 1 g/L MnSO₄·H₂O, 0.2 g/L NaMoO₄·2H₂O, 0.2 g/L ZnSO₄·7H₂O, 0.1 g/L AlCl₃·6H₂O, 0.1 g/L CuCl₂·2H₂O, and 0.05 g/L H₃BO₄). Sucrose and lactose were sterilized separately and added to the sterilized shake flask to a

final concentration of 20 and 10 g/L (changed to 80 and 20 g/L after optimization), respectively. If the induction of the xylose promoter P_{xyLA} was required, 20 g/L xylose was added as an inducer. *B. subtilis* strains were cultivated on LB solid culture medium for 10–12 h at 37 °C, and a single colony was inoculated into 20 mL liquid LB medium in 250 mL shake flasks at 37 °C and 220 rpm for 10–12 h. The seed cultures were further inoculated into 30 mL of the fermentation medium at the rate of 10% in a 250 mL baffled flask at 37 °C and 220 rpm for 72 h, and three replicates were set for each strain. Sampling was performed every 12 h for OD₆₀₀, 2'-FL, carbon source, and by-products measurements.

Fed-batch culture in 3-L bioreactor

The fermentation medium used for the fed-batch culture consisted of 24 g/L yeast extract, 24 g/L tryptone, 13.1 g/L K₂HPO₄·3H₂O, 3 g/L KH₂PO₄, 6.7 g/L urea and 10 mL/L trace metal solution (composition: 4 g/L FeSO₄·7H₂O, 4 g/L CaCl₂, 1 g/L MnSO₄·H₂O, 0.2 g/L NaMoO₄·2H₂O, 0.2 g/L ZnSO₄·7H₂O, 0.1 g/L AlCl₃·6H₂O, 0.1 g/L CuCl₂·2H₂O, and 0.05 g/L H₃BO₄). Seed culture was carried out in baffled 500 mL shake flasks containing 75 mL LB medium at 37 °C with shaking at 220 rpm for 12 h. The seed culture (75 mL) was inoculated into a 3-L bioreactor (T&J Bioengineering Co., Ltd., Shanghai, China) with an initial 1.5 L fermentation medium. The pH was kept at 6.0–6.5 by adding 29% NH₃·H₂O, and the temperature was maintained at 37 °C. The aeration rate and the agitation speed were 2.0 vvm and 1000 rpm, respectively. Sucrose and lactose were maintained at 10–30 g/L and 10–20 g/L by feeding concentrated sucrose (800 g/L) and lactose (300 g/L), respectively.

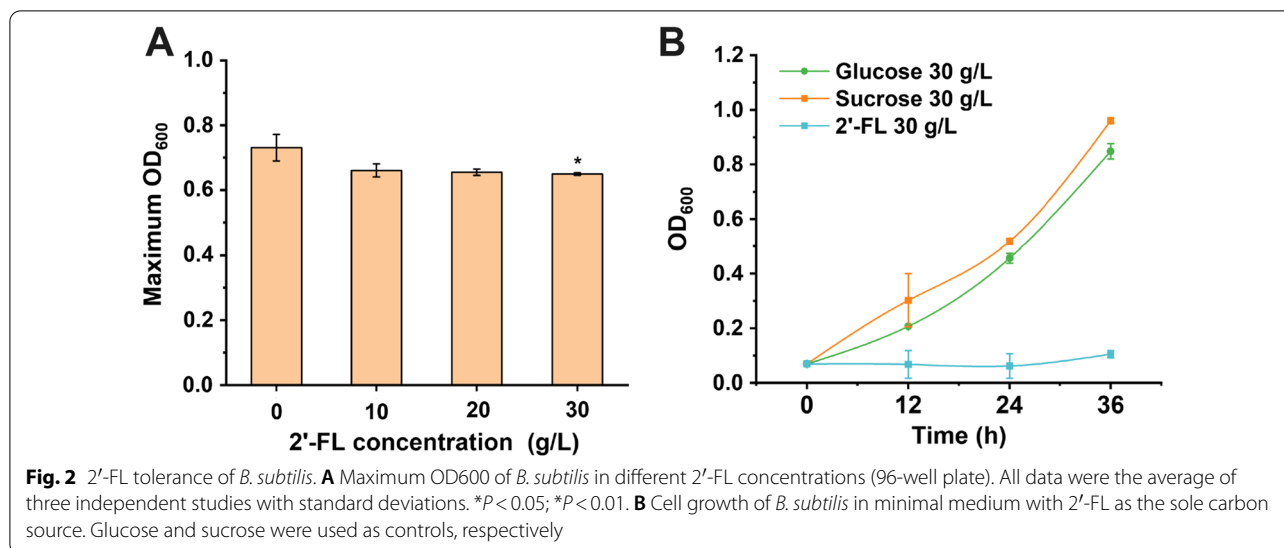
Statistical analysis

All data were the average of three independent studies with standard deviations. The * and ** indicate P < 0.05 and P < 0.01 relative to control, respectively.

Results and discussion

2'-FL tolerance of the *B. subtilis* strain

Previous studies indicated that 2'-FL had growth-inhibiting and adhesion-reducing effects on certain microorganisms [25, 26], and thereby the tolerance of *B. subtilis* to 2'-FL needs to be examined. *B. subtilis* was cultured in LB medium containing different concentrations of 2'-FL with 96-well plates. The maximum OD₆₀₀ of all experimental groups added with 10, 20, and 30 g/L 2'-FL decreased by 9.62%, 10.40%, and 11.17%, respectively (Fig. 2A). Although the maximum OD₆₀₀ was slightly lower than that in the control group without 2'-FL addition, it did not significantly decrease with higher 2'-FL concentrations. In order to identify whether the tolerance



of *B. subtilis* to 2'-FL comes from its ability to utilize 2'-FL, *B. subtilis* was cultured in minimal medium with 2'-FL as the sole carbon source, and the minimal medium with glucose or sucrose as the sole carbon source was used as the control. As shown in Fig. 2B, *B. subtilis* cannot grow in the minimum medium with 2'-FL as the sole carbon source, while can grow normally in the presence of glucose or sucrose. These results suggested that *B. subtilis* could be used as a host for 2'-FL production.

2'-FL production by introducing the de novo pathway

GDP-*L*-fucose is a key precursor for 2'-FL biosynthesis [27]. The de novo pathway could utilize low-cost carbon sources (glucose, sucrose, and so on) to synthesize 2'-FL. To produce 2'-FL utilizing sucrose and lactose, the de novo pathway was constructed by introducing *manB*, *manC*, *gmd*, and *wcaG* genes from *E. coli* BL21 (DE3) for synthesizing GDP-*L*-fucose and the *futC* gene from *H. pylori* into *B. subtilis* 168 (Fig. 1). To express the heterologous genes efficiently in *B. subtilis*, the original promoters of *manB*, *manC*, *gmd*, *wcaG*, and *futC* genes were respectively replaced by constitutive promoter P₄₃ and integrated into the genome of *B. subtilis*, yielding the strain BS0. The shake flask culture of BS0 was performed, while the extracellular 2'-FL and intracellular GDP-*L*-fucose cannot be detected, implying that one or more genes in the GDP-*L*-fucose synthesis pathway were not normally transcribed or translated. In addition, protein structure instability may also lead to the above results.

To explore the reasons for the above unexpected results, the plasmid pP43NMK was used to respectively express the *manB*, *manC*, *gmd*, and *wcaG* genes. Four strains (BS-MB, BS-MC, BS-GD, and BS-WG) were constructed to verify the expressions of phosphomannomutase (ManB;

50.19 kDa), mannose-1-phosphate guanylyltransferase (ManC; 53.81 kDa), GDP-mannose-4,6-dehydratase (Gmd; 42.05 kDa), and GDP-*L*-fucose synthase (WcaG; 36.15 kDa). SDS-polyacrylamide gel electrophoresis results showed that BS-MB, BS-GD, and BS-WG strains displayed single bands of approximate 51, 42, and 37 kDa, respectively (Additional file 1: Fig. S1), while the gel electrophoresis result of strain BS-MC was similar to the control group. The aforementioned results suggested that the target genes in strains BS-MB, BS-GD, and BS-WG were transcribed and translated but not the target gene in strain BS-MC.

The P₄₃ promoter is a strong constitutive promoter used to express various heterologous genes in *B. subtilis* [28, 29]. The above results indicated that the *manC* gene was not expressed, and eventually 2'-FL cannot be synthesized normally. It was speculated that the expression strength of the P₄₃ promoter was not appropriate to ManC. Therefore, the inducible promoter P_{xyIA} was used based on earlier reports to optimize the expression level of *manC* [30]. The accumulation of 1.12 g/L 2'-FL in the shake flask fermentation supernatant of strain BS2 (P_{xyIA}-*manC*) was achieved, whereas no 2'-FL was detected in the fermentation supernatant of the control strain BS (Fig. 3A). The LC/MS identification results are shown in Fig. 3B and C. Results showed that the inducible promoter P_{xyIA} enabled the transcription and translation of *manC*, and 2'-FL was eventually synthesized in *B. subtilis*.

Improving lactose utilization by knocking out β-galactosidase

As an important precursor for 2'-FL de novo synthesis, lactose is extremely important for the overproduction

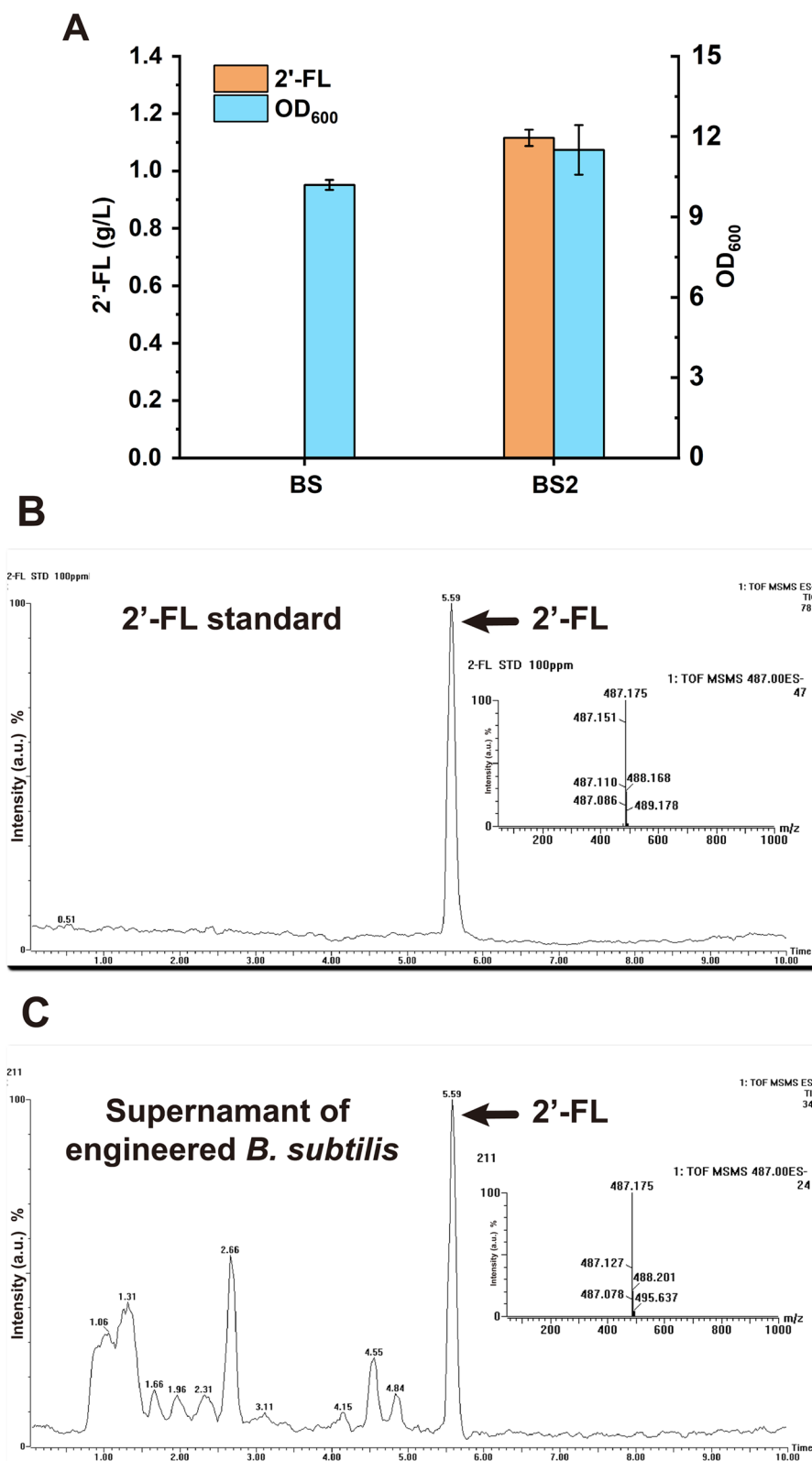
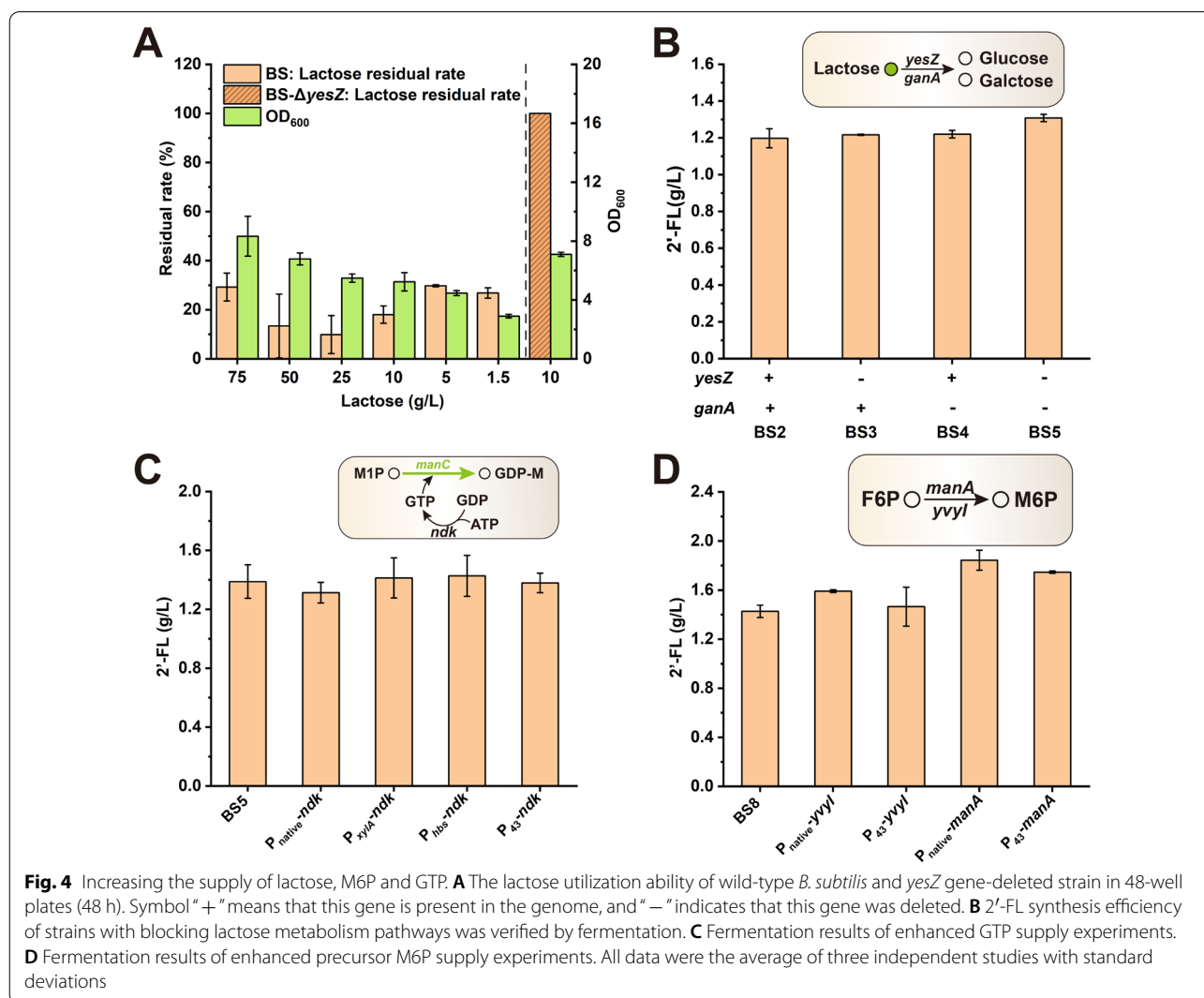


Fig. 3 Production of 2'-FL in the strain BS2. **A** 2'-FL titer and OD₆₀₀ of 20 g/L xylose-induced strain BS2. **(B and C)** 2'-FL standard and BS2 strain shake flask fermentation supernatant were analyzed by HPLC and LC/MS, respectively. Cells were cultivated for 36 h at 37 °C and 220 rpm. All data were the average of three independent studies with standard deviations

of 2'-FL. The lactose transportation in *B. subtilis* is dependent on the phosphotransferase system [31]. The β -galactosidase encoded by *yesZ* and *ganA* was able to hydrolyze lactose in *B. subtilis* [32, 33]. To test the lactose utilization ability of *B. subtilis*, *B. subtilis* was cultured in LB medium containing different concentrations of lactose (1.5, 5, 10, 25, 50, and 75 g/L). Figure 4A shows that *B. subtilis* consumed 70–90% of the lactose added in the medium. Among them, the experimental group with an initial lactose concentration of 75 g/L had the maximum residual lactose (~21.93 g/L) but with the consumption of >50 g/L lactose. The final OD₆₀₀ of *B. subtilis* gradually increased with the increase of lactose concentration, indicating that excess lactose was catabolized for cell growth (Fig. 4A). In addition, the effect of blocking the endogenous lactose metabolic pathway was tested by knocking out the *yesZ* gene, and results showed that *yesZ*

deletion resulted in the cessation of lactose consumption in *B. subtilis* (Fig. 4A). According to the chemical reaction equation of 2'-FL synthesis, the stoichiometric ratio of lactose to 2'-FL was 1:1 (Fig. 1). Therefore, results indicated that the lactose transportation system of *B. subtilis* sufficed the needs of 2'-FL synthesis.

Although the lactose transport capacity was demonstrated, endogenous lactose metabolic pathways reduced the effective supply of lactose in *B. subtilis* (Fig. 1). β -Galactosidase could catalyze lactose hydrolysis; two β -galactosidase genes (*yesZ* and *ganA*) were found in *B. subtilis* [34–36]. Next, the effect of blocking the lactose catabolic pathway by knocking out different genes on 2'-FL accumulation was investigated. The results of shake flask fermentation (Fig. 4B) showed that knockout of the *yesZ* or *ganA* gene alone and in combination increased the 2'-FL titer to 1.22, 1.22, and 1.31 g/L, respectively. An earlier study suggested that the endogenous lactose



transport system in *B. subtilis* was insufficient for the availability of 2'-FL production [20]. Results confirmed that the endogenous lactose transport system of *B. subtilis* sufficed the 2'-FL production with sufficient lactose, but a large amount of lactose was used for cell growth. Therefore, two β -galactosidase genes needed to be knocked out to improve lactose availability in *B. subtilis*.

Enhancing 2'-FL production by engineering GTP and M6P supply

GTP and nicotinamide adenine dinucleotide phosphate were required as GDP donors and energy sources in the GDP_L-fucose de novo synthesis pathway (Fig. 1). 2'-FL production could be enhanced by increasing the GTP supply [20, 37, 38]. To increase GTP availability for optimal 2'-FL production, the *B. subtilis ndk* gene encoding a nucleoside-diphosphate kinase that converted GMP into GTP was respectively overexpressed by the original promoter (P_{native-ndk}), inducible promoter (P_{xyLA-ndk}), and constitutive promoters (P_{hbs-ndk} and P_{43-ndk}). Shake flask fermentation results showed that the 2'-FL titer of strains BS7 (P_{xyLA-ndk}) and BS8 (P_{hbs-ndk}) increased to 1.41 and 1.43 g/L, respectively, whereas the titers of the remaining strains decreased (Fig. 4C). Therefore, strain BS8 was selected for subsequent research.

The direct precursor of the de novo synthesis of GDP_L-fucose was M6P, which was catalyzed by the M6P isomerase encoded by *manA* and *yvyI* (putative) genes [39]. *manA* expression was regulated by mannose and a transcription activator (*manR*), and the endogenous promoter of *manA* was subjected to carbon catabolite repression [40]. To increase the supply of the precursor M6P, the original *manA* and *yvyI* were retained in the genome of strain BS8, and *manA* and *yvyI* expression cassettes harboring the original and strong promoters were introduced (P_{native-yvyI}, P_{43-yvyI}, P_{native-manA} and P_{43-manA}). Shake flask cultivation was performed for these strains with BS8 as a control. In Fig. 4D, the titers of the four strains (BS10–BS13) integrating the *manA* or *yvyI* gene increased to 1.59, 1.47, 1.84, and 1.75 g/L, respectively. Among these, with the P_{native-manA} expression cassette, the titer increased most significantly up to 1.84 g/L. All gene insertions exhibited a positive effect on 2'-FL accumulation. Results indicated that the precursor M6P supply played an important role in 2'-FL production.

Noninducible 2'-FL production and fermentation optimization

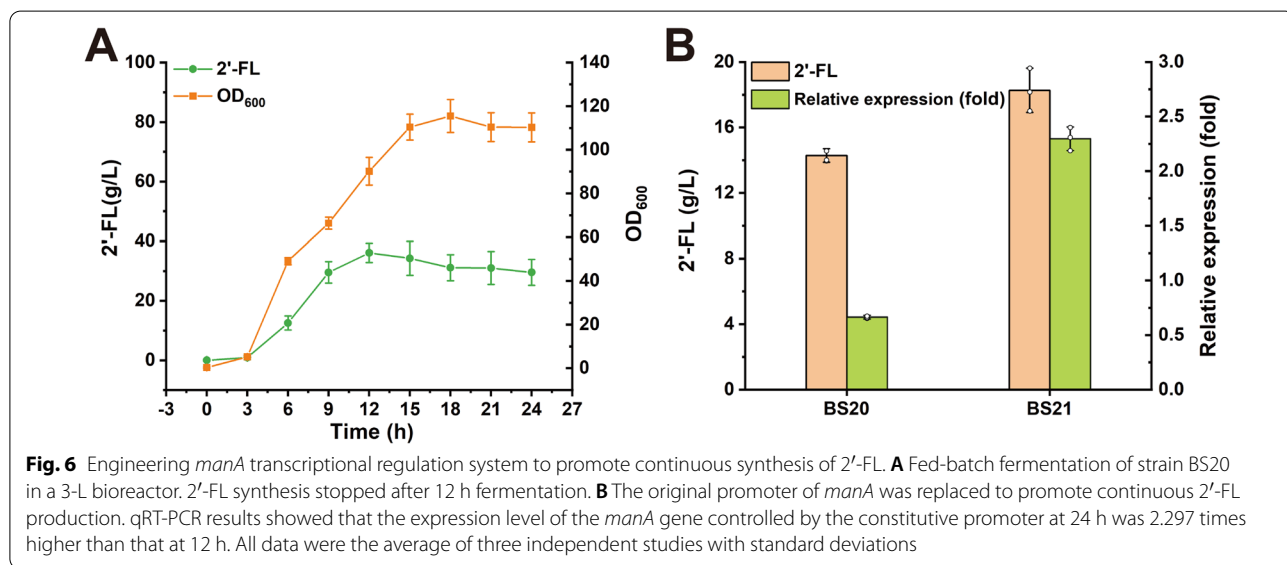
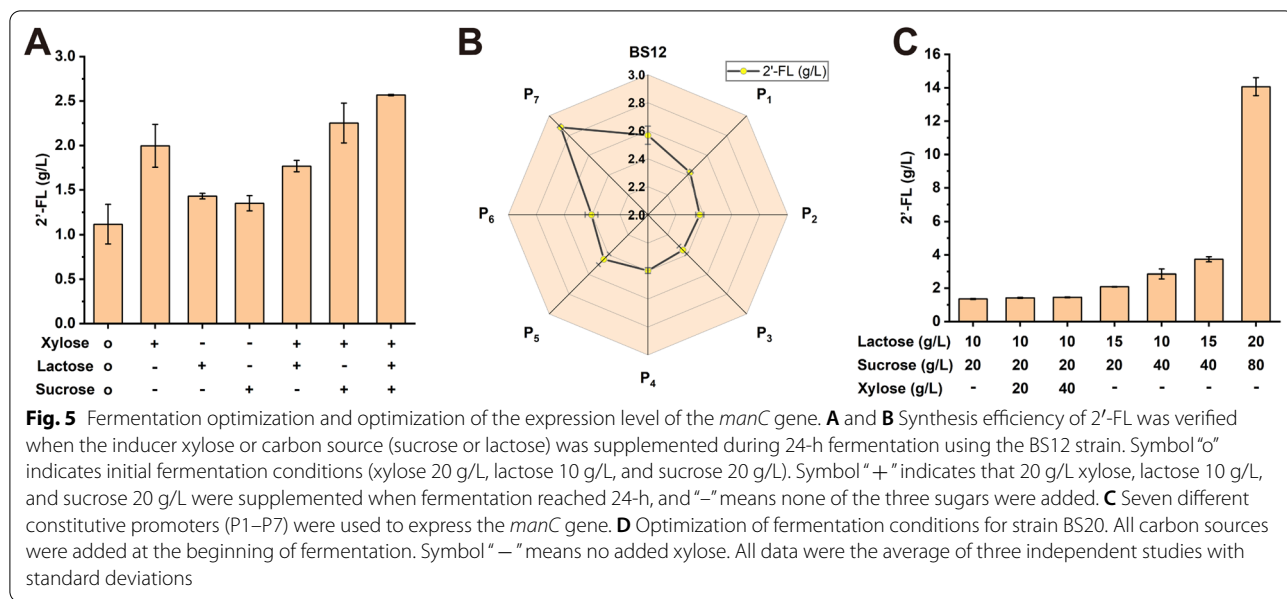
As mentioned in the preceding sections, a 2'-FL-producing strain with an improved titer was obtained. The titer and OD₆₀₀ curves during the fermentation process (Additional file 1: Fig. S2) showed that 2'-FL accumulated

slowly in the later stages of fermentation. Considering that this engineered strain required xylose as an inducer for 2'-FL production, there could be two possible reasons for the reduction in productivity volume: (1) *B. subtilis* could use xylose as a carbon source for growth and metabolite synthesis [41]. Therefore, xylose depletion as an inducer might have led to the inhibition of 2'-FL synthesis. (2) With the prolongation of the fermentation time, the residual carbon source was gradually consumed, and the lack of carbon source resulted in a decrease in production volume. Therefore, carbon source and inducer supplemental fermentation were performed. Results showed that adding the inducer xylose alone considerably increased the titer by 78.59% (2.00 g/L), and supplementing the lactose or sucrose alone increased the 2'-FL titer by 27.91% (1.43 g/L) and 20.77% (1.35 g/L), respectively (Fig. 5A). Results demonstrated that adding the inducer dose was the most effective in increasing the titer. In Fig. 5A, the 2'-FL titer increased by 129.72% with the addition of lactose and sucrose by increasing the inducer dose, and 2.57 g/L 2'-FL was obtained in the shake flask.

Although xylose supplementation significantly increased the 2'-FL titer, the high cost was unsuitable for an industrial-level production economy. To avoid using xylose, seven constitutive promoters (P1–P7) from *B. subtilis* with gradually increasing expression levels were used to replace inducible promoter P_{xyLA}. The fermentation results shown in Fig. 5B indicated that the titers of the strains harboring the P1 to P6 promoters decreased by 5.47%, 7.63%, 8.33%, 6.63%, 4.67%, and 6.36%, respectively. The strain BS20 harboring the strongest promoter had the highest 2'-FL titer, reaching 2.88 g/L (Fig. 5B). The highest 2'-FL titer was obtained by optimizing the addition of sucrose and lactose. In Fig. 5C, 80g/L sucrose and 20 g/L lactose were added at the beginning of the fermentation process, and the 2'-FL titer reached 14.06 g/L in 72 h (Fig. 5C). Meanwhile, considering that xylose could be consumed by *B. subtilis* as a carbon source, the effect of xylose on the 2'-FL titer was tested. Results showed that adding xylose did not affect 2'-FL accumulation (Fig. 5C).

Enhancing 2'-FL production by engineering the transcriptional regulation of M6P isomerase

To further validate the 2'-FL production capacity of the engineered strain BS20, fermentation was performed in a 3-L bioreactor (Fig. 6A). Surprisingly, with the extension of fermentation time, the 2'-FL titer stopped increasing at 12 h, whereas the OD₆₀₀ continued to increase, and a final 2'-FL titer of 36.09 g/L was obtained. All the exogenous genes in the 2'-FL de novo synthesis pathway was expressed with constitutive promoters, so there is no



transcriptional repression for the expression of these genes. It was reported that, the endogenous *manA* gene (encoding the M6P isomerase) belongs to the mannose operon, and there is a phosphorylated transcription activator *manR* upstream of the mannose operon [40]. Therefore, we speculate that the stop of 2'-FL synthesis after 12 h may be due to the transcriptional inhibition of the endogenous *manA* gene.

Based on the above analysis, the original promoter (P_{native}) of the *manA* gene of the BS20 strain was replaced with the constitutive promoter P7, generating the strain BS21. The cells of BS20 and BS21 cultured

in shake flask for 12 and 24 h were collected, and the relative transcription level of the *manA* gene was analyzed by qRT-PCR (Fig. 6B). The results showed that the relative expression of *manA* at 24 h in the strain BS20 decreased to 0.664 times that at 12 h, whereas the relative expression of *manA* at 24 h in the strain BS21 increased to 2.297 times that at 12 h. Accordingly, the strain BS21 produced the highest 2'-FL titer of 18.27 g/L, which was 1.28-fold higher than that of the strain BS20 (14.29 g/L). This result suggested that the transcriptional regulation of *manA* hinders the continuous synthesis of 2'-FL in the later stage of

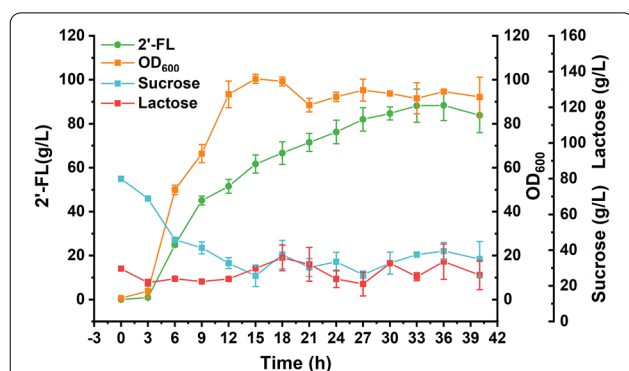


Fig. 7 2'-FL production in a 3-L bioreactor. Fed-batch culture of the strain BS21 in a 3-L bioreactor. Sucrose and lactose were maintained at 10–30 g/L and 10–20 g/L, respectively. All data were the average of three independent studies with standard deviations

fermentation, and the expression of *manA* gene with the constitutive promoter can solve this issue.

The production of 2'-FL by the finally engineered strain BS21 was performed in a 3-L bioreactor. The pH was kept at 6.0–6.5 by adding NH_4OH ; sucrose and lactose concentrations were maintained at 10–30 g/L and 10–20 g/L, respectively. Figure 7 shows that the strain BS21 could produce 88.3 g/L 2'-FL with a yield of 0.61 g/g lactose, representing the highest 2'-FL titer reported so far.

Conclusions

This study constructed the 2'-FL de novo synthesis pathway in *B. subtilis* and further improved the 2'-FL titer by increasing the supply of M6P, lactose and cofactor GTP. In addition, the native promoter of *manA* gene was replaced with the constitutive promoter P7, leading to the further increase of 2'-FL titer. In a 3-L bioreactor, the 2'-FL titer of the finally engineered strain BS21 reached 88.3 g/L with a yield up to 0.61 mol/mol lactose. In the future, the dynamic regulation of central carbon metabolism with genetic circuits can be performed to further improve the synthesis efficiency of 2'-FL.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12934-022-01838-w>.

Additional file 1: **Table S1.** Plasmids used in this study. **Table S2.** Primers and promoters used in this study. **Figure S1.** The SDS-PAGE analysis of cell extracts from single-gene expression strain. **Figure S2.** Production of 2'-FL in the shake flasks by the engineered strain BS2.

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Author contributions

Long Liu conceived, supervised and coordinated the project. Quanwei Zhang, Yonglian Zhu, and Linfeng Xu executed related research. Quanwei Zhang conceived drafted the first manuscript which was later revised and corrected by Xueqin Lv, Yanfeng Liu, Jianghua Li and Guocheng Du. Ziyang Huang, Zhenmin Liu, and Hongzhi Xia gave us some advice from the perspective of industrialization. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Competing interests

The authors declare no competing financial interest.

Consent for publication

Not applicable.

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