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Original Research Article

Efficient production of 2′-fucosyllactose in *Pichia pastoris* through metabolic engineering and constructing an orthogonal energy supply system

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ABSTRACT

2′-fucosyllactose (2′-FL) holds significant role in the infants' nutrition. While microbial production of 2′-FL has predominantly utilized *Escherichia coli* and *Saccharomyces cerevisiae*, the potential of *Pichia pastoris*, renowned for its robust NADPH regeneration capability, remains underexplored. Herein, we systematically engineered the metabolism of *P. pastoris* to develop an efficient 2′-FL-producing cell factory. We first constructed the *de novo* biosynthesis pathway for 2′-FL in *P. pastoris*, achieving an initial titer of 0.143 g/L. By optimizing enzyme selection and solubility of α-1,2-fucosyltransferase (FutC), 2′-FL production was enhanced by nearly ten folds. Subsequently, engineering NADPH supply further increased the 2′-FL production by 170 %. Furthermore, we enhanced energy supply by incorporating an orthogonal energy module based on the methanol dissimilation pathway and increasing GTP availability, resulting in a 32 % improvement in 2′-FL production. Finally, through the optimization of fermentation condition, we realized the production titer of 2′-FL to 3.50 g/L in shake-flask, representing the highest titer in *P. pastoris*. These findings highlight the potential of *P. pastoris* as a chassis to produce chemicals by providing abundant NADPH and utilizing methanol as co-substrate to supply sufficient energy.

1. Introduction

Human milk oligosaccharides (HMOs) are unique constitute of human milk, comprising a diverse array of sugars that confer health benefits to infants through breastfeeding [1]. Increasing evidence highlights the vital role of HMOs as prebiotics, fostering the growth of beneficial intestinal bacteria (e.g., *Lactobacillus* spp. and *Bifidobacterium* spp.) while inhibiting the proliferation of pathogenic bacteria [2]. To date, HMOs have been approved as functional ingredients in advanced infant formula in many countries. Among HMOs, 2′-fucosyllactose (2′-FL) is the most abundant component, serving as a regulator of intestinal flora, an anti-adhesive agent to protect pathogens infection, and more [3–5]. Given the fast-growing market demand, there is an urgent need for efficient production of 2′-FL. Traditional chemical synthesis and enzymatic synthesis methods requires labor-intensive multi-step process or complex engineering of enzymes, which limited the industrial production of 2′-FL. Microbial synthesis endows the advantage of simpler operational process, cost-effectiveness and ease of scale up production, gradually becoming the dominant industrial production method [6].

The *de novo* pathway and salvage pathway are the two routes constructed for microbial synthesis of 2′-FL, with the key difference being the source of the precursor guanosine 5′-diphosphate-L-fucose (GDP-L-fucose) [7]. *E. coli* has made significant progress as the most commonly used chassis for developing 2′-FL cell factories. Pioneering research by Samain et al. in *E. coli*. chassis utilized endogenous GDP-L-fucose pathway and achieved 2′-FL production through the overexpression of *Helicobacter pylori* α-1,2-fucosyltransferase (FutC) [8]. Additionally, overexpression of the *RcsA* gene and deletion of the *WcaJ* gene were implemented to further enhance 2′-FL production. Wang et al. realized the highest 2′-FL titer of 11.2 g/L in shake flask in *E. coli* to date reported

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in the literature [9]. Despite the significant progress made in *E. coli*, issues such as phage contamination during large-scale production, endotoxin production, and safety concerns cannot be overlooked. In addition to *E. coli*, *Corynebacterium glutamicum* and *Bacillus subtilis*, two commonly used prokaryotic model organisms, have also been employed as cell factories for the production of 2′-FL [10,11]. Zhang et al. successfully introduced a lactose synthesis module into *B. subtilis*, enabling the efficient production of 2′-FL with glucose as the sole carbon source, which reduced production costs and metabolic burden [12].

Yeast, as a robust eukaryotic model organism, offers enhanced safety and greater resistance to phage contamination. To produce 2′-FL in engineered *Saccharomyces cerevisiae*, Yu et al. genetic introduced the salvage pathway using L-fucose and lactose as substrates by expressing *Lac12*, *FKP*, and *FutC* genes and successfully achieved 2′-FL production [13]. Xu et al. engineered a *de novo* biosynthesis pathway and introduced a dynamic regulation system to rewire the metabolic flux, achieving the highest 2′-FL titer of 3.37 g/L in shake-flask in *S. cerevisiae* [14]. The biosynthesis of 2′-FL requires substantial NADPH, and enhancing NADPH regeneration through strengthening the pentose phosphate (PP) pathway could promote the synthesis of GDP-L-fucose [15,16]. *Pichia pastoris*, with its higher PP pathway flux compared to *S. cerevisiae*, is better suited for 2′-FL production by providing sufficient NADPH [17]. In addition, *P. pastoris* possesses natural ability to efficiently utilize methanol, a cheaper and sustainable carbon source, for energy supply and biosynthesis [18,19]. Although 2′-FL synthetic pathway has been constructed in *P. pastoris* [20], production improvement remain urgently required especially taking advantage of methanol utilization.

In this study, we enhanced 2′-FL production in *P. pastoris* by utilizing glucose and methanol as a co-substrate, through metabolic engineering and the integration of an orthogonal energy module (Fig. 1). First, by introducing a *de novo* synthesis pathway for 2′-FL and optimizing the expression and solubility of key enzyme, we successfully achieved the *de novo* production of 2′-FL from glucose and lactose in *P. pastoris*. Subsequently, we enhanced the yield of 2′-FL through NADPH regeneration engineering. Furthermore, we facilitated GTP availability and engineered an orthogonal energy module by interrupting the methanol assimilation pathway and refactoring a constitutive expressed methanol dissimilation pathway, resulted in a more productive strain, with the 2′-FL production reached 3.12 g/L in shake-flask from glucose and methanol. Finally, by optimizing the fermentation conditions, we further enhanced the titer of 2′-FL to 3.50 g/L. Overall, we achieved efficient production of 2′-FL in *P. pastoris* through metabolic engineering and enhanced energy supply, representing the highest titer reported to date.

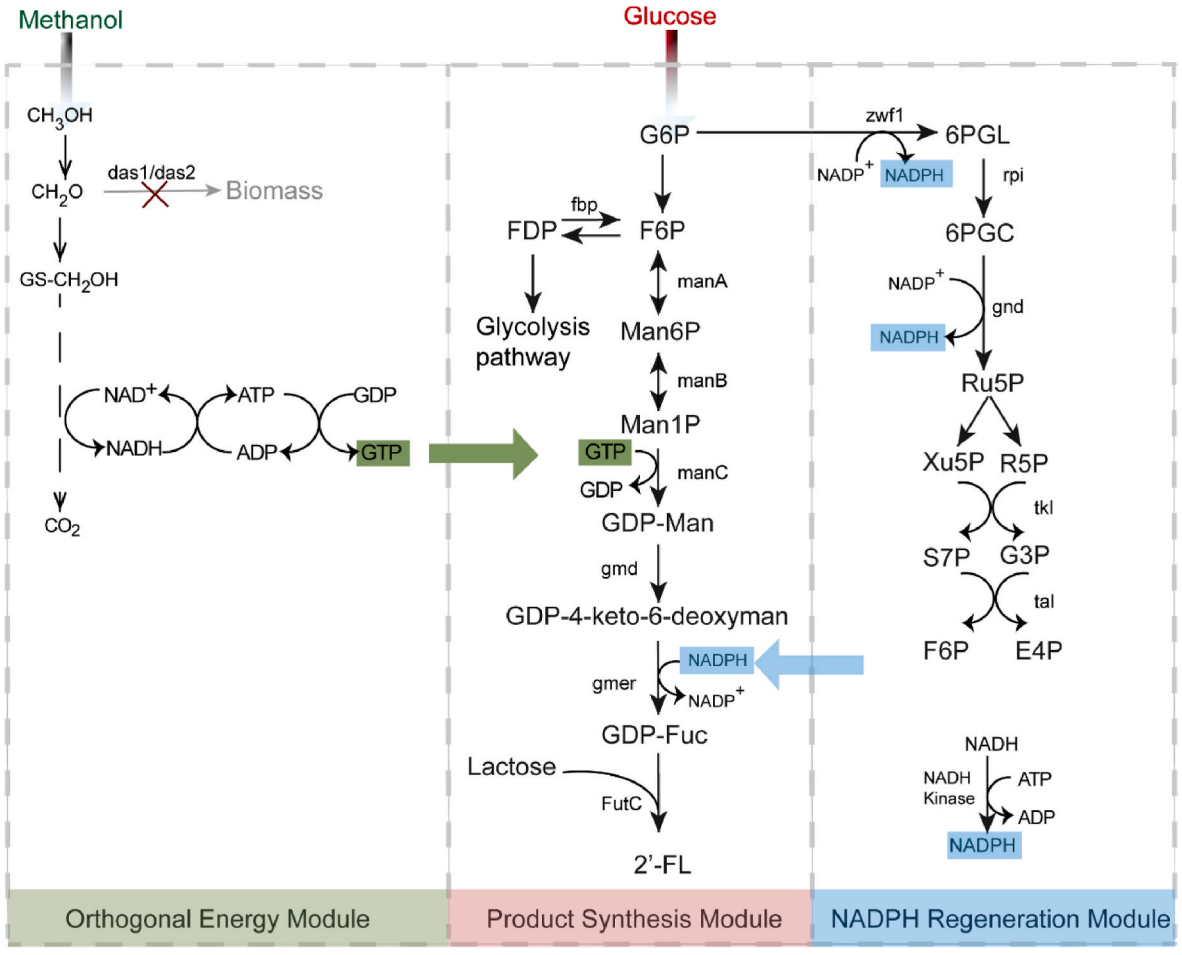

Fig. 1. An overview of the reconstructed pathways for the biosynthesis of 2'-FL in *P. pastoris* and the key optimization strategies across three modules [1]: orthogonal energy module [2]; product synthesis module [3]; NADPH regeneration module. Abbreviations: CH_2O, formaldehyde; GS-CH_2OH, S-hydroxymethyl glutathione; G6P, glucose-6-phosphate; FDP, fructose-1,6-bisphosphate; F6P, fructose-6-phosphate; GDP-Fuc, guanosine diphosphate fucose; GDP-Man, guanosine diphosphate mannose; GDP-4-keto-6-deoxyman, GDP-4-keto-6-deoxy-D-mannose; Man6P, mannose-6-phosphate; Man1P, mannose-1-phosphate; 6PGL, 6-Phospho-d-glucono-1, 5-lactone; 6PGC, 6-Phospho-D-gluconate; Xu5P, xylulose 5-phosphate; Ru5P, Ribulose 5-phosphate; R5P, Ribose 5-phosphate; S7P, Sedoheptulose 7-phosphate; E4P, Erythrose 4-phosphate; G3P, Glyceraldehyde 3-phosphate; ATP, adenosine triphosphate; GTP, guanosine triphosphate.

2. Methods

2.1. Strains, plasmids and reagents

All plasmids, strains and synthetic gene sequences after codon optimization used in this study are listed in Supplementary Table 1, Supplementary Table 2 and Supplementary Table 4.

The 2 × Phanta® Max Master Mix was obtained from Vazyme (Nanjing, China). Premix Taq™ (Ex Taq™ Version 2.0 plus dye), and PrimeSTAR® Max DNA Polymerase were sourced from Takara. CutSmart Buffer (10×), *Bpi*I (*Bbs*I-HF), T4 DNA Ligase, and 10 mM ATP were purchased from NEB. Hygromycin was acquired from Yeasen (Shanghai, China). The DNA Cycle Pure Kit, Plasmid Purification Kit, and DNA Gel Purification Kit were also purchased from Vazyme. d-Sorbitol, dl-Dithiothreitol (DTT), Ethylene Glycol, and Dimethyl Sulfoxide (DMSO) were obtained from Sigma-Aldrich.

Trace metals stock solution contained $FeSO_4 \cdot 7H_2O$ 3.0 g/L, $ZnSO_4 \cdot 7H_2O$ 4.5 g/L, $CaCl_2 \cdot 2H_2O$ 4.5 g/L, $MnCl_2 \cdot 4H_2O$ 1 g/L, $CoCl_2 \cdot 6H_2O$ 300 mg/L, $CuSO_4 \cdot 5H_2O$ 300 mg/L, $Na_2MoO_4 \cdot 2H_2O$ 400 mg/L, H_3BO_3 1 g/L, KI 100 mg/L, $Na_2EDTA \cdot 2H_2O$ 19 g/L. Vitamins solution contained d-Biotin 50 mg/L, D-Pantothenic acid hemicalcium salt 1.0 g/L, Thiamin-HCl 1.0 g/L, Pyridoxin-HCl 1.0 g/L, Nicotinic acid 1.0 g/L, 4-Aminobenzoic acid 0.2 g/L, Myo-Inositol 25 g/L.

BEDS solution: 10 mmol/L bicine-NaOH, pH 8.3, 3 % (v/v) ethylene glycol, 5 % (v/v) DMSO and 1 mol/L sorbitol.

2.2. Genetic manipulation

We used strain Gsy002 as the start strain for all genetic manipulation. Supplementary Table 5 provides a compressive list of all primers used in this study. To facilitate the deletion of genes and the integration of expression cassettes, we employed the CRISPR-Cas9 system. For the identification of potential gRNAs for targeting gene, we utilized the CRISPR direct webtool (http://crispr.dbcls.jp). The vector backbone containing the hygromycin resistance gene was obtained from the plasmid pLAT1 gifted by Professor Xiang Gao, and their accuracy was verified by sequencing.

All gRNA plasmids were constructed by Golden gate method. Seven exogenous enzymes, lac12 (XP_452193), cdt2 (EAA34637), gmd (ADC54120), gmer (WP_001002442), BKHT (PAF50342.1), WcfB (CAH06753.1), and HP-FutC (ABO61750), were codon optimized for *P. pastoris* and synthesized by Genescript Co., Ltd. (Jiangsu, China).

To amplify native promoters, genes, homology sequences and terminators of *P. pastoris*, we used GS115 genomic DNA as a template. Expression cassette construction and gene deletion repairs were carried out by fusion PCR.

2.3. Yeast transformation

Yeast transformation was performed according to the manufacturer's protocol [21]. In detail, 4.5 mL of ice-cold BEDS solution supplemented with 0.5 mL of 1 M DTT was added to the yeast cells, which were then incubated for 5 min at 200 rpm at 30 °C. After centrifugation at 5000 rpm for 5 min, the cells were resuspended in BEDS solution. For DNA transformation, condensed electroporation (Bio-Rad Laboratories, Hercules, CA, USA) was used after adding 1 μg of Cas9 plasmid containing different sgRNA fragments and 1 μg of repair dsDNA into competent cells using the following parameters (Gene Pulser® II electroporator: cuvette gap, 2.0 mm; charging voltage, 1500 V). The transformed cells were cultivated for 3 days on YPD plates containing 200 μg/mL hygromycin.

2.4. Strain cultivation

E. coli strain DH5α was used for plasmid construction and cultured in Luria-Bertani (LB) medium containing 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, and 100 μg/mL hygromycin at 37 °C.

For the preparation of competent cells, yeast strains were cultivated in YPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract). From frozen stock, streak yeast for isolation on YPD media agar to obtain single colony isolates. Subsequently, a single colony of the strain was inoculated into a 10 mL tube with 1 mL of YPD medium. Finally, the cells were seeded at an initial 0.2 OD in 100 mL flasks containing 15 mL of YPD medium until the OD_{600} reached 0.6–0.8.

For shake flask fermentation, the basal medium used was minimal Delft medium, containing 7.5 g/L $(NH_4)_2SO_4$, 14.4 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L histidine, trace metals, and vitamin solutions. Initially, a single colony of the strain was inoculated into a 10 mL tube with 1 mL of YPD medium. Subsequently, the cells were seeded at an initial 0.2 OD in 100 mL flasks containing 15 mL of Delft medium with 2 % glucose and 0.5 % lactose and culture at 200 rpm and 30 °C for 48 h. After 2 days of fermentation, the fermentation broth was supplemented with 1 % glucose and 1 % methanol. Noted that, to avoid the toxicity of methanol to the strain, methanol was replenished after 48 h.

2.5. Measurement of growth curve

Yeast cultures are prepared in the same way as for shake flask fermentation. To measure the growth of yeast, 200 μL of the prepared yeast cultures were transferred to the microplate wells. The microplates were covered with the gas permeable sealing membrane and placed in a microplate reader (BioTek LogPhase 600) to monitor the OD at 600 nm of the yeast in the wells. The plates were maintained at 30 °C and rotated at 600 rpm. The OD_{600} of each well were read every 60 min for 288 h.

2.6. Analytical methods

For analysis of metabolites and 2′-FL in the fermentation broth, 500 μL of the fermentation broth was taken and centrifuged at 13,000 rpm for 1 min and then filtered through a 0.22 μm filter. To disrupt cell wall and collect intracellular 2'-FL, we harvested 300 μL of cell culture by centrifugation at 13,000 rpm for 1 min, washed twice with distilled water, and re-suspended pellet with 300 μL of distilled water. To ensure that all of the 2′-FL was released, the cells were then further disrupted by continuous boiling with DKT-100 Dry Bath Incubator (MIULAB, Hangzhou) for 30 min. After centrifugation at 13,000 rpm for 1 min. We collected super supernatant and then filtered through a 0.22 μm filter. The concentration of 2′-FL, glucose, and lactose were detected by HPLC. The Agilent 1260 Infinity II Prime and a Aminex HPX-87H Organic Acid H + (8 %) Column were used. The column temperature was set at 50 °C and the refractive index detector temperature was set at 40 °C. 5 mM H_2SO_4 was used as the mobile phase (0.6 mL/min). The extracellular 2′-FL and lactose were measured from the supernatant of the fermentation broth. Cell density was estimated by measuring the optical density at 600 nm with a spectrophotometer.

3. Results

3.1. Construction and optimization of the 2'-FL production pathway in P. pastoris

Constructing the *de novo* biosynthesis pathway of 2'-FL in *P. pastoris* requires four key components [1]: the precursor GDP-L-fucose biosynthesis pathway [2]; the supply and uptake of lactose as a substrate [3]; the FutC enzyme for converting of GDP-L-fucose and lactose to 2'-FL [4]; the 2′-FL transporter, which facilitate the export of 2' -FL from the cell to prevent its accumulation in the cytosol and alleviating product inhibition. To construct the synthetic pathway, we first integrated the 2'-FL

transporter coding gene *CDT2* and *Lac12*, which encodes the lactose permease from *Kluyveryomyces lactis*, into the parental strain Gsy002, resulting the strain HK01. We further overexpressed endogenous *ManA*, *ManB*, and *ManC* to increase the supply of GDP-D-mannose. Finally, we heterologously expressed GDP-D-mannose-6-dehydrogenase (gmd) and GDP-L-fucose synthase (gmer) and FutC from *Helicobacter pylori* (HP-FutC, Table 1), which is a commonly used enzyme for constructing the synthetic pathway of 2′-FL. This enzyme facilitates the transfer of a fucosyl residue to the substrate lactose, resulting in the production of 2′-FL (Fig. 2A). The resulting strain HK20 successfully produced 2′-FL at a concentration of 0.143 g/L, which is comparable to previous study [20]. Additionally, no 2'-FL was detected in HK10 (Fig. 2B). Cell growth was not affected compared to the wild-type strain Gsy002 (Supplementary Fig. 2).

FutC is generally regarded as a rate-limiting enzyme in the 2′-FL biosynthetic pathway [22–24], owing to its poor solubility in the cytosol and low substrate specificity for lactose. To obtain a more functional FutC in *P. pastoris,* we selected two other FutCs from *Helicobacter* sp. *11S02629-2* and *Bacteroides fragilis*, named BKHT, WcfB (Table 1). We found that expression of BKHT and WcfB significantly increased 2'-FL production by 8.6 folds and 8.8 folds, respectively (Fig. 2C). To improve the solubility of these FutCs [25,26], we also constructed SUMO fusion versions by fusing a SUMO-tag to the N-terminus of the FutCs via a GS linker. As shown in Fig. 2C, SUMO-tag increased 2'-FL production when it fused to HP-FutC and BKHT. While the OD_{600} of HK25 with SUMO-BKHT integrated decreased, it exhibited the highest 2'-FL titer, reaching 1.41 g/L. In contrast, the 2'-FL yield of HK23 with SUMO-WcfB is lower than that of HK22 which contained WcfB without SUMO tag. This may be ascribed to the introduction of the SUMO tag adversely affected the folding of WcfB, subsequently impacting its enzymatic activity. Based on these results, we selected HK25 for the next round of engineering.

The growth performance of the recombinant strains was assessed and the results indicated that the introduction of different FutC variants significantly impact cell growth, with the overexpression of BKHT and WcfB notably reducing cell growth (Fig. 2D). Additionally, the expression of SUMO-tagged HP-FutC and BKHT further reduced cell growth while simultaneously increasing 2′-FL production. These results indicate that cell growth is compromised by 2′-FL production, potentially due to the excessive consumption of cofactors, including NADPH and ATP, in strains HK21 and HK25.

3.2. Increasing NADPH supply improved 2-FL production

The biosynthesis of 1 mol of GDP-L-fucose requires 1 mol cofactor NADPH, highlighting the importance of an adequate supply of NADPH for GDP-L-fucose production. Previous studies have demonstrated that overexpression of genes involved in NADPH regeneration pathways (Fig. 3A) including the PP pathway and NADH kinase could facilitate the synthesis of GDP-L-fucose in *Yarrowia lipolytica* and *E. coli* [15,16]. Therefore, we overexpressed NADPH regeneration-related genes individually, including 6-phosphogluconate dehydrogenase (GND), transketolase (TKL), transaldolase (TAL), ribose-5-phosphate isomerase (RPI), fructose-bisphosphatase (FBP), glucose-6-phosphate dehydrogenase (ZWF1) and endogenous mitochondrial NADH kinase (PAS_chr1-4_0124), in the strain HK25, resulting the strains HK30-HK36 (Supplementary Fig. 1). As shown in Fig. 3B, HK31 overexpressing *ZWF1* slightly increased the 2′-FL production, with titer increasing to 1.49 g/L (Fig. 3B). Conversely, the overexpression of *GND* and *RPI* did not have a significant negative impact on 2′-FL production. The overexpression of other genes showed no detectable changes in 2′-FL levels.

To analysis whether the growth of recombinant strains were influenced by the engineering of NADPH supply pathways, cell growth curves were performed (Fig. 3C). These results indicate that increasing NADPH supply by strengthening PP pathway may primarily support restoring cell growth, further confirming that the reduced growth of the HK21-HK25 strain observed earlier is likely due to competition between 2′-FL synthesis and cell growth for NADPH.

To further enhance the supply of NADPH, we overexpressed genes that can promote cell growth without negatively impacting 2'-FL production, including *FBP*, *GND*, *TKL* and *PAS_chr1-4_0124* in the strain HK31, respectively, resulting in the strains HK40-HK43 (Supplementary Fig. 1). Strains HK42 with *TKL* overexpression and HK43 with *FBP* overexpression significantly increased 2'-FL titer by 58.7 % and 60.1 % compared to the parent strain HK31, respectively. Strain HK43 achieved the highest 2'-FL titer of 2.39 g/L. The strain HK41 showed a slightly increased 2'-FL titer, while the strain HK40 showed a reduced 2'-FL titer (Fig. 3D). These results indicate that metabolic engineering aimed at increasing NADPH supply is an effective strategy for enhancing the 2'-FL biosynthesis in *P. pastoris*, which is consistent with that in *Y. lipolytica* and *E. coli* [15,16].

3.3. Engineering an orthogonal methanol dissimilation pathway to enhance energy supply

The production of 1 mol of 2'-FL requires the consumption of 1 mol of guanosine triphosphate (GTP), which can be converted from ATP to generate precursor GDP-L-fucose. Additionally, lactose permease also requires energy supply [27]. Enhancing GTP availability has been shown to effectively improve the biosynthesis of GDP-L-fucose [28–30], indicating insufficient energy supply is a rate-limiting step during 2'-FL biosynthesis. Theoretically, one molecule of methanol entering the dissimilatory pathway produces two molecules of NADH, which then generate five molecules of ATP through the mitochondrial electron transport chain [31]. To augment GTP supply, we designed an orthogonal energy supply module utilizing methanol as a co-substrate. Initially, we interrupted the methanol assimilation pathway by knocking out the genes coding for dihydroxyacetone synthase, DAS1 and DAS2. This modification was intended to redirect methanol into the dissimilatory pathway, thereby enhancing NADH production and subsequently increasing ATP supply, resulting in the strain HK50 (Supplementary Fig. 1). The HK50 strain showed no growth with methanol as sole carbon source, confirming the successful blocking of the methanol assimilation pathway (Supplementary Fig. 3). The 2'-FL titer of strain HK50 increased by only 2.9 % to 2.46 g/L compared to HK43. This limited enhancement may be attributed to the inefficient conversion of ATP to GTP. To facilitate this conversion, we overexpressed nucleoside diphosphate kinase (NDK), the native enzyme in *P. pastoris* responsible for catalyzing the interconversion between ATP and GTP, in the HK50 strain, resulting in the HK60 strain. The 2'-FL titer of strain HK60 increased by 17.9 % to 2.90 g/L compared with HK50. This result suggests that overexpression of NDK in HK50 enhanced the availability of GTP, consistent with the literature reported [11,30]. We hypothesized that enhancing ATP production capacity could further boost GTP availability and ensure a sufficient energy supply. To test this, we further strengthened the orthogonal energy module. Considering the glucose repression effect, the endogenous methanol dissimilation pathway can be inhibited by the presence of glucose [32,33]. To mitigate this issue, we constructed the strain HK70 by constitutively expressing the components of the methanol dissimilation pathway, specifically alcohol oxidase (AOX1), formaldehyde dehydrogenase (FLD), S-formylglutathione hydrolase (FGH) and formate dehydrogenase (FDH), utilizing constitutive promoters. The 2′-FL yield of the HK70 strain reached 3.12 g/L, which is an improvement over that of HK60

Table 1
Heterologous α-1,2-fucosyltransferase information involved in this paper.

FutC	Species Origin	GenBank accession number
HP-FutC	*Helicobacter pylori*	ABO61750
BKHT	*Helicobacter* sp. *11S02629-2*	PAF50342.1
WcfB	*Bacteroides fragilis*	CAH06753.1

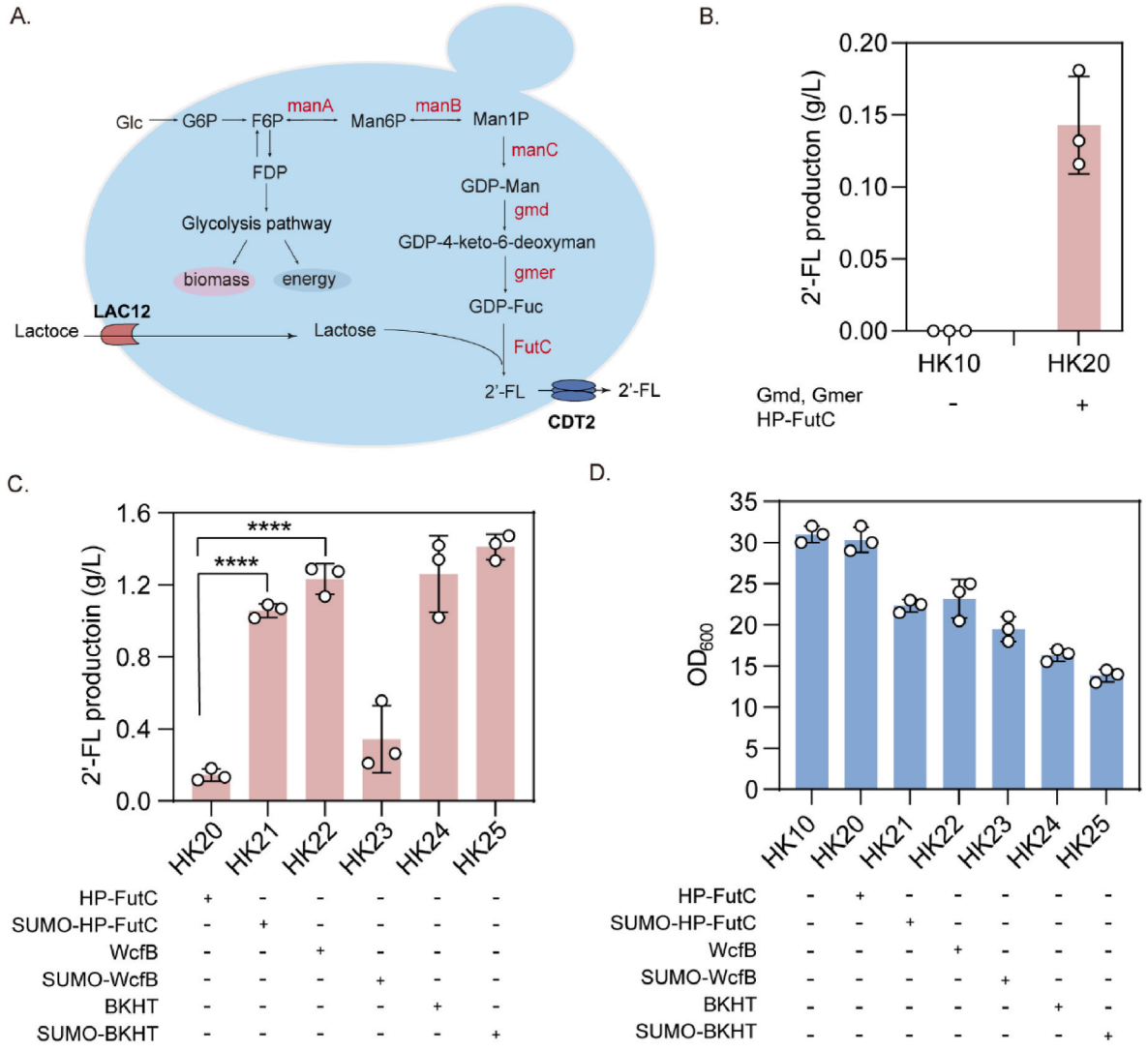

Fig. 2. Production of 2′-FL by engineered *P. pastoris*. (A) Co-overexpression of *Lac12* and *CDT2* to uptake the substrate lactose into the cell and export the product 2′-FL out of the cell. Furthermore, the genes associated with the 2′-FL production pathway, including *ManA*, *ManB*, *ManC*, *Gmd*, *Gmer* and *FutC*, were also overexpressed. *ManA*, *ManB*, and *ManC* were overexpressed to enhance the GDP-mannose supply, while *Gmd, Gmer,* and *FutC* were expressed to catalyze the conversion of GDP-mannose and lactose into 2′-FL. (B) The engineered strain HK20, which overexpresses *Gmer*, *Gmd*, and *HP-FutC*, was evaluated for 2′-FL production, with strain HK10 as a control. (C) A comparison of the 2′-FL production capacities of FutC from different sources and forms in *P. pastoris*. (D) The final OD_{600} values of the series of 2′-FL-producing strains HK20-25 and the control strain HK10. Cells were cultured in 15 mL of minimal medium with 2 % glucose as the sole carbon source and 0.5 % lactose. Data represent the mean ± SD from biological triplicates.

(Fig. 4B). The constitutive expression of the methanol dissimilation pathway has reduced the inhibitory effect of glucose. These results demonstrated that ensuring an adequate supply of energy may be crucial for efficient production of 2'-FL in *P. pastoris*. Therefore, the strategy of introducing an orthogonal energy supply module is effective for producing products that require substantial amounts of energy in *P. pastoris*.

Finally, we tested the intracellular and extracellular production of 2′-FL after 120 h of fermentation with strain HK70 to assess the feasibility of industrial-scale production. The results demonstrated that the extracellular yield of 2′-FL from strain HK70 was 2.88 g/L, while the intracellular residual amount was 0.25 g/L (Fig. 4C). Approximately 92 % of the 2′-FL was secreted into the extracellular space, which is a significant improvement compared to the performance of the CDT2 transporter in *S. cerevisiae* [25,26]. HK70 shows promise for scaled-up fermentation processes, which will facilitate downstream separation and purification steps.

3.4. Optimization of fermentation conditions

We optimized the fermentation conditions for the highest-yielding strain HK70. To optimize the carbon source ratios for 2′-FL production, we fixed the methanol concentration at 1 % and adjusted the glucose concentration to 2 %, 3 %, and 4 % for fermentation testing. As shown in Fig. 5A, optimal carbon source ratio effectively enhanced the conversion rate of 2′-FL, with the highest yield observed at a glucose to methanol ratio of 3:1. Considering that cell growth and product formation may compete for the substrate glucose, the growth state of the strain could impact yield. Therefore, we set initial OD_{600} of 0.1, 0.2, and 0.6 to investigate their effects on 2′-FL yield. As shown in Fig. 5B, inoculating HK70 at an initial OD_{600} of 0.1 resulted in the highest 2′-FL yield. The yield of 2′-FL reached 3.38 g/L in shake-flask fermentation, which increased 8 % compared to the yield before optimization. Further optimization of pH, RPM, and lactose concentration did not result in a higher yield (Supplementary Fig. 4). Through interval samplings, we observed the accumulation of a certain concentration of ethanol during

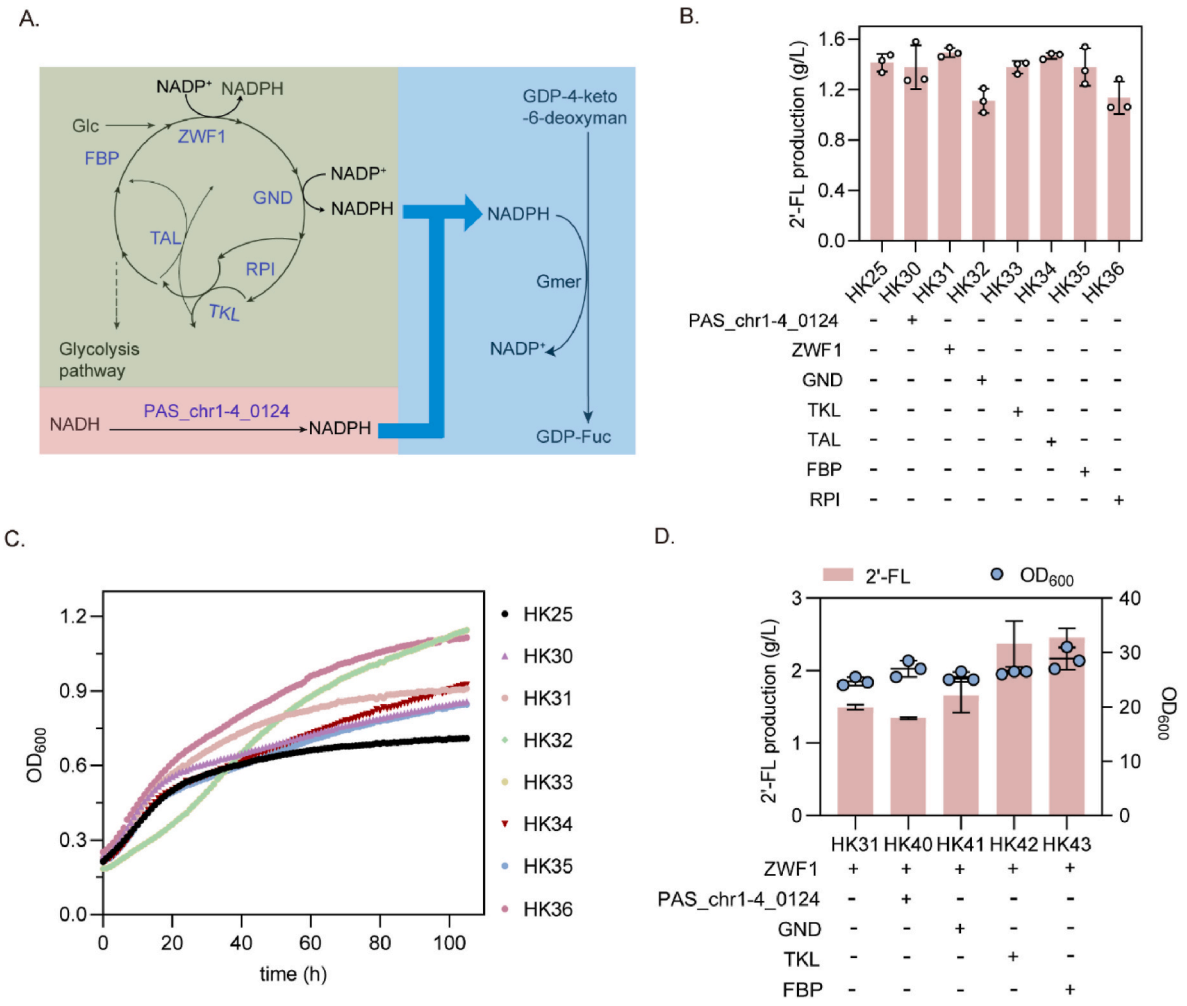

Fig. 3. Enhanced 2'-FL production by boosting the NADPH regeneration capacity. (A) Schematic representation of the PP pathway for NADPH regeneration. (B) 2'-FL production of the strains HK30-36 overexpressing *PAS_chr1-4_0124*, *ZWF1*, *GND*, *TKL*, *TAL*, *FBP*, and *RPI*, respectively. The strain HK25 as a control. (C) Cell growth curves of strains HK25 and HK30-35 (D) 2'-FL production and OD_{600} of the strains HK40-43 overexpressing of *PAS_chr1-4_0124*, *GND*, *TKL*, and *FBP* in the strain HK31. The strain HK31 as a control. Cells were cultured in 15 mL of minimal medium with 2 % glucose as the sole carbon source and the fermentation broth was supplemented with 1 % glucose at 48 h. Data represent the mean ± SD from biological triplicates.

the mid-fermentation phase. As fermentation progressed, the accumulated ethanol was consumed, after the fermentation was completed, only trace amounts of ethanol was detected in the fermentation broth, while the residual methanol content was quantified at 0.356 %. (Fig. 5C). To further refine the analysis of methanol supplementation effects, we fixed the glucose concentration at 3 % and varied the methanol concentration for testing. As shown in Fig. 5D, within the methanol supplementation range of 0 %–1.25 %, the 2′-FL yield increased as the methanol concentration was raised, while cell growth gradually declined (Supplementary Fig. 5). Compared to the group without methanol, the addition of 1.25 % methanol increased the 2′-FL yield from 2.97 g/L to 3.50 g/L, which demonstrates that the introduction of an orthogonal energy module can effectively enhance 2′-FL production. When the methanol concentration exceeded 1.5 %, the 2′-FL yield decreased, likely due to the inhibitory effects of methanol-induced cytotoxicity. Additionally, we analyzed the residual methanol content under methanol supplementation levels of 0.5 %, 1 %, and 1.5 %. The results showed that reducing the methanol supplementation significantly decreased the residual methanol content. When the methanol concentration was reduced to 0.5 %, the residual methanol content was less than 0.1 %, as shown in Fig. 5E. These results suggest that by reducing the methanol addition, we can address the methanol residue issue, making the cell factory more suitable for the production of food-grade 2′-FL. Unfortunately, the current conversion rate of HK70 glucose to 2′-FL is relatively low, at approximately 11.3 %. We hypothesize that the competition for carbon sources between cellular growth and product formation is excessively limiting the yield. Consequently, future efforts will be directed towards optimizing the balance between cell growth and product synthesis to achieve a higher yield of 2′-FL.

4. Discussion

2'-FL is a high-value nutritional additive approved for use in infant formula. To meet the growing demand for 2'-FL, microbial biosynthesis is gradually replacing traditional methods owing to the simpler operational processes, cost-efficiency and ease of scale-up for production. Currently, *E. coli.* is the most widely used chassis for 2′-FL production; however, issues such as phage contamination, endotoxins, and safety concerns remain. To circumvent these challenges, yeast chassis, including *S. cerevisiae* and *P. pastoris* have been explored as alternative production hosts. *P. pastoris*, with its inherent ability to utilize methanol, presents the additional advantage of using a low-cost substrate for production. Despite its strong NADPH regeneration capacity, *P. pastoris* has not yet to achieve the high levels of 2′-FL production seen in *S. cerevisiae*. In this study, we developed an efficient 2′-FL production strain in *P. pastoris* through metabolic engineering and the introduction

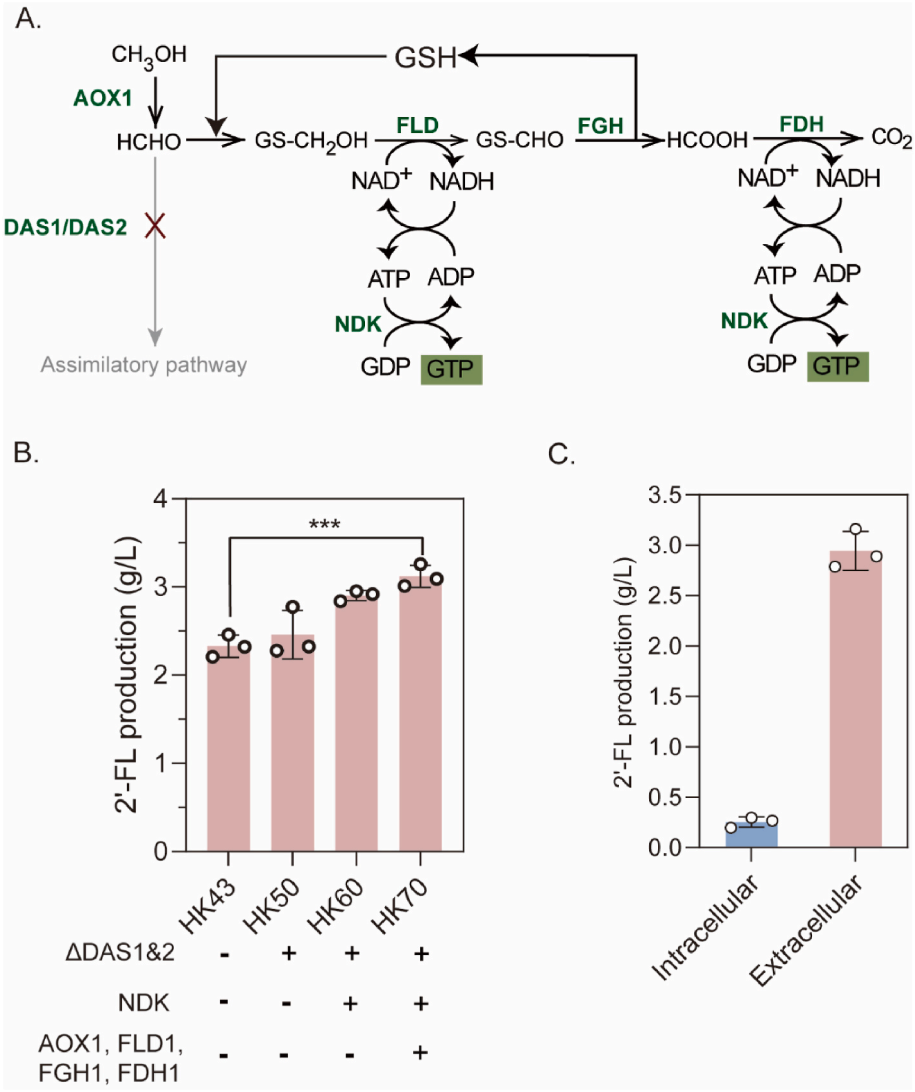

Fig. 4. The construction of an orthogonal energy supply module to enhance 2'-FL production. (A) Engineering strategies for constructing orthogonal energy module, including disrupting the methanol assimilation pathway, constitutively expressing methanol dissimilation pathway, and improving GTP availability. (B) 2'-FL production of the strains HK50, HK60, HK70. The strain HK43 as a control. Cells were cultured in 15 mL of minimal medium with 2 % glucose and 0.5 % lactose. The fermentation broth was supplemented with 1 % glucose and 1 % methanol at 48 h. (C) The intracellular and extracellular 2′-FL yields of HK70. Data represent the mean ± SD from biological triplicates.

of an orthogonal energy supply module.

We systematically engineered three modules to improve 2'-FL production [1]: optimizing the product biosynthetic pathway by screening the rate-limiting enzyme [2]; engineering the PP pathway to increase NADPH regeneration capability [3]; introducing an orthogonal energy supply module. First, FutC has been reported as a rate-limiting enzyme in the 2'-FL biosynthesis [22–24]. To optimize the enzyme performance, we screened a series of FutCs derived from different species and identified the most suitable candidate for the *P. pastoris* platform. Satisfyingly, SUMO-BKHT demonstrated the best performance, achieving a titer of 1.41 g/L. Second, since the production of 1 mol 2'-FL requires 1 mol NADPH, adequate NADPH supply is essential for efficient biosynthesis. To ensure sufficient NADPH availability, we engineered the PP pathway by overexpressing key genes involved in NADPH regeneration. As expected, the production capacity significantly improved, reaching a titer of 2.39 g/L. Finally, the production of 1 mol 2'-FL requires more than 1 mol GTP, to ensure adequate energy supply is also crucial. To leverage the methanol-utilizing capability of *P. pastoris,* we constructed an orthogonal energy supply module by blocking the methanol assimilation pathway and constitutively expression of methanol assimilation pathway. Additionally, we enhanced the interconversion of ATP and GTP by increasing the copy number of *NDK*. All these energy supply-related engineering efforts led to improved growth and production capability, resulting in a strain with a yield of 3.12 g/L. In the future, the production capacity of 2'-FL could be further improved through multicopy expression of key pathway enzymes and rewiring of metabolic flux. As climate change intensifies, the urgency to achieve carbon neutrality targets grows. Therefore, developing efficient production systems that utilize methanol as a sole carbon source for 2'-FL and other products will become increasingly important, despite the challenges involved.

In conclusion, through systematically metabolic engineering, a highly efficient 2'-FL producing microbial cell factory was constructed using the *P. pastoris* chassis, achieving the highest 2'-FL titer in *P. pastoris*. These results demonstrate the capability of *P. pastoris* for the production of high value-added chemicals, and the applied engineering strategies can be extended to the production of a broader range of chemicals.

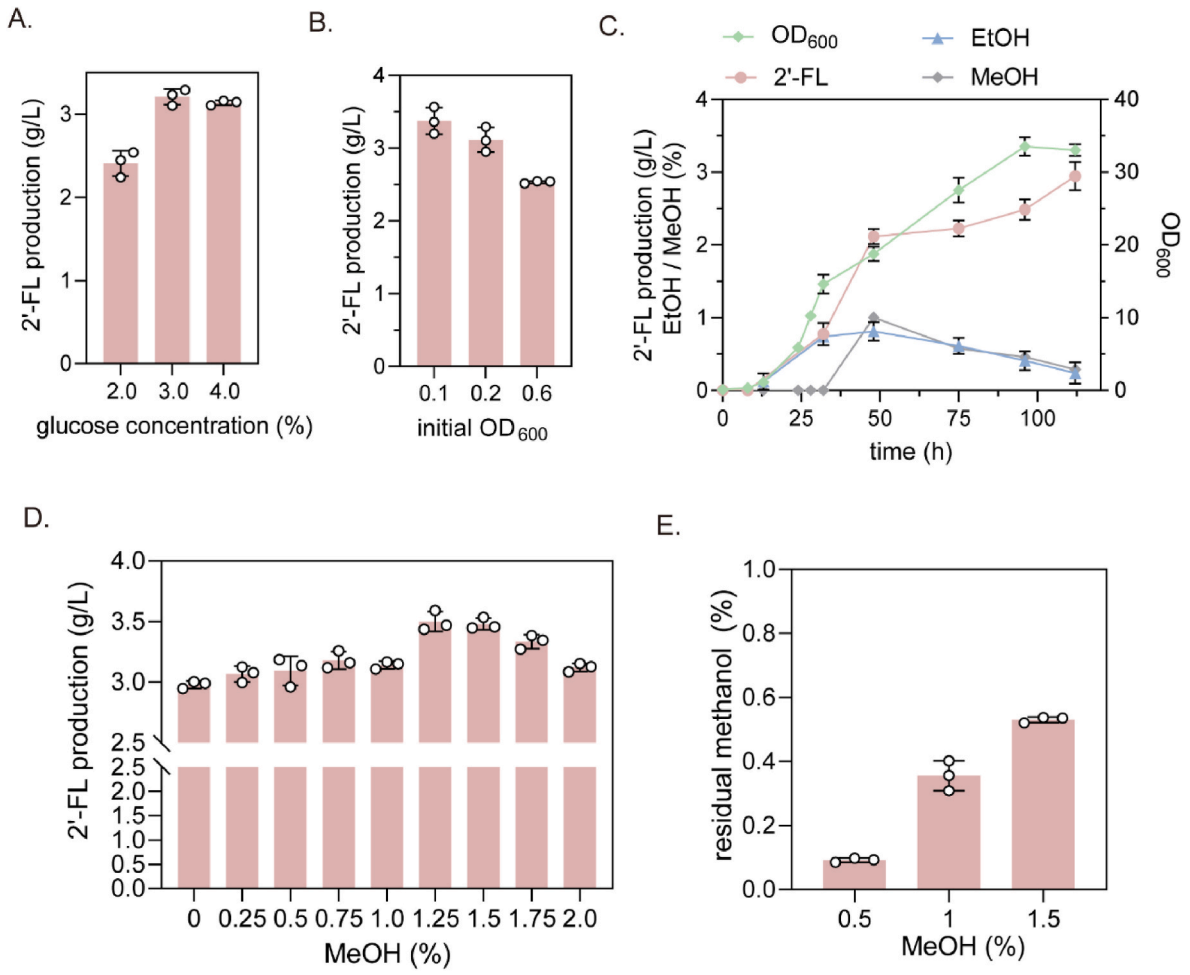

Fig. 5. Optimization of fermentation conditions. (A) Effect of varying glucose concentrations at a fixed methanol concentration of 1 % on 2′-FL production. HK70 were inoculated at 0.2 OD and cultured in 15 mL of minimal medium with different glucose concentrations. (B) Effect of different initial OD_{600} on 2′-FL yield. HK70 were inoculated at different initial ODs and cultured in 15 mL of minimal medium with 3 % glucose and 0.5 % lactose. The fermentation broth was supplemented with 1 % glucose and 1 % methanol at 48 h. (C) Analysis of metabolic profiles and cell growth via multi time-point sampling of the HK70 strain under shake flask cultivation. The fermentation conditions were as follows: 3 % glucose, 1 % methanol, an initial OD_{600} of 0.1, and 0.5 % lactose supplementation. (D) Effect of varying methanol concentrations at a fixed glucose concentration of 3 % on 2′-FL production. (E) Methanol residue at different methanol supplementation levels in the fermentation broth. Data represent the mean ± SD from biological triplicates.

CRediT authorship contribution statement

Yi Li: Writing – review & editing, Writing – original draft, Data curation. **Xiang Wang:** Investigation. **Kaidi Chen:** Investigation. **Zhoukang Zhuang:** Data curation. **Hongting Tang:** Supervision. **Tao Yu:** Funding acquisition. **Wenbing Cao:** Writing – original draft, Funding acquisition.

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Conflict of interest

No potential conflict of interest was reported by the authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2025.04.002.

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