Effect of acetate metabolism modulation on 2'-fucosyllactose production in engineered Escherichia coli

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\section*{ABSTRACT}
2'-Fucosyllactose (2'-FL) is one of the most important oligosaccharides in human milk. In this study, a glucose–glycerol mixed carbon source and the deletion of the genes \textit{arcA} and \textit{iclR} were attempted for the first time in engineered \textit{Escherichia coli} to improve 2'-FL production. The highest 2'-FL titre of 5.04 g/L, almost 1.4 times higher than that in the control strain, was obtained in the \textit{arcA} mutant strain using shake-flask culture. In addition, the \textit{arcA} mutant showed a significantly reduced accumulation of acetate. However, the deletion of \textit{iclR} and the double deletion of \textit{arcA/iclR} provided no benefit in terms of 2'-FL biosynthesis. Overall, these results suggest that deletion of \textit{arcA} could efficiently increase the 2'-FL yield in \textit{E. coli} and could lay a foundation for more efficient and larger-scale production. Moreover, in shake-flask culture under optimal conditions, the 2'-FL titer reached a maximum value of 7.14 g/L.

\section*{Introduction}
Human milk contains a class of carbohydrates with unique structure, called human milk oligosaccharides (HMOs), which are the third most abundant component in breast milk after lactose and lipids. The existence of a correlation between these human milk oligosaccharides (HMOs) and infant health is undoubtful [1,2]. In particular, 2'-fucosyllactose (2'-FL) is not only the most abundant but also one of the most important HMOs involved in biological functions [3,4]. There are several great benefits of 2'-FL supplementation in infant care; for instance, this compound can improve digestion in infants and promote infant brain development as well as exert prebiotic, antibacterial, anti-inflammatory, and antiviral effects [1,5]. These properties make 2'-FL an attractive molecule for nutritional and pharmaceutical applications [4,6,7]. Moreover, 2'-FL has attracted considerable commercial interest as an additive to infant formula [8]. As a result, cost-effective synthesis of 2'-FL has become a research hotspot, since the commercial promotion of this molecule reached a climax around the world [9].

Currently, some approaches [10] for 2'-FL production have been reported in the literature, such as chemical [11–15], chemo-enzymatic [16,17] and enzymatic [18,19]. A substantial body of work has been performed on the microbial synthesis of 2'-FL, and most studies were conducted in \textit{Escherichia coli} due to the simple culture conditions and availability of advanced genetic tools [20,21]. In our previous research, several attempts have been undertaken to improve the 2'-FL production in \textit{E. coli} C41 (DE3)\textit{ΔZ} (Figure 1) [22]. Some genes mediating the biosynthesis of by-products such as colanic acid, leading to consumption of the lactose substrate, were removed [23]. In addition, some other genes enhancing the biosynthesis of guanosine 5'-diphosphate (GDP)-L-fucose were either overexpressed or removed [24]. Moreover, our recombinant strains use glycerol as the main carbon source. Huang et al. [25] reported using glucose as the carbon source, their engineered strain accumulated more 2'-FL than that obtained using glycerol as the carbon source. However, the presence of glucose inhibited the transcription of the \textit{E. coli lac} operon in a lactose-containing medium [24]. Therefore, for to
Improve the 2'-FL yield, we use glucose and glycerol as co-carbon sources for cell growth and maintenance and 2'-FL production. As expected, shake-flash fermentations in mixed carbon sources of glucose and glycerol improved the production of 2'-FL. However, it caused the strain to produce some acetate. Acetate excretion can affect the cell density, biomass accumulation and macromolecule biosynthesis even at concentrations as low as 0.5 g/L [26,27]. As previously reported [28], we found the similarity in our recombinant strains which produced some acetate in the biosynthesis pathway of 2'-FL [22]. Here, we describe another strategy for improving 2'-FL yield, that is, to reduce the accumulation of acetate.

Anaerobic redox control (ArcA) is an important global regulator in *E. coli*. In particular, phosphorylated ArcA has an inhibitory effect on the expression of related genes (*gltA, acnA, icdA, sucABCD, sdhCDAB, fumAB, mdh* and *aceBAK*) in the TCA cycle and the glyoxylate shunt (Figure 1) [29–32]. Knockout of *arcA* alleviates the repression of the TCA cycle, decreases acetate formation and enhances the expression of heterologous genes [33–35]. Isocitrate lyase regulator (IclR) is a local regulator is a local regulator in *E. coli*. IclR are negatively regulates the gene expression of the *aceBAK* operon that codes for the glyoxylate pathway enzymes (Figure 1) [36,37]. The glyoxylate pathway might be activated by the knockout of *iclR*, resulting in a decrease in acetate formation [38–40]. Acetate overflow was significantly decreased and the production of target products such as D-lactate, Poly(3-hydroxyalkanoates), 1,3-propanediol and L-threonine was increased by deleting *arcA* or *iclR* [34,35,41–43]. The effects of single deletions of genes encoding global regulators such as ArcA have been widely studied [44]. However, the outcomes of their combined knockouts have rarely been investigated, although some reports have highlighted that the combined deletion of *arcA* and *iclR* has profound effects on metabolism [45–47]. It is, therefore, plausible to enhance production of 2'-FL by *arcA* or *iclR* deletion.

In this work, *arcA*, *iclR*, and *arcA/iclR* double mutant strains were constructed using λ Red homologous recombination [48], and the production of 2'-FL in these mutant strains was investigated using shake-flask fermentation. As expected, the engineered strain with *arcA* mutation hardly produced acetate and presented an increase of 38.8% in the 2'-FL production.

**Figure 1.** Metabolic pathways of *E. coli* for 2'-Fucosyllactose (2'-FL) production and microbial growth. The intracellular enzymes are abbreviated as follows: ManB, phosphomannomutase; ManC, α-D-mannose-1-phosphate guanylyltransferase; Gmd, GDP-mannose-6-dehydrogenase; WcaJ, UDP-glucose: undecaprenyl-phosphate glucose-1-phosphate transferase; LacZ, β-galactosidase; FutC, α-1,2-fucosyltransferase.

Note: The acronyms, DHA, G3P, DHAP, PEP and TCA, refer to dihydroxyacetone, glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, phosphoenolpyruvate and tricarboxylic acid, respectively. Genes involved in those pathways are in italics.
compared with the control strains. The work presented here demonstrates the first use of \textit{arcA} deletion mutant to improve the biosynthesis of 2'-FL. Finally, to maximize the production performance of the selected high-yield strain, we systematically optimized the medium components and induction conditions. In shake-flask culture under optimal conditions, the 2'-FL titer reached a maximum value of 6.95 g/L.

Materials and methods

\textbf{Bacterial strains and culture conditions}

The strains used for this study are listed in Table 1. All strains were grown at 37 or 30°C in Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone, and 10 g/L NaCl) with rotary shaking at 230 rpm. Media were supplemented with kanamycin (50 mg/L), spectinomycin (50 mg/L), isopropyl-\(\beta\)-d-thiogalactopyranoside (IPTG; 0.3 mmol/L), or arabinose (10 mmol/L) when necessary.

\textbf{Gene deletions}

The complete list of primers used for this study is shown in Table 1. The gene \textit{arcA} was deleted from the genome of the \textit{E. coli} FL-B2 strain through a \(\lambda\) Red recombinase protocol using the primers listed in Table 1. First, a knockout cassette (a kanamycin resistance gene flanked by FLP recognition sites and an \textit{arcA}-targeting region) was generated using polymerase chain reaction (PCR) with the primers \textit{arcA}-Kan-F/R, using the kanamycin resistance marker from the plasmid pKD4 as template (Table 1). Second, bacteria were transformed using electroporation with the plasmid pKD46, allowing the expression of the \(\lambda\) Red recombinase and the knockout cassette, and kanamycin-resistant strains were selected. Successful recombination was confirmed using PCR with check primers (\textit{arcA}-DF/DR) (Table 1). Then, antibiotic resistance was removed through expression of the FLP recombinase from the pCP20 plasmid, and successful deletions were determined by loss of antibiotic resistance and using PCR with loci check primers (\textit{arcA}-DF/DR) (Table 1). The resultant strain was named FL-01. Using the same method, the strain FL-02 was constructed from FL-B2 by deleting \textit{iclR}, whereas FL-03 was constructed from FL-01 by deleting \textit{iclR}.

\textbf{Construction of recombinant plasmids}

The plasmids used in this study are listed in Table 1. The pRSF-CBGW plasmid was constructed from the vector pRSF-Duet-1 to express mannose-1-phosphate guanylyltransferase (ManC, E.C. 2.7.7.22), phosphomannomutase (ManB, E.C. 5.4.2.8), GDP-D-mannose-4,6-dehydratase (Gmd, E.C. 4.2.1.47), and GDP-L-fucose

\begin{table}
\centering
\caption{Strains, plasmids and primers in this study.}
\begin{tabular}{|l|l|l|}
\hline
\textbf{Name} & \textbf{Description} & \textbf{Source} \\
\hline
\hline
\textit{E. coli} DH5\textalpha & \textit{F} endA1 glnV44 thi-1 recA1 gyrA96 deoR80 lacZΔM15 lacY1 araL-leu2-6His6 gal dcm (DE3) & Invitrogen \\
\hline
\textit{E. coli} C41(DE3) & \textit{F} ompT hsdSB (rB- mB-) gal dcm (DE3) & Thermo Fisher Scientific \\
\hline
C41 (DE3)ΔZ & \textit{E. coli} C41 (DE3): ΔlacZ & Lab stock \\
\hline
FL-01 & C41 (DE3)ΔZ\textit{muCl}\textiΔwcaj & Lab stock \\
\hline
FL-01 & FL-01ΔarcA & This study \\
\hline
FL-02 & FL-02ΔiclR & This study \\
\hline
FL-03 & FL-03ΔarcAΔiclR & This study \\
\hline
FL-011 & FL-01/pRSF-CBGW/ pfET-fucC & This study \\
\hline
FL-012 & FL-02/pRSF-CBGW/ pfET-fucC & This study \\
\hline
FL-031 & FL-03/pRSF-CBGW/ pfET-fucC & This study \\
\hline
\hline
\textbf{Plasmids} & & \\
\hline
pKD46 & Expressed \(\lambda\)-red recombinase (gam, bet, exo), repA101(Ts) bla arac ParaB-Red, AmpR & [48] \\
\hline
pRD4 & FRT aph FRT P57 P52 ornR6K, KanR & [48] \\
\hline
pCP20 & cI857 IRES fop pSC101 oriT5, Amp\textiR Cm\textiR & [48] \\
\hline
pRSF-Duet-1 & Two T7 promoters, RSF 1030 replicon, Kan\textiR, high copy number, \(\sim\)100 copies/cell & Novagen \\
\hline
pETDuet-1 & Two T7 promoters, pBR 322 replicon, Amp\textiR, medium copy number, \(\sim\)40 copies/cell & Novagen \\
\hline
pRS-CBGW & pRSF-Duet-1 + manC-manB-T7 terminator (Ncol/NotI) + gmd-wcaG (Ndel/AvrII), Kan\textiR & This laboratory \\
\hline
pfET-fucC & pETDuet-1 + fucC (Ndel/AvrII), Amp\textiR & This laboratory \\
\hline
\textbf{Primers}\textsuperscript{a,b} & Sequence (\textit{S}\textsuperscript{\(\prime\)} to \textit{T}) & \textsuperscript{a,b} \\
\hline
\textit{arcA}-Kan-F & ACCCCCGACATCTCTATCAGTTAAGACGGTTGAACAAGCAACGTGATGGCTGAGTCGCTTCC & This study \\
\hline
\textit{arcA}-Kan-R & TACACCAAGAACGTAACCTTCACCCAGGAGTGGTGGCGGATGATGTTGAATTACGCACTGATCC & This study \\
\hline
\textit{arcA}-F & CATGCATCAGGCAGGTCCAG & This study \\
\hline
\textit{arcA}-R & GAGCAAGATCAATCAGTATGG & This study \\
\hline
\textit{iclR}-Kan-F & CCCGCCGTTGTCACCGCCACCAA CCCGGCACTGGAACAGGTTCAGCTTCATTTAGTCTAGGGCTGAGTCGCTTCC & This study \\
\hline
\textit{iclR}-Kan-R & CGATCCACCGATGCGCGACCGTACCTCTCCTGCGGTTTAACTTGAATTACGCACTGTGCC & This study \\
\hline
\textit{iclR}-F & TACCATCGGATCATGTCAGCAAC & This study \\
\hline
\textit{iclR}-R & ATGCCACTCAGTATGATG & This study \\
\hline
\end{tabular}
\end{table}
Shake-flask cultivation of recombinant E. coli strains

The initial medium for 2'-FL production was prepared as described by Huang et al. [25] with minor modifications. In brief, this medium consisted of 30 g/L glycerol, 17.9 g/L Na₂HPO₄·12H₂O, 3.1 g/L KH₂PO₄, 2.0 g/L NH₄Cl, 1.0 g/L (NH₄)₂HPO₄, 1.7 g/L citric acid, 15 mg/L CaCl₂, 2 g/L yeast extract, 2.2 g/L C₆H₅Na·2H₂O, 1 g/L MgSO₄·7H₂O, 0.3 mL/L Triton-X 100, 10 mg/L vitamin B₁ and 10 mL/L trace element solution (25 g/L FeCl₃·6H₂O, 2 g/L CaCl₂·2H₂O, 2.0 g/L ZnCl₂, 1.9 g/L CuSO₄·5H₂O, 0.42 g/L MnCl₂·H₂O, 2 g/L Na₂B₄O₇·10H₂O and 2 g/L Na₃MoO₄·2H₂O). For flask fermentation, E. coli strains were streaked from frozen stocks onto LB agar plates, which were incubated overnight at 37°C. Then, one loop of colonies was then inoculated in 5 mL of LB medium and cultivated overnight. Next, the seed culture was transferred into a 250-mL flask containing 100 mL of fermentation medium with appropriate antibiotics (100 g/mL ampicillin and 50 g/mL kanamycin) at an initial OD₆₀₀ of 0.1 and cultivated until it reached an OD₆₀₀ of ~0.8. Then, the culture was transferred into a 500-mL flask containing 100 mL of fermentation medium with appropriate antibiotics (100 g/mL ampicillin and 50 g/mL kanamycin) at an initial OD₆₀₀ of 0.2 and cultivated at 37°C. Finally, when the OD₆₀₀ of the culture reached about 0.8, lactose and IPTG were added to a final concentration of 8 g/L and 0.3 mmol/L, respectively, and cultures were further incubated for another 96 h at 26°C with rotary shaking (250 rpm).

Analytical methods

Growth of E. coli was monitored by measuring OD₆₀₀ using an UV-1800 spectrophotometer. During fermentation, 2 mL of the culture broth was sampled at different time points. A 1-mL aliquot was boiled for 5 min and filtered for metabolite analyses. Another 1-mL aliquot was centrifuged at 17 800 × g for 10 min at 4°C. Harvested cells were washed with 10 mL of phosphate-buffered saline (PBS; 10 mmol/L Na₂HPO₄·12H₂O, 10 mmol/L NaH₂PO₄·2H₂O and 500 mmol/L NaCl, pH 7.4) and centrifuged at 17 800 × g for 10 min at 4°C. The cell pellet was dried to constant weight at 80°C for 4 h and then the dry cell weight (DCW) was measured. The supernatant was collected and measured by using an Agilent high-performance liquid chromatography (HPLC) system (1,200 Series) equipped with an Aminex HPX-87H column (Bio-Rad) and a refractive index (RI) detector (Agilent). The column heated at 60°C was applied to analyse 100 μL of diluted culture broth. Sulphuric acid (H₂SO₄) 5 mmol/L solution was used as a mobile phase at a flow rate of 0.5 mL/min. The standard curves were generated using standard 2'-FL (Carbosynth Ltd, CAS: 41263-94-9), glycerol (Solarbio, CAS: 56-81-5), lactose (Sigma, CAS: 64044-51-5), and acetate (Aladdin, CAS: 64-19-7) and a sensitive and reliable external standard HPLC method for determining the content of metabolites (2'-FL, glycerol, lactose and acetate) was set up.

Results and discussion

Effects of glucose on 2'-FL production

The main carbon source of the initial medium was glycerol (30 g/L). But glucose, as a ‘fast’ carbon source, would be more suitable for cell growth and recombinant protein expression. According to Huang et al. [25], their engineered strain using glucose as a carbon source produced more 2'-FL than that using glycerol as a carbon source. However, the presence of glucose inhibited the transcription of the E. coli lac operon as a fast carbon source, whereas 2 g/L of glucose exerted stronger repression on 2'-FL production than on cell growth. Indeed, increasing glucose concentrations triggered a trend of early increase and late decrease in the maximum DCW and the production of 2'-FL. Hence, different concentrations of glucose (5–25 g/L) were added to the initial fermentation medium to investigate their effects on the production of 2'-FL in the strain FL-B21 and to identify the optimal carbon source to use in combination with glycerol. As shown in Figure 2, the glucose concentration obviously affected the maximum DCW and the production of 2'-FL. Indeed, increasing glucose concentrations triggered a trend of early increase and late decrease in the maximum DCW and 2'-FL production. In particular, 15 g/L of glucose showed an inhibitory effect on cell growth and 2'-FL production, whereas 20 or 25 g/L of glucose exerted stronger repression on 2'-FL production than on cell growth. The maximum DCW and 2'-FL production were 6.42 and 3.63 g/L, respectively, and were both obtained at a glucose concentration of 10 g/L. Thus, the best carbon source combination was found to consist of a mixture of 10 g/L of glucose and 30 g/L of glycerol.

However, as shown in Table 2, strain FL-B21 produced 1.8 g/L acetate in the initial fermentation medium supplemented with 10 g/L of glucose. As previously reported [28], the strains for producing 2'-FL
also produced acetate. The concentration of acetate in cells, even as low as 0.5 g/L, will affect cell density, biomass accumulation and macromolecular biosynthesis [26,27]. So, an attempt was made to delete arcA and iclR gene in strain FL-B21, to reduce the accumulation of acetate and boost 2'-FL production.

**Effects of arcA and iclR gene knockout on cell growth**

The arcA, iclR and arcA/iclR double mutant E. coli strains were constructed using the λ Red recombinase protocol, transformed with recombinant plasmids, and then tested for 2'-FL production. The control strain (FL-B21) and arcA, iclR and arcA/iclR mutants (namely FL-011, FL-021 and FL-031, respectively) were cultivated in Erlenmeyer flasks at 26°C for 96 h, after induction via addition of IPTG at a final concentration of 0.3 mmol/L. As shown in Figure 3(A), compared with that of the control FL-B21, the maximum DCW of the mutant strains FL-021 and FL-031 was slightly increased (by 6.2% and 3.1%, respectively), whereas that of the arcA mutant FL-011 was decreased by 3.9%. Although FL-011 grew better than FL-B21 for the first 32 h of culture, it grew slower than FL-B21 later. Consistent with its growth status, FL-011 consumed glycerol faster than FL-B21 during the first 32 h of culture (Figure 3(B)). However, after 32 h, the glycerol consumption of FL-011 slowed down, resulting in a residual glycerol content slightly higher than that of FL-B21. In addition, the cell yield of each strain was also calculated. FL-011 achieved 2.06 g DCW/g glycerol, a 3.7% lower yield than that of the control strain FL-B21 (Figure 4). And, the cell yields of FL-021 and FL-031 were slightly higher than that of FL-B21. These
results indicate that deletion of *arcA*, *iclR*, or both did not significantly affect the growth of the *E. coli* cells.

**Deletion of arcA or iclR gene overcomes acetate overflow**

Next, we examined the effect of *arcA* and *iclR* knockout on the production of acetate. As shown in Figure 5(A), the control strain FL-B21 produced acetate continuously throughout the period of culture. After 96 h of cultivation, the production of acetate reached the highest yield of 1.80 g/L. In contrast, the *arcA* mutant FL-011, the *iclR* mutant FL-021, and the *arcA/iclR* mutant FL-031 hardly produced acetate. These results indicated that *arcA* and *iclR* mutants could dramatically decrease acetate accumulation during 2′-FL production. This decrease of acetate accumulation may be due to the increase in TCA cycle flux. Nizam et al. [51] reported the effect of *arcA* knockout on acetate formation in *E. coli* K12 strain, and their results were not as significant as shown in this study. This could be a result of different host strains. In addition, as studied by Liu et al. [47], the ability to overcome acetate formation of *arcA/iclR* double mutants was less than that of the *arcA* single mutants. In our study, however, *arcA*, *iclR* and *arcA/iclR* double mutant strains all showed reduced acetate production remarkably as compared to the control strains. The regulatory mechanisms remained unclear and needed to be resolved.

**Knockout of arcA and/or iclR increases 2′-FL production**

The effect of *arcA*, *iclR* and *arcA/iclR* knockout on 2′-FL production by *E. coli* FL-B21 was also investigated (Figure 5(B)). The 2′-FL yield of the *arcA* mutant FL-011 was 5.04 g/L, approximately 1.4 times higher than that of the control strain FL-B21. Notably, the conversion efficiency of glycerol to 2′-FL was increased by 1.4-fold by *arcA* knockout, from 13.6% to 19.4% (Figure 4). Lactose is another substrate for the production of 2′-FL. Similarly, upon *arcA* knockout, the efficiency of lactose conversion to 2′-FL also increased by 1.6-fold, from 79.2% to 122.5%. These data suggest that

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**Figure 4.** The effects of *arcA* and/or *iclR* knockout on the cell phycology and metabolism of recombinant *E. coli* strains in shake-flask cultures.  
Note: The lactose conversion efficiencies, glycerol conversion efficiencies, cell yields of strains and 2′-FL production per gram of DCW. The lactose and glycerol conversion efficiencies are the mass ratio of 2′-FL production and lactose and glycerol consumption, respectively. All experiments were conducted at least thrice and the error bars represent the standard deviations.

**Figure 5.** Metabolite production profiles of FL-011, FL-021 and FL-031 strains cultivated in flasks. (A) Acetate, (B) 2′-FL for FL-011, FL-021 and FL-031.  
Note: All experiments were conducted at least thrice and the error bars represent the standard deviations.
knockout of arcA significantly improved the production of 2'-FL as well as the glycerol and lactose conversion efficiency. Moreover, it is speculated that the genetic modification of arcA knockout could result in a highly efficient production of 2'-FL and reduce the manufacturing cost in industrial applications. The engineering design of global regulatory agencies may show phenotypes and changes in carbon fluxes and metabolic pathways. It is hard to determine which particular genes or pathways were affected by the knockout of the global regulator ArcA. So, at present, the regulatory mechanisms of global regulator ArcA are unclear and need to be further explored.

On the contrary, compared with that in the control FL-B21, 2'-FL production sharply decreased in the iclR mutant FL-021. Indeed, only 1.81 g/L of 2'-FL was accumulated by FL-021 (Figure 5(B)), and the conversion efficiency of glycerol and lactose to 2'-FL were also decreased by 63.7% and 37.1%, respectively (Figure 4). In addition, the arcA/iclR mutant FL-031 produced 3.89 g/L of 2'-FL, a 114.9% higher yield than that observed in FL-021, but 22.8% lower than that observed in FL-011 (Figure 5(B)). The conversion efficiency of glycerol and lactose to 2'-FL were increased by 110.4% and 56.0%, respectively, compared with those of FL-021 (Figure 4). These results further indicate that deletion of arcA could improve the production of 2'-FL and the conversion efficiency of glycerol and lactose to 2'-FL, while the deletion of iclR resulted in no benefit for the biosynthesis of 2'-FL. On the one hand, the maximum DCW of the iclR mutant increased by 6.2%, which may affect the cell physiology and metabolism. On the other hand, the deletion of iclR may promote the growth of the cells instead of the biosynthesis of 2'-FL, but the molecular mechanisms are not clear completely.

Besides, compared with FL-B21, FL-011 displayed an enhanced 2'-FL production per gram of DCW, which increased by 0.5-fold, from 0.56 to 0.82 g 2'-FL/g DCW (Figure 4). Furthermore, compared with FL-021, FL-031 showed an enhanced 2'-FL production per gram of DCW, which increased by 1.2-fold, from 0.27 to 0.59 g 2'-FL/g DCW (Figure 4). These data show that deletion of arcA led to improved 2'-FL production per gram of DCW. Therefore, the arcA mutant FL-011 clearly contributed to the production of 2'-FL and was selected for further study.

Optimization of culture medium compositions

The optimization of culture medium composition can effectively enhance cell density and the expression of recombinant proteins in E. coli and consequently increase the yield of target products [52]. Therefore, the effects of different culture medium compositions on the production of 2'-FL by FL-011 were investigated with respect to the original fermentation medium.

Effect of phosphate on 2'-FL production

Phosphate, one of the crucial elements for E. coli growth and metabolism, is essential for the growth of cells [53]. In the initial fermentation medium, phosphate was supplied in the form of Na2HPO4·12H2O, KH2PO4, and (NH4)2HPO4, among which Na2HPO4·12H2O was the most concentrated compound at 50 mmol/L. To study the effect of phosphate on 2'-FL production, the concentration of Na2HPO4·12H2O was changed through a series of small increments within the range of 20–200 mmol/L. As indicated in Figure 6(A), when the Na2HPO4·12H2O concentration increased from 0 to 50 mmol/L, the production of 2'-FL was enhanced from 2.01 to 5.04 g/L and the final OD600 increased from 14.04 to 17.00. However, with concentrations higher than 50 mmol/L, both the production of 2'-FL and OD600 gradually decreased. Therefore, Na2HPO4·12H2O significantly affected the cell growth and 2'-FL production of E. coli. For shake-flask culture, the optimum Na2HPO4·12H2O concentration was found to be 50 mmol/L, corresponding to the concentration of the original medium.

Effect of nitrogen sources on 2'-FL production

During the growth of recombinant E. coli, the nitrogen and carbon sources in the fermentation medium act as pivotal elements in the synthesis of the recombinant product. The nitrogen source is one of the most important components of microbial culture media, since nitrogen is mainly used for the synthesis of cell macromolecules, such as proteins and nucleic acids, as well as of some other nitrogen-containing metabolites. Nitrogen sources include organic and inorganic sources. It is acknowledged that proper combinations of organic and inorganic nitrogen sources can promote cell growth and enhance the expression of recombinant proteins [54].

The original fermentation medium contained Oxoid yeast extract as an organic nitrogen source and NH4Cl and (NH4)2HPO4 as inorganic nitrogen sources. Thus, at first, different concentrations of Oxoid yeast extract were used to investigate their effects on 2'-FL production. The results are shown in Figure 6(B). The highest OD600 of FL-011 (16.99) was reached at an Oxoid yeast extract concentration of 2 g/L. Simultaneously, the
highest 2'-FL production (5.04 g/L) also occurred at this Oxoid yeast extract concentration. Therefore, the optimum Oxoid yeast extract concentration was determined as 2 g/L, corresponding to the concentration of the original shake-flask medium.

Next, various concentrations of organic nitrogen sources such as Oxoid tryptone and fish peptone were added to the fermentation medium. The results are shown in Figure 6(C,D). The highest 2'-FL production (5.49 g/L) and OD<sub>600</sub> (20.43) were obtained at a fish peptone concentration of 10 g/L. The highest OD<sub>600</sub> (20.92) of FL-011 was achieved at an Oxoid tryptone concentration of 5 g/L. However, such high OD<sub>600</sub> did not lead to high 2'-FL production. The highest 2'-FL production (6.17 g/L) appeared at an Oxoid tryptone concentration of 15 g/L. Therefore, Oxoid tryptone may be the most suitable nitrogen source for 2'-FL production because it led to higher 2'-FL yields (5.85 and 6.17 g/L, respectively) than those triggered by fish peptone (5.49 and 5.18 g/L, respectively) at the same concentrations (10 and 15 g/L).

**Optimization of induction conditions**

In addition to the culture medium composition, the conditions that induce recombinant protein production by *E. coli* also influence cell growth and synthesis of target products. Therefore, the induction conditions, such as initial induction OD<sub>600</sub>, induction temperature and inducer concentration, were optimized systematically.

Temperature is not only one of the most important factors associated with the growth and reproduction of microorganisms, but also the key factor affecting the expression of recombinant proteins. Thus, its influence on the production of 2'-FL by *E. coli* FL-011 was evaluated at five different induction temperatures (24, 26, 28, 30 and 32°C). The results (Figure 7(A)) showed that neither the highest nor the lowest induction
temperatures tested promoted 2'-FL production or cell growth in the recombinant strain FL-011. However, among the intermediate temperatures tested, it was observed that, with the increase in the induction temperature, OD_{600} and 2'-FL production was first increased and then decreased. The highest OD_{600} (19.41) and 2'-FL production (6.58 g/L) were obtained at an induction temperature of 28°C.

The lac promoter and its derivatives are widely used in the expression of exogenous genes. IPTG can inhibit the repression of the lac promoter; therefore, the level of recombinant proteins is regulated by the concentration of IPTG in the medium. Considering the high cost of IPTG, lactose is often a valid substitute for this inducer to induce protein expression [55]. However, lactose can be used not only as an inducer but also as a carbon source and a substrate in the process of 2'-FL production. Thus, using lactose as an inducer is more complex than using IPTG. Therefore, the induction of protein expression for 2'-FL biosynthesis by FL-011 was conducted at various concentrations of lactose (8–130 g/L). The results showed that the yields of 2'-FL were between 0.2 and 0.7 g/L when lactose alone was supplied as an inducer (data not shown). Therefore, lactose cannot be used as an effective inducer to achieve high yields in strain FL-011. This could be because lactose is the carbon source for cell metabolism and the substrate for 2'-FL production. Its concentration changes dynamically. So, there is not enough lactose in the cell as an inducer. On the contrary, IPTG, as an inducer, is not degraded and metabolized in *E. coli* and has a long-lasting induction effect.

Consequently, different concentrations of IPTG (0.2–0.7 mmol/L) were used to investigate their effects on the production of 2'-FL. The results are shown in Figure 7(B). The highest 2'-FL production (7.14 g/L) and OD_{600} (21.24) were obtained at an IPTG concentration of 0.5 mmol/L. Since IPTG is toxic to bacteria and at high concentrations it may even kill cells, IPTG concentrations of 0.6 and 0.7 mmol/L inhibited the growth of the strain and reduced the 2'-FL yield. Furthermore, the 2'-FL production by strain FL-011 was induced at different initial induction OD_{600} values (0.5, 0.7, 0.9, 1.1 and 1.3). The production of 2'-FL was almost the same at all induction OD_{600} tested (data not shown).

Based on the above optimization, compared with the initial culture conditions, the optimum fermentation medium was supplemented with 10 g/L glucose and 15 g/L Oxoid tryptone. The optimum concentration of the inducer IPTG was increased from 0.3 to 0.5 mmol/L and the optimum induction temperature was enhanced from 26 to 28°C. Finally, the optimum culture conditions for 2'-FL production were as follows: medium supplemented with 30 g/L glycerol, 10 g/L glucose, 2 g/L yeast extract, 15 g/L Oxoid tryptone, 17.9 g/L Na_{2}HPO_{4}·12H_{2}O, 3.1 g/L KH_{2}PO_{4}, 2.0 g/L NH_{4}Cl, 1.0 g/L (NH_{4})_{2}HPO_{4}, 1.7 g/L citric acid, 15 mg/L CaCl_{2}, 2.2 g/L C_{6}H_{12}Na_{2}H_{3}O, 1 g/L MgSO_{4}·7H_{2}O, 0.3 mL/L Triton-X 100, 10 mg/L vitamin B1 and 10 mL/L trace element solution (25 g/L FeCl_{3}·6H_{2}O, 2 g/L CaCl_{2}·2H_{2}O, 2.0 g/L ZnCl_{2}, 1.9 g/L CuSO_{4}·5H_{2}O, 0.42 g/L MnCl_{2}·H_{2}O, 2 g/L Na_{2}SO_{4}·10H_{2}O and 2 g/L Na_{2}MoO_{4}·2H_{2}O); induction with 0.5 mmol/L IPTG at an initial induction OD_{600} of 0.7–0.9; and growth at 28°C for 96 h. Cultivation in
shake flasks was performed in the optimized medium under the optimized induction conditions and resulted in a high 2'-FL production of 7.14 g/L. Such yield was 1.42 times higher than that (5.04 g/L) before optimization.

The production levels of 2'-FL in shake-flask fermentation in previous studies were compared in Table 3 [23,28,56–59]. Currently, the highest yield of 2'-FL in shake-flask fermentation is 15.4 g/L [28]. The difference from this study is that the strain uses WcfB instead of FutC as an α-1,2-fucosyltransferase. In the reports using FutC as the α-1,2-fucosyltransferase for production of 2'-FL, the highest reported yields were about 6-7 g/L [20,23,28,57–59]. Our engineered strains with a deletion of the arcA gene used a mixed carbon source of glucose and glycerol and under optimized fermentation conditions reached high 2'-FL production of 7.14 g/L. 2'-FL, being one of the most abundant oligosaccharides in human milk, is involved in many biological functions beneficial for infant health. 2'-FL has a great potential in applications for functional food materials and pharmaceuticals.

Conclusions

In this work, we first used glucose and glycerol as co-carbon sources to improve 2'-FL production from 2.67 to 3.62 g/L. Then, the effect of the deletion of the genes arcA or iclR on 2'-FL production by E. coli FL-B21 was investigated. Knockout of arcA and iclR was surprisingly useful for avoiding acetate formation. Moreover, the arcA mutant FL-011 displayed higher 2'-FL production and higher lactose and glycerol utilization efficiency than the control strain FL-B21. The 2'-FL production of the arcA mutant FL-011 was 5.04 g/L. To maximize 2'-FL production by FL-011, systematic optimization of the culture medium composition and induction conditions was performed. As a result, the production of 2'-FL reached 7.1 g/L in shake-flask culture.

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Author contributions

Yingxue Liao conceived and designed the experiments. Yingxue Liao and Yuanfei Ge performed the experiments. Zhijian Ni, Jinyong Wu and Zhongkui Li contributed to the experiment plan and discussion of the manuscript. Yingxue Liao wrote the manuscript under the guidance of Xiangsong Chen and Jianming Yao. All authors reviewed and approved the manuscript.

Disclosure statement

The authors have no financial conflicts of interest to declare.

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