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Fine-Tuning the Expression of the Glycolate Biosynthetic Pathway in *Escherichia coli* Using Synthetic Promoters

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Abstract: Glycolate plays an important role as a platform chemical in both polymeric material and cosmetic industries. However, the microbial production of glycolate often encounters challenges associated with unbalanced metabolic flux, leading to a notably low titer. Additionally, the use of expensive inducers, such as IPTG, contributes to an increase in the overall production cost. To address these issues, the key enzymes involved in the glycolate biosynthetic route, including citrate synthase (*gltA*), isocitrate lyase (*aceA*), isocitrate dehydrogenase kinase/phosphatase (*aceK*) and glyoxylate reductase (*ycdW*), were overexpressed in *E. coli* under the control of inducible promoters with varying strengths in order to determine the optimal combination. Subsequently, the glycolate pathway was further modulated by replacing inducible promoters with various constitutive synthetic promoters. Through this systematic optimization, the best strain, named Mgly4T1562, produced 3.02 g/L glycolate with 97.32% theoretical yield in shake-flask cultivation. The titer further increased to 15.53 g/L in a fed-batch experiment. Notably, this study marks the first successful utilization of synthetic promoters in tuning the glycolate biosynthetic pathway for glycolate biosynthesis. The strategy presented in this research holds significant promise for facilitating the cost-effective and industrially viable production of glycolate without the need for expensive inducers.

Keywords: glyoxylate shunt; promoter library; constitutive promoter; metabolic engineering; biotechnology

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1. Introduction

Glycolate, known as the simplest hydroxy acid, functions as both alcohol and acid [1–4]. It is an important platform chemical in the cosmetic industry and polymeric materials, such as poly(glycolate) and poly(lactate-co-glycolate) [5,6]. Projections indicate that the global glycolate market will experience gradual growth at an annual rate of 6.6% from 2022, ultimately reaching a value of USD 450 million by 2027 [7]. Currently, industrial glycolate production primarily involves the chemical treatment of formaldehyde in petrochemical feedstocks with carbon monoxide and water. However, this high-temperature and high-pressure reaction requires sophisticated equipment and expensive and toxic precursors, raising significant environmental and economic issues [3]. In the pursuit of sustainable and eco-friendly glycolate production, there has been considerable focus on exploring its biosynthesis.

Glycolate is biosynthesized naturally by some chemolithotrophic iron- and sulfur-oxidizing bacteria, or it can be derived from glycolonitrile through hydrolysis by the nitrilase enzyme, albeit at a very low titer. For example, efforts have been made to produce glycolate using a variety of microorganisms, such as *Escherichia coli*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae* and *Kluyveromyces lactis* [1]. These microorganisms utilize the naturally occurring glyoxylate shunt, including isocitrate lyase (*aceA*), isocitrate dehydrogenase kinase/phosphatase (*aceK*) and glyoxylate reductase (*ycdW*). Notably,

E. coli is favored for glycolate production due to its rapid growth, well-established biological background and mature gene editing tools [8]. In recent years, several pathways have been designed for improving glycolate production from glucose or other substrates, such as D-xylose, ethanol, L-arabinose, glycerol, acetate, and formaldehyde. These pathways include modified glyoxylate shunt, D-xylulose-1-phosphate, D-ribulose-1-phosphate, L-xylulose-1-phosphate and the Dahms pathway [1,2,9–12]. Among them, glycolate synthesis via a modified glyoxylate shunt using glucose as a substrate is extensively studied and proven to be the most efficient pathway [1,3]. Basically, the modified glycolate pathway often includes the overexpression of a citrate synthase (*gltA*) in the TCA cycle to enhance the carbon flux of glyoxylate, thereby improving the glycolate titer (Figure 1A). Following this approach, an engineered *E. coli* strain (AG0956), overexpressing *aceA* and *ycdW*, successfully obtained 52.2 g/L glycolate from glucose with 45% theoretical yield [13]. Another engineered *E. coli* strain (Mgly434) with the overexpression of *aceA*, *aceK*, *ycdW*, and *gltA* obtained 65.5 g/L glycolate with 90% of the theoretical yield [14]. Despite these advancements, there remains scope for further enhancement, particularly with regard to achieving the theoretical maximum yield (0.85 g/g-glucose) [15].

Furthermore, the aforementioned studies [13,14] rely on expensive inducers, resulting in driving up cultivation costs. Moreover, the overexpression of the modified glycolate pathways in these studies using inducer-dependent promoters like T7 and Tet promoter has been proven to surpass the requirements for glycolate production. This disparity often results in imbalances in protein synthesis, cell growth, and product synthesis, leading to a severe metabolic burden, inconsistent gene expression and flow distribution, as well as the squandering of the cellular building blocks and energy [16–19]. Therefore, in order to solve the above problems, strategies for metabolic engineering and synthetic biology like balancing heterologous gene expression and promoter engineering are often used to balance the metabolic flow of cells and promote the maximum flow of carbon sources and energy to glycolate synthesis [20]. Notably, it is imperative to fine-tune the gene expression levels in the modified glycolate pathways under the action of constitutive promoters to balance the metabolic flux and avoid the use of expensive inducers.

A promoter is the primary regulatory element in the biosynthesis of target compounds and plays a crucial role in fine-tuning gene expression at the transcriptional level [21–23]. In order to obtain the required constitutive promoter library, Rao et al. constructed a gradient-strength P_{bacA} promoter with various 5′-UTR in *Bacillus licheniformis*, and the expression ranged from 32.6 to 741.8% [24]. Recently, Zhao et al. used the trc promoter as a template by error-prone PCR and obtained a constitutive synthetic promoter library of 3665 mutants in *E. coli*, with ~454-fold difference between the strongest and weakest expression levels [25]. This synthetic promoter can be further used for the precise fine-tuning of the gene expression associated with the glycolate biosynthetic pathway.

In this study, we first identified the gene expression combination of the modified glyoxylate pathway for glycolate production. Subsequently, we established the optimal synthetic promoter replacement strategy without induction. Following this, the optimal synthetic promoter was selected for glycolate production. Finally, the best strain was cultivated in batch or fed-batch experiments. So far, this is the first effort regarding glycolate production using self-developed synthetic promoters.

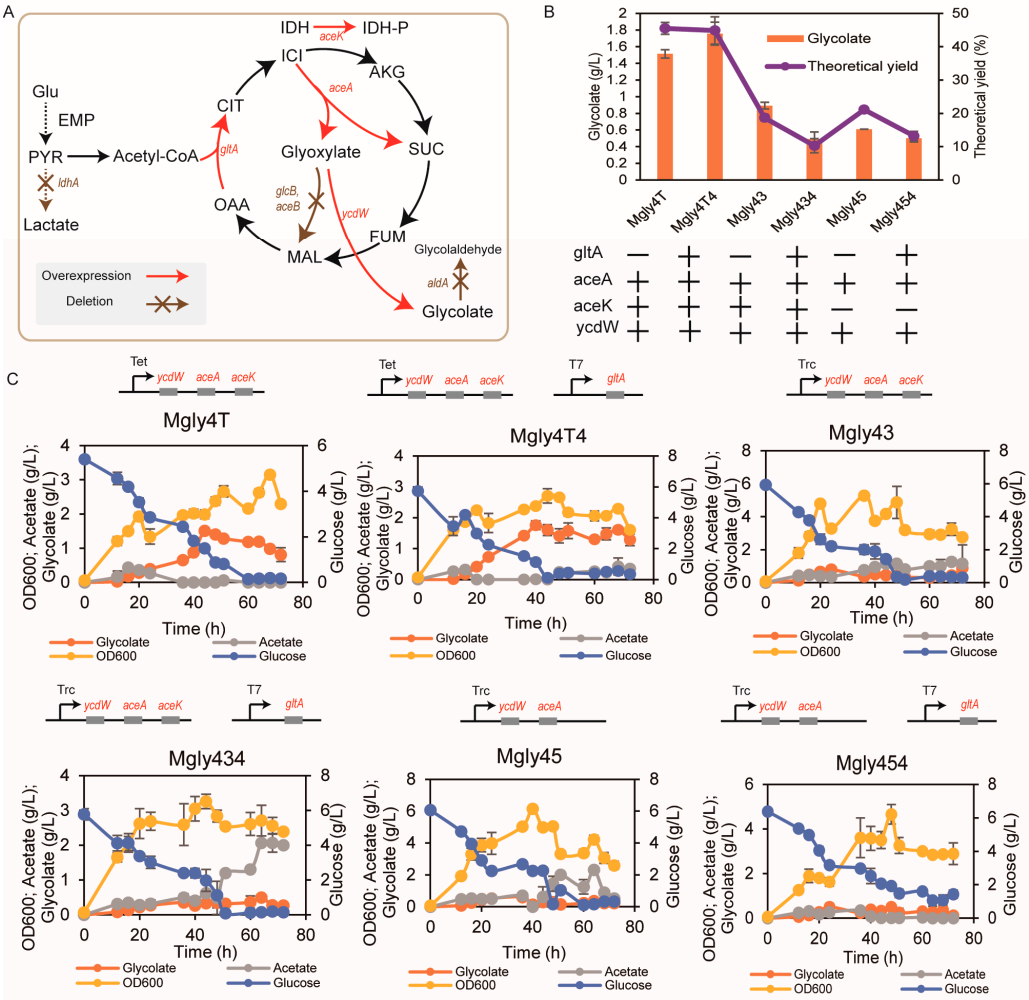

Figure 1. Engineering and optimization of the glyoxylate cycle. (**A**) Glycolate pathway with possible genetic modification for redirecting carbon flux to glycolate in *E. coli*. Glu, glucose; PYR, pyruvate; EMP, glycolysis pathway; CIT, citrate; ICI, isocitrate; AKG, α-ketoglutarate; SUC, succinate; FUM, fumarate; MAL, malate; OAA, oxaloacetate; *gltA*, citrate synthase; *aceA*, isocitrate lyase; *ycdW*, glyoxylate reductase; *ldhA*, D-lactate dehydrogenase; *glcB*, *aceB*, malate synthases; *aldA*, aldehyde dehydrogenase. (**B**) Flask cultivation with recombinant strains Mgly4T, Mgly4T4, Mgly43, Mgly434, Mgly45, Mgly454. Mgly4T: Mgly4 strain harboring pGly-2; Mgly4T4: Mgly4 strain harboring pGly-2, pCDF-*gltA*; Mgly434: Mgly4 strain harboring pTrc-*aceA*-*aceK*-*ycdW*(pJNU-3); Mgly434: Mgly4 strain harboring pTrc-*aceA*-*aceK*-*ycdW*(pJNU-3), pCDF-*gltA*(pJNU-4); Mgly45 strain harboring pTrc-*aceA*-*ycdW*; Mgly454: Mgly4 strain harboring pTrc-*aceA*-*ycdW*, pCDF-*gltA*(pJNU-4). (**C**) Comparison of the glycolate titer and yield for individual strains.

2. Materials and Methods

2.1. Strains and Growth Conditions

The strains utilized in this work are presented in the Supplementary Materials (Table S1). *E. coli* JM109 (obtained from Invitrogen, Carlsbad, CA, USA) was utilized as a cloning host to construct and amplify plasmids. The MG1655 strain (Invitrogen) was used as a host to express Superfolder Green Fluorescent Protein (sfGFP) [26] using different promoters to compare promoter strength. The Mgly4 (MG1655(DE3) Δ*ldhA*Δ*glcB*Δ*aceB*Δ*aldA*) strain was obtained from Deng et al. [14], which was used as the starting strain for producing glycolate. This strain had the capability of reducing the titer of by-products and glycolate degradation and promoting the flow of carbon sources to glycolic acid synthesis.

Strains were cultivated overnight in LB medium with yeast extract (5 g/L), sodium chloride (10 g/L) and peptone (10 g/L) for the pre-culture. The medium was supplemented

with kanamycin (50 μg/mL) or ampicillin (100 μg/mL) when required. Solid media were produced by adding 2% (*w*/*v*) agar to LB broth. For the cultivation of strains in the flask, 0.5 mL of *E. coli* pre-culture was transferred to 250 mL flasks containing 50 mL of M9 minimal medium with a composition of disodium hydrogen phosphate (6.78 g/L), potassium dihydrogen phosphate (3.0 g/L), ammonium chloride (1.0 g/L), sodium chloride (0.5 g/L), glucose (6 g/L), magnesium sulfate (0.241 g/L) and calcium chloride (0.011 g/L). The initial conditions of shaking flask cultivation were 250 rpm orbital shaking and 37 °C, which were changed to 250 rpm and 30 °C when optical density at 600 nm (OD_{600}) reached 0.6. The isopropyl-β-D-thiogalactopyranoside (1 mM) or anhydrotetracycline (250 ng/μL) was added when required for the induction of gene expression and OD reached 0.6. In addition, some commonly used chemicals and reagents were purchased from Sangon Biotech (Shanghai, China).

2.2. Genetic Manipulations and Plasmid Construction

The plasmids utilized in this study were provided for the expression of sfGFP, and the modified glyoxylate pathway for glycolate production are presented in the Supplementary Materials (Table S1). The primers listed in the Supplementary Materials (Table S2) were mainly used for gene and promoter amplification and validation. Molecular-biology-related operations were carried out according to the standardized methods, and all molecular reagents were purchased from Takara (Shiga, Japan). The constructed plasmids were sent to Genewiz (Suzhou, China) for sequencing. Once the sequencing was correct, the next experiment was carried out.

The plasmids pGly-2 [27], pJNU-3 [14], pJNU-4 [14] and pTrc-aceA-ycdw [28] were used to produce glycolate. The pL0-sfGFP and pL3429-sfGFP from Zhao et al. [25] were used to compare promoter strength. The pTet-sfGFP using pL0-sfGFP as the template was constructed by inserting a Tet promoter from pGly-2. The pRep1 plasmid using pGly-2 as the template was assembled by whole-plasmid PCR [29] using primers pRep1 F and pRep1 R. The PCR products were digested by *Dpn* I and CIAP and ligated by T4 ligase. The ligated production was transferred into JM109 (Invitrogen) for screening by colony PCR and Sanger sequencing (veri-pGly3429 F and veri-ycdW R). The plasmids pRep2 and pRep3 were constructed in a similar way. The plasmids pRep1, pRep2 and pRep3 were transformed into Mgly4 to produce strains Mrep1, Mrep2 and Mrep3, respectively, for evaluating the replacement of P_{Tet} with the synthetic promoter PL3429. In order to find the most suitable promoter for producing glycolate, the promoter PL3429 in pRep1 was replaced with PL3153, PL1562, PL3262, PL2353, PL3090, PL2917, PL3149, PL3195, PL2538 and PL1993 from Zhao et al. [25] to form a new plasmid library consisting of pGly3153, pGly1562, pGly2113, pGly2353, pGly3090, pGly2917, pGly1411, pGly3195, pGly2538 and pGly1993. The detail sequence information of these promoters from Zhao et al. [25] are provided in the Supplementary Materials (Table S3). The plasmids pGly3153, pGly1562, pGly2113, pGly2353, pGly3090, pGly2917, pGly1411, pGly3195, pGly2538 and pGly1993 were transformed into Mgly4 to produce strains Mgly4T3153, Mgly4T3429, Mgly4T1562, Mgly4T3262, Mgly4T2353, Mgly4T3090, Mgly4T2917, Mgly4T3149, Mgly4T3195, Mgly4T2538 and Mgly4T1993, respectively, to assess the glycolate production under different synthetic promoters.

2.3. Fluorescence Analysis

The plasmid pTet-sfGFP, pL3429-sfGFP, pL1562-sfGFP, pL3262-sfGFP, pL2353-sfGFP, pL3090-sfGFP, pL2917-sfGFP, pL3149-sfGFP, pL3195-sfGFP, pL2538-sfGFP and pL1993-sfGFP from Zhao et al. [25] were transformed to MG1655 to produce strain MLTet, ML3429, ML1562, ML3262, ML2353, ML3090, ML2917, ML3149, ML3195, ML2538 and ML1993 to test the expression of sfGFP [26] under the control of different promoters. The single colony was picked separately into 96-well plates containing 200 μL of LB medium at 250 rpm and 37 °C, and about 20 μL of overnight cultures was inoculated with 180 μL of M9 medium. The cultures were grown at 250 rpm and 37 °C in a shaking incubator for 2 h. Then, the cells were cultured at 250 rpm and 30 °C in a shaking incubator for 4–6 h.

Next, in order to detect cell growth and whole-cell fluorescence on a Tecan plate reader (Zurich, Switzerland), about 150 µL of the sample was diluted 3 times with PBS buffer. The whole-cell fluorescence was measured under the excitation wavelength (485/60 nm) and emission wavelength (528/60 nm), while optical density was determined by reading absorbance at 600 nm (OD_{600}) [25,30]. The activity of sfGFP was determined by using sfGFP fluorescence/OD_{600} [25].

2.4. Real-Time Quantitative PCR (RT-qPCR)

The total RNA was first extracted by treating the cells during their logarithmic growth phase according to the Ultrapure RNA Kit (CWbiotech, Beijing, China). Next, the obtained RNA was catalyzed by reverse transcriptase from the cDNA Synthesis Kit with gDNA Clean (CWbiotech, China) to synthesize cDNA. Finally, the RT-qPCR reaction was performed in a 96-well optical reaction plate with a total volume of 10 µL of reaction mixture containing 0.5 µL of cDNA as a template, 9.5 µL of SYBR Premix Ex Taq, and two primers (each primer had a 0.5 µM final concentration) on a Bio-Rad CFX RT-qPCR Detect System (Hercules, CA, USA). The 16 s rDNA (an internal reference gene) and the key genes (*aceA*, *aceK*, and *ycdW*) of the modified glyoxylate pathway were amplified for fluorescence quantification. After obtaining Ct values, the transcription levels of target genes were calculated by $2^{-\Delta\Delta Ct}$ [31,32] and evaluated by referring to previous studies [25]. The details of specific primers were provided for RT-qPCR in the Supplementary Materials (Table S2).

2.5. Batch and Fed-Batch Cultivation

For the batch cultivation in a 5 L bioreactor (Baoxing, Shanghai, China), the recombinant strain Mgly4T1562 (Mgly4 carrying pGly1562) was inoculated into M9 minimal medium. The medium contained glucose (10 g/L) and was supplemented with 50 µg/mL of kanamycin (Km) and a seed culture at a concentration of 2% of the total working volume. In order to balance between acetate and glycolate production, the optimum agitation (400 rpm) and aeration (1 vvm) was constant throughout the fermentation process, which provided a higher glycolate titer than that obtained from controlled condition [3,14,28,33]. In addition, the initial culturing temperature was set to 37 °C for cell growth and later adjusted to 30 °C after an OD_{600} reached 0.6 for inducing gene expression or reducing metabolic burden. A pH meter was used to monitor the pH status during the cultivation process, and sodium hydroxide (2 mol/L) was automatically pumped to maintain pH at 7.0.

The significant difference between fed-batch and batch cultivation was that when the glucose concentration reached about 3 g/L, 40% of glucose was further added to the bioreactor in a slow stream, and the concentration of glucose was controlled to maintain a low level to reduce the production of by-products [14]. The rest of the operations are consistent with batch cultivation.

2.6. Analytical Methods

To analyze metabolite concentrations, the supernatant of the cultivation broth was periodically extracted using high-speed centrifugation and then filtered with a 0.22 µm nylon membrane. The various metabolites (glucose, acetate and glycolate) in the supernatant were quantified using an HPLC system (Agilent, Waldbronn, Germany). The specific detection technique was the same as that previously reported [28]. The theoretical maximum yield for glycolate production was calculated to be from D-glucose 0.67 C-mol/C-mol (0.85 g/g) [15]. Therefore, 1 g of glucose could produce a maximum of 0.85 g of glycolate. The glycolate yield is equal to glycolate titer divided by glucose consumption. The percentage of glycolate theoretical yield is equal to glycolate yield divided by theoretical yield (0.85 g/g-glucose). For example, the glycolate yield achieved from glucose in Mgly4T, 0.38682 g/g, is about 45.51% of the theoretical yield.

2.7. Data Availability

The error bars in this study show the standard deviation and correspond to the triplicate measurements made for every experiment.

3. Result

3.1. Gene Expression and Construction of the Modified Glycolate Pathway

The ycdW, aceA, aceK or gltA of the modified glycolate pathway were overexpressed in the MG1655(DE3) ΔldhA ΔglcB ΔaceB ΔaldA strain (Mgly4) under the control of Tet, T7 and Trc promoters to form recombinant strains Mgly4T (Mgly4 carrying pGly-2), Mgly4T4 (Mgly4 carrying pGly-2 and pJNU-4), Mgly43 (Mgly4 carrying pJNU-3), Mgly434 (Mgly4 carrying pJNU-3 and pJNU-4), Mgly45 (Mgly4 carrying pTrc-aceA-ycdw) and Mgly454 (Mgly4 carrying pTrc-aceA-ycdw and pJNU-4), respectively. The Mgly4T strain, under the Tet promoter regulating ydcW, aceA and aceK, produced 1.51 g/L glycolate and 0.0026 g/L acetate, with 45.51% of the theoretical yield of glycolate (Figure 1B,C). Exhibiting very close results, the Mgly4T4 strain, under Tet regulating ydcW, aceA and aceK and the T7 promoter regulating gltA, produced 1.76 g/L glycolate and 0.0058 g/L acetate, with 44.88% of the theoretical yield (Figure 1B,C). Although both strains exhibited similar growth, titer and yield, Mgly4T, lacking overexpressed gltA, faced less metabolic burden in glycolate synthesis compared to that exhibited by the Mgly4T4 strain. The glycolate production by strain Mgly43 under the Trc promoter regulating ydcW, aceA and aceK was 0.89 g/L glycolate and 1.15 g/L acetate, with 18.67% of the theoretical yield (Figure 1B,C). The Mgly434 strain with Trc regulating ydcW, aceA and aceK and the T7 promoter regulating gltA produced 0.49 g/L glycolate and 2.06 g/L acetate, with 10.27% of the theoretical yield (Figure 1B,C). Consequently, Mgly43 demonstrated superior performance in terms of growth, glycolate titer and yield. The Mgly45 strain, under the Trc promoter regulating aceA and ycdW, produced 0.61 g/L glycolate and 0.70 g/L acetate, with 21.11% of the theoretical yield (Figure 1B,C). The Mgly4 strain harboring pTrc-aceA-ycdW and pCDF-gltA(pJNU-4) was named Mgly454. This strain with Trc regulating aceA and ycdW and the T7 promoter regulating gltA produced 0.50 g/L glycolate and 0.013 g/L acetate, with 13.22% of the theoretical yield (Figure 1B,C). Notably, the main difference was in the promoter that made the production of glycolate 1.70 times higher in Mgly4T than that of Mgly43. Compared to the Trc promoter, the Tet promoter was stronger in tensity [17] and may be more conducive to the expression of ycdW, aceA and aceK. After expressing the gltA, the production of glycolate in Mgly4T4 was found to be 3.57 times higher than that of Mgly434, with a higher proportion of the theoretical yield (44.88%) in Mgly4T4. Mgly454 exhibited a lower capacity for glycolate accumulation compared to Mgly45 in terms of cell growth and yield. Considering the results, the glycolate production and yield of Mgly45 and Mgly454 were notably lower relative to Mgly4T and Mgly4T4. The production of glycolate in Mgly4T4 was found to be 1.17 times higher than that of Mgly4T, albeit with a slightly higher proportion of the theoretical yield and less acetate in Mgly4T. Therefore, the Mgly4T strain was further considered for its greater potential to improve glycolate production as it exhibited the highest theoretical yield of glycolate and minimal by-products.

3.2. Replacement of P_{Tet} with the Synthetic Promoter PL3429

As discussed in the previous section, Mgly4T required a single antibiotic to produce glycolate and provided a relatively high yield and titer. However, related genes were expressed in this strain under the P_{Tet} promoter, which needed anhydrotetracycline (aTc) as an inducer [34]. Given the high cost of aTc, its usage would not suitable for industrial production [19]. To circumvent the reliance on aTc, the P_{Tet} promoter was replaced by a PL3429 promoter of equivalent fluorescence strength from the constitutive synthetic promoter library developed by Zhao et al. [25] (Figure 2A). Meanwhile, in order to reduce the impact of the replacement promoter on glycolate production, we first evaluated the replacement strategy (Tet^R, P_{Tet}, RBS) of PL3429 on pGly-2. In the case of the Mgly4T strain expressing genes under the Tet promoter, the Tet^R and RBS elements were also required.

In order to assess the approaches of replacing P_{Tet} with the synthetic promoter PL3429, the Tet^R and Tet promoter-related sequences of pGly-2 were replaced by PL3429 to form pRep1 plasmid. The Tet^R, Tet promoter and RBS-related sequence were replaced by PL3429 to form the pRep2 plasmid. Only the Tet promoter was replaced by PL3429 to form the pRep3 plasmid. The pRep1, pRep2 and pRep3 plasmids were transformed to Mgly4 to produce the Mrep1, Mrep2 and Mrep3 strains for evaluating the replacement of P_{Tet} with the synthetic promoter PL3429. The glycolate production levels by the resulting strains of Mrep1, Mrep2, Mrep3 and Mgly4T were 2.24 g/L (64.11% of the theoretical yield), 0.42 g/L (11.64% of the theoretical yield), 0.75 g/L (18.23% of the theoretical yield) and 2.42 g/L (45.46% of the theoretical yield), respectively (Figure 2B,C).

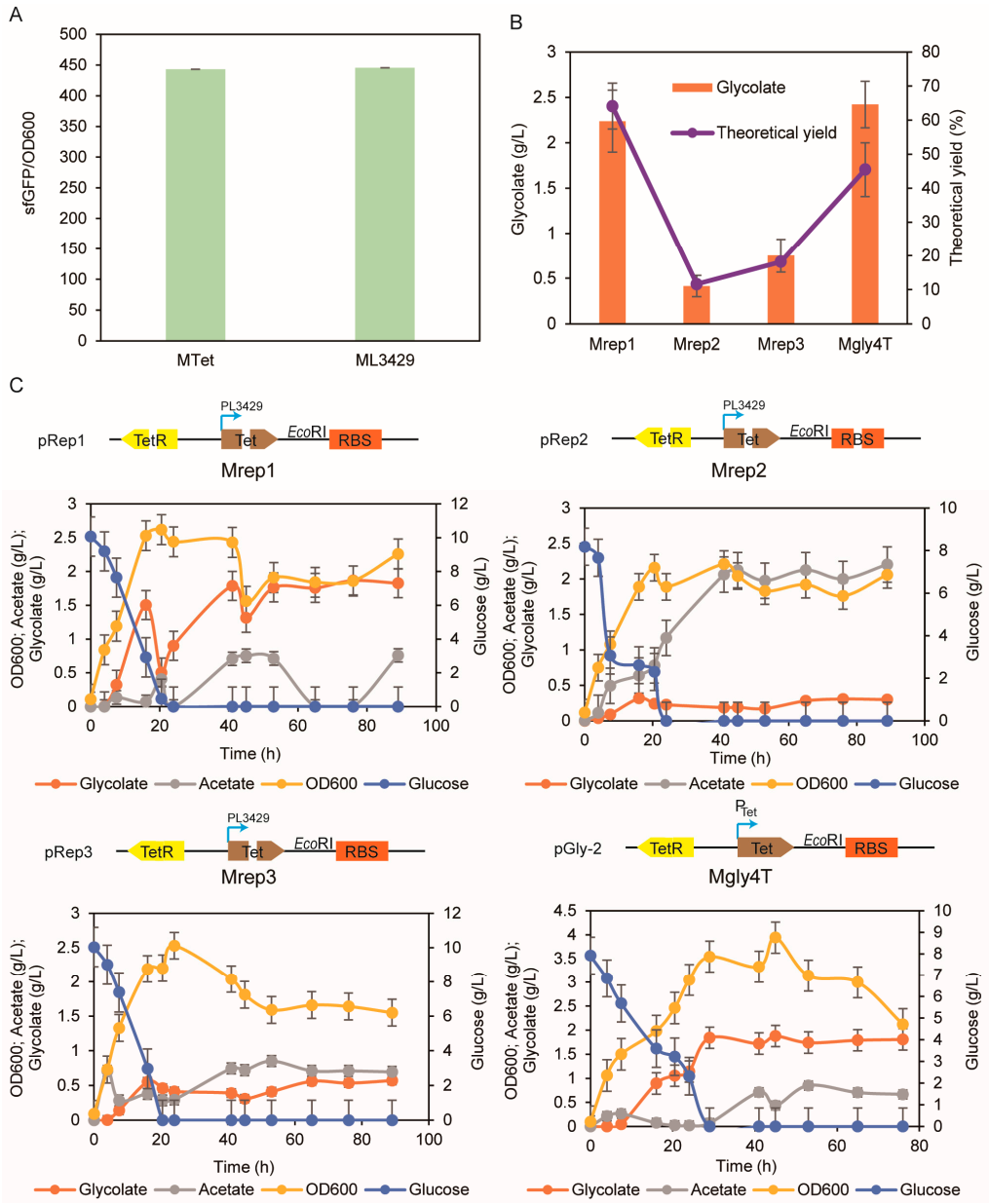

Figure 2. Replacement of P_{Tet} with PL3429 for glycolate production. (**A**) Fluorescence intensity of Mtet and ML3429. MTet: MG1655 carrying pTet-sfGFP. ML3429: MG1655 carrying pL1-sfGFP. (**B**) Cultivation process of Mrep1, Mrep2, Mrep3 and Mgly4T strains. Mrep1: Mgly4 strain harboring pRep1; Mrep2: Mgly4 strain harboring pRep2; Mrep3: Mgly4 strain harboring pRep3; Mgly4T: Mgly4 strain harboring pGly-2. (**C**) Comparison of the glycolate titer and yield.

The above results indicated that glycolate could be synthesized by the three replacement methods, signifying the successful substitution of the inducible P_{Tet} with the synthetic promoter PL3429 through the pRep1 replacement. However, the Mrep1 strain, in which Tet^R and Tet promoters were removed, produced higher glycolate than the strain removing either the Tet promoter or RBS (Mrep2 and Mrep3 strain). The possible reasons were as follows: (1) the elements related to the Tet promoter need to be removed thoroughly, otherwise the expression of subsequent genes would be affected; (2) the addition of RBS is also conducive to gene expression. In addition, the glycolate titer produced by the Mrep1 strain was close to that produced by the Mgly4T strain requiring induction. Nevertheless, the yield provided by the Mrep1 strain was 1.41 times higher than that of Mgly4T. To further compare the differences in the expression of genes between Mrep1 and Mgly4T, the transcriptional levels of *aceA*, *aceK* and *ycdW* were determined (Table S4). Compared to Mgly4T, Mrep1 had a higher transcription level of *aceK*, whereas the transcriptional levels of *ycdW* and *aceA* were similar. This might also be the direct reason for their similar glycolate titer.

3.3. Optimizing the Different Synthetic Promoters for Glycolate Production

Although the Mrep1 strain exhibited and improved the glycolate titer by replacing the Ptet promoter with the synthetic promoter PL3429, its glycolate yield was still low, with only 64.1% of the theoretical yield. In order to further increase glycolate yield and balance metabolic flux, we selected 11 synthetic promoters with varying intensities to fine-regulate the expression of the glycolate metabolic pathway using the pRep1 replacement strategy. The 11 synthetic promoters (PL3153, PL3429, PL1562, PL3262, PL2353, PL3090, PL2917, PL3149, PL3195, PL2538, PL1993) from Zhao et al. [25] were compared with 0.5, 1, 6, 10, 14, 25, 29, 34, 42, 44 and 79 multiple of fluorescence intensity of P_{Tet}. We found that the glycolate production increased with the enhancement of promoter strength up to a certain level but a drop followed thereafter (Figure 3A,B). The glycolate titer reached the highest value under the action of the PL1562 promoter (Figure 3B). Correspondingly, Mgly4T1562 produced a maximum of 3.02 g/L glycolate with 97.32% of the theoretical yield (Figure 3B). The performances of Mgly4T3153, Mgly4T3429, Mgly4T1562, Mgly4T3262, Mgly4T2353, Mgly4T3090, Mgly4T2917, Mgly4T3149, Mgly4T3195, Mgly4T2538 and Mgly4T1993 for their glycolate and acetate titers are illustrated in Figure S1. Additionally, we assessed the transcription of *aceA*, *aceK* and *ycdW*, as outlined in Table S5. The transcriptional patterns of *ycdW* among the strains were found to be similar, with *aceA* and *aceK* exhibiting different transcriptional intensities in different strains. However, these transcriptional results seemed to have minimal correlation with the final titer of the product. These synthetic promoters independently express only one gene (lacZ, ldhA or sfGFP); we found a close correlation between activity, transcription levels and the sfGFP/OD600 ratios, both on the plasmid and the genome, as described in the previous article [25]. However, when these promoters (synthetic promoter, T7 promoter, Tet promoter, etc.) express multiple genes of metabolic pathways, due to complex intracellular metabolic regulation, the strength of the promoter is not closely related to the activity of expression transcription, which has been shown in the literature [3,14,28]. Based on the titer and yield of glycolate, Mgly4T1562 was the optimal producer as it exhibited 97.32% of the theoretical yield with only 0.92 g/L acetic acid accumulation (Figure S1).

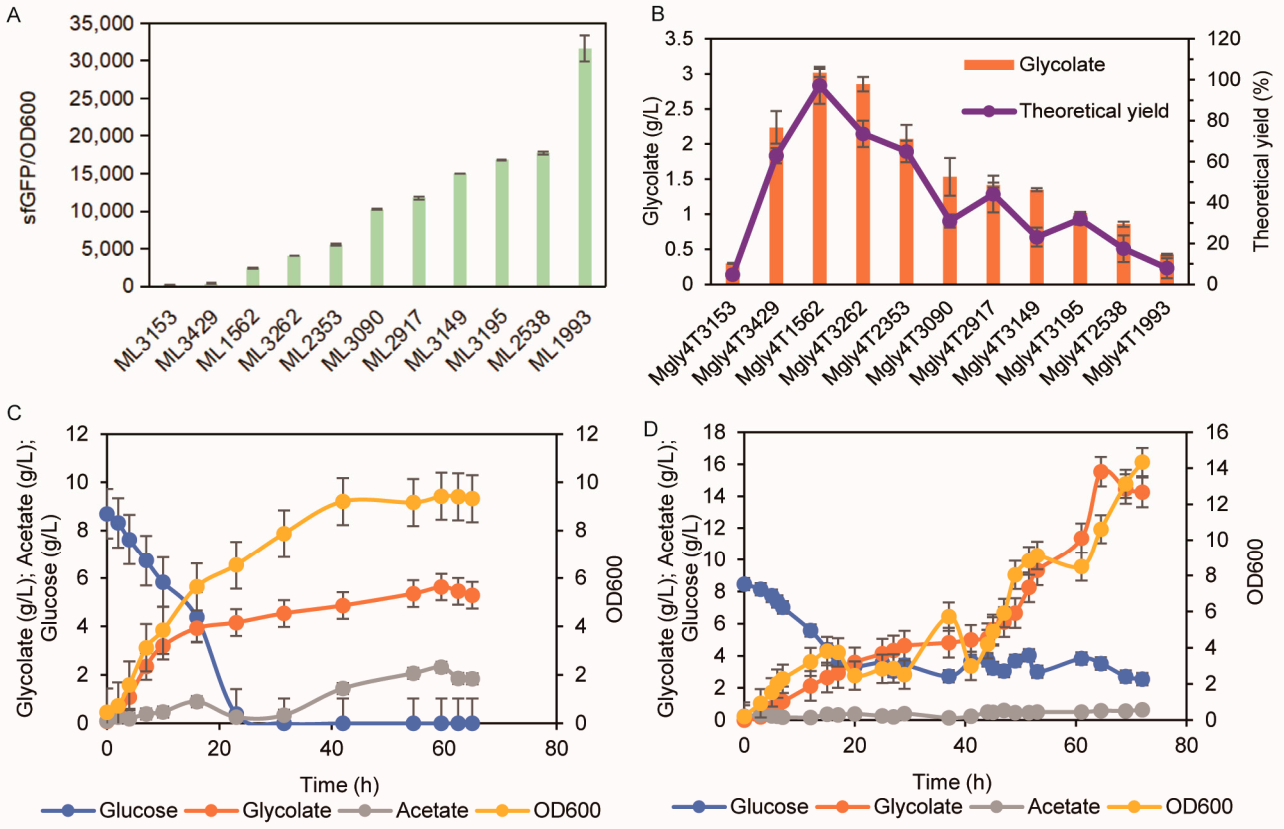

Figure 3. Promoter optimization results and glycolate cultivation. (**A**) Fluorescence intensity of different synthetic promoters. Different synthetic promoters from the constitutive promoter library were selected to replace P_{Tet} promoter with the different multiples of fluorescence intensity. (**B**) Comparison of the highest glycolate titer and yield. (**C**) Batch cultivation of glycolate by Mgly4T1562 strain. Glucose consumption, metabolites and cell growth during fed-batch cultivation. (**D**) Fed-batch cultivation of glycolate by Mgly4T1562 strain. Glucose consumption, metabolites, and cell growth during fed-batch cultivation. Error bars represent the s.d. from three independent assays.

3.4. Production of Glycolate in 5-L Bioreactor

To streamline the adaptation process for industrial glycolate production, it is imperative to thoroughly assess the cultivation capabilities of the metabolically optimized strain. Therefore, the finally selected engineered strain (Mgly4T1562) was further studied for cultivation in a 5 L bioreactor using 10 g/L glucose in M9 minimal medium under 1 vvm aerations and 400 rpm agitation (Figure 3C). Under these conditions, we observed rapid cellular growth within the initial 42 h, followed by a stationary phase when OD reached above 9. The concentration of glucose ran out after 32 h. Notably, the glucose demonstrated here was extracellular, while the intracellular accumulation of the up-taken glucose might be still have been there to continue related metabolism as well as improve the titer of glycolate and acetate [10,14,19,35,36].The glycolate titer gradually increased and reached a maximum of 5.63 g/L with 76.2% of the theoretical yield, and the titer of acetate was 2.32 g/L after 60 h. The production of glycolate had a certain relationship with the growth. To achieve a higher titer of glycolate, glucose was employed for fed-batch cultivation with a low concentration (~3 g/L) for about 17 h [14]. We observed that glycolate production gradually increased with the growth of cells and reached a maximum glycolate titer of 15.53 g/L with 63.6% of the theoretical yield after 64.5 h. It is noteworthy that under the action of a low concentration of glucose, the titer of the by-product acetic acid exhibited a significant decrease compared with batch cultivation.

4. Discussion

A promoter is an essential genetic element for regulating gene expression both in natural and metabolically rewired strains. Given that there are certain differences in the expression of heterologous genes under different promoters, we fine-tuned the expression of genes of the glycolate pathway. This optimization aimed to strike a balance in metabolic flux, ultimately aiming to obtain a high titer. To explore these dynamics, various strains were cultured under the same conditions for optimization and to test glycolate production capacity (Figure 1). The pGly-2 was used to express genes like *aceA*, *aceK* and *ycdW* under the Tet promoter, while pJNU-3 expressed the same genes under the Trc promoter. On the other hand, two genes, *aceA* and *ycdW*, were expressed in pTrc-*aceA*-*ycdW*. The glycolate production and theoretical yield provided by the Mgly45 and Mgly454 strains were lower than those exhibited by the Mgly4T and Mgly4T4 strains. Strikingly, it was revealed that the mere variance in the promoter led to a 1.70-fold increase in glycolate production by Mgly4T compared to Mgly43. After expressing the *gltA*, the production of glycolate in Mgly4T4 was 3.57 times that of Mgly434. However, the theoretical yield of the Mgly4T strain was slightly higher than that provided by Mgly4T4. Therefore, the Mgly4T strain was further considered for its greater potential to produce more glycolate.

Due to the exorbitant cost of inducers, the aforementioned inducible expression systems would not be popular in industrial processes, leading to the finding of constitutive expression systems [17]. The best-match constitutive promoter (PL3429) was screened from a synthetic promoter library developed by Zhao et al. [25] with an intensity similar to that of the Tet promoter. In order to reduce the impact of the replacement of the promoter on glycolate production, the replacement modes (tetR, P_{Tet}, RBS) of PL3429 on pGly-2 were further screened out (Figure 2). The glycolate titer produced by the Mrep1 strain was very close to the titer produced by the Mgly4T strain. Meanwhile, the theoretical yield of glycolate shown by the Mrep1 strain was 64.11%. However, there is still room for improvement in relation to the theoretical yield. In order to further improve the titer and yield, the Tet promoter was replaced by 11 different synthetic promoters (0.5, 1, 6, 10, 14, 25, 29, 34, 42, 44, 79 multiples of fluorescence intensity of P_{Tet}). Through the optimization, we found PL1562 presented a great advantage and Mgly4T1562 could produce 3.02 g/L glycolate with 97.3% of the theoretical yield (Figure 3). The possible reason is that under the action of the PL1562 promoter, the expression of heterologous genes of the glycolate pathway reaches a balance so that the yield reaches a high level. Recently, Xu et al. optimized the glycolate pathway using the gradient-strength PUTR complexes and eventually achieved 78.2% of the theoretical yield [3]. But the scope of the PUTR promoter library is relatively narrow, which limits the fine regulation and optimization of glycolate pathway. And few self-developed synthetic promoters have been used to regulate glycolate metabolic pathways. The resulting recombinant strain, Mgly4T1562, did not exceed the highest glycolate titer (65.5 g/L) in fed-batch cultivation. Thus, the Mgly4T1562 strain was selected as the best strain by shaking flask culture step by step and the titer was not only related to the strain, but also closely related to cultivation conditions such as the composition of the medium, which optimizes the carbon/nitrogen (C/N) ratio [14]. But the yield of Mgly4T1562 was extremely high. Therefore, it is worth noting that the strain in our study employed the constitutive expression promoter and does not require the addition of inducers. In this way, it could be an important reference for cost-effective glycolate production.

5. Conclusions

In this work, we report glycolate production in *E. coli* expressing the pathway fine-tuned with a synthetic promoter to avoid the use of an inducer. The glycolate metabolic pathway in *E. coli* Mgly4 was constructed by overexpressing *aceA*, *aceK*, *ycdW* or *gltA*, which resulted in various strains. Comparing these glycolate production strains, the Mgly4T strain (Mgly4 carrying pGly-2 and harboring ycdW-aceA-aceK under the Tet promoter) produced a maximum of 1.51 g/L glycolate with a 45.51% theoretical yield in shake flasks. In order to further enhance yield, we replaced P_{Tet} with synthetic promoters for glycolate

cultivation in a 5 L bioreactor. The final selected strain (Mgly4T1562) produced 3.02 g/L glycolate with 97.32% theoretical yield in shake flasks and 15.53 g/L glycolate in fed-batch cultivation. This is the first time that glycolate was produced using synthetic promoters. The strategy reported here could be valuable for cost-effective glycolate production without requiring any inducer.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10010067/s1, Table S1: Strains and plasmids used in this study; Table S2: Primers used in this study; Table S3: Synthetic promoter sequence used in this study; Table S4: The transcription levels of *aceK*, *aceA* and *ycdW* genes in Mgly4T and Mgly4T3429; Table S5: The transcription levels of *aceK*, *aceA* and *ycdW* genes in different strains; Figure S1: Cultivation process of strain Mgly4T3153, Mgly4T3429, Mgly4T1562, Mgly4T3262, Mgly4T2353, Mgly4T3090, Mgly4T2917, Mgly4T3149, Mgly4T3195, Mgly4T2538 and Mgly4T1993 and comparison of the highest glycolate and acetate titer [14,25,27,28].

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