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Metabolic engineering of *E. coli* for efficient production of glycolic acid from glucose

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ABSTRACT

Glycolic acid is the smallest member of the α-hydroxy acid family. In order to produce glycolate from glucose via the glyoxylate shunt stably, one malate synthase gene *aceB* in *Escherichia coli* BW25113 was deleted by homologous recombination; another malate synthase gene *glcB* was then replaced by a DNA cassette WAK harboring isocitrate lyase gene (*aceA*), glyoxylate reductase gene (*ycdW*) and isocitrate dehydrogenase kinase/phosphatase gene (*aceK*). The above three genes were over-expressed in the chromosome of *E. coli* EYX-1WAK. This strain was then transferred 20 times on M9 medium to have a mutant strain: EYX-2 with a significantly improved growth rate. The glycolate yields of EYX-2 in the shaken flasks and the 5-L bioreactor using batch fermentation strategy under 2 vvm aeration and 800 rpm stirring speed were 0.33 g/g-glucose and 0.48 g/g-glucose, respectively. The fed-batch fermentation of EYX-2 on 120 g/L glucose had the highest titer of 56.44 g/L with 0.52 g/g-glucose yield in 120 h, and this is the highest reported glycolate yield ever.

1. Introduction

Glycolic acid ($HOCH_2COOH$), or glycolate, is the smallest member of the α-hydroxy acid family [1]. Glycolic acid has dual functionalities with both alcohol and moderately strong acid groups on a very small molecule. Its properties make glycolic acid ideal for a broad spectrum of consumer and industrial applications, including the leather industry, the oil and gas industry, the laundry and textile industry and personal care products [2]. Glycolic acid is also used together with lactic acid to produce a co-polymer (PLGA) for medical applications, e.g., drug delivery [3]. Glycolic acid market was USD 93.3 million (40 million kg) in 2011, and expected to reach USD 203 million in 2018 [1].

Glycolic acid is naturally produced by a variety of microorganisms from ethylene glycol by oxidation [2], or from glycolonitrile by hydrolyzation [4]. Chemolithotrophic iron- and sulphur oxidizing bacteria were also used for producing glycolate [5]. However, the above methods required expensive and highly pollutant precursors. Recently, microorganisms were engineered to over-express glyoxylate shunt to direct carbon flux from isocitrate to glycolate. The highest titer of glycolate: 56 g/L was reported by Dischert et al. using *Escherichia coli* by adding ~500 g/L glucose [6]. Koivistoinen et al. engineered *Saccharomyces cerevisiae* and *Kluyveromyces lactis* to produce glycolate at 15 g/L using D-xylose and ethanol as substrates [1]. However, the previous work used replicating plasmids to over-express genes responding to glycolate synthesis. The stability of those plasmids was not reliable for industrial purpose.

In this study, to achieve glycolate synthesis from glucose via the glyoxylate shunt stably, we deleted the malate synthase gene *aceB* and replaced malate synthase gene *glcB* with WAK cassette harboring *ycdW* and *aceA-aceK* under the control of a constitutive promoter in the chromosome [7]. The adaptive evolution was used to improve the growth of the engineered strain. The final strain was characterized under optimized fermentation parameters.

2. Materials and methods

2.1. Strains and plasmids

The strains and plasmids are shown in Table 1. *E. coli* BW25113, which was the strain used for chromosome modification with

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Table 1
Strains and plasmids used in this study.

Strains and plasmids	Relevant genotype	Reference
Strains		
E. coli BW25113	F-, DE(araD-araB) 567, lacZ4787(del)::rrnB-3, LAM-, rph-1, DE(rhaD-rhaB) 568, hsdR514	[8]
BW25113-pGLY-1	BW25113 carring pGLY-1	This study
EXY-1	Δ*aceB* (BW25113)	This study
EXY-1-pGLY-1	EXY-1 with plasmid pGLY-1	This study
EYX-1-glcB-	Δ*aceB*, Δ*glcB* (BW25113)	This study
EYX-1-glcb-1-pGLY-1	EXY-1-glcB- with plasmid pGLY-1	This study
EYX-1WAK	Δ*aceB*, Δ*glcB*::*WAK* cassette	This study
EYX-2	Evolved strain of EYX-1WAK	This study
hhh		
Plasmids		
pGLY-1	pCOLADuet-1harboring ycdW and aceA-aceK from *E. coli* MG1655	[7]
pGLY-PRBSaceAK	pGLY-1 harboring PRBSaceAK cassette	This study
pGLY- PRBS	pGLY- PRBSaceAK harboring PRBS cassette	This study
pGLY-PRBS-FRT	pGLY- PRBS harboring FRTterminator cassette	This study
pGLY-WAK	pGLY-1 harboring *WAK* cassette	This study
pKD46	Temperature sensitive replication (repA101ts); encodes lambda Red genes (exo, bet, gam); native terminator (tL3) after exo gene; arabinose-inducible promoter for expression (ParaB); encodes araC for repression of ParaB promoter; Ampicillin resistant.	[8]
pKD4	This is a template plasmids for frt-flanked kan cassette	[8]
pCP20	pCP20 has the yeast Flp recombinase gene, FLP, chloramphenicol and ampicillin resistant genes, and temperature sensitive replication	[8]

the help of pKD46 and pCP20 [8]. The plasmid pGLY-1 for over-expressing *ycdW*, *aceA-aceK*, was a gift from Dr. Prather's group of MIT [7]. pGLY-1 was transformed to BW25113 strain forming BW25113-pGLY-1. All the primers used in this study are shown in Table S1.

2.2. Culturing conditions

The *E. coli* strains used for transformation and chromosome modifications were cultured in Luria Broth (LB) medium. The fermentation medium was M9 minimal medium with the addition of glucose and NH_4Cl. The first round of strain tests were done in the 500 mL shaken flasks with addition of $CaCO_3$. The fermentations were done in the 5-L bioreactor (Eppendorf Bioflo 310). The pre-cultures were grown in the shaken flasks for overnight and 10% culture was inoculated into the bioreactor and then grew for 24–120 h. Cultures were carried out at 37 °C. The pH was adjusted at pH 6.8 by addition of base (NH_4OH 7.5% W/W). The volumetric oxygen transfer coefficient (K_La) was described previously [9]. The fermentation was in discontinuous fed-batch mode, with a feed stock solution of concentrated glucose [10].

2.3. Construction of EYX-1 and EYX-1-glcb-strains

The general strategy for engineering *E. coli* to accumulate glycolate is shown in Fig. 1A. The protocol of deleting *aceB* was described previously [8]. The brief process is: the *aceB* deletion cassette was amplified from pKD4 plasmid [8] by PCR using primers aceB-KO-F and aceB-KO-R flanked by 39-nt DNA oligos homologous to the upstream and downstream of *aceB* gene, respectively. The *aceB* deletion cassette was transformed to *E. coli* BW25113 with pKD46 by electroporation. The homologous recombination was to replace *aceB* gene by the *aceB* deletion cassette, and the antibiotic marker was deleted by FLP recombinase expressed on plasmid pCP20 [11]. The deletion of *aceB* was verified by PCR using primers aceB-veri-F and aceB-veri-R. The resulting strain was designated as EYX-1. In EYX-1, *glcB* was deleted by using the same method as deleting *aceB*, forming a new strain EYX-1-glcb-. The primers used for *glcB* deletion are shown in Table S1.

2.4. Construction of WAK cassette for integration of genes into the chromosome

All sequences of the synthetic cassette and primers were shown in Table S1. pGLY-1 was used for over-expressing glycolate pathway genes and its backbone was pCOLADuet-1. In order to replace *glcB* gene in EYX-1 strain, a DNA cassette WAK was constructed to replace *glcB* in the chromosome. The order of genes in the WAK cassette was (5′→3′): constitutive promoter P(Lac) IQ (tggtacaaaac-ctttcgcggtatggcatgatagcgcc), ribsome binding site (RBS), *aceA-aceK*, P(Lac) IQ promoter, ribsome binding site (RBS), *ycdW*, terminator, FRT sequence, kanamycin resistant gene region and FRT sequence (Fig. 1B). The procedures of constructing WAK cassette are shown below:

The first step was to insert P(Lac) IQ and ribosome binding site (RBS) to the upstream of *aceA-aceK* on pGLY-1 plasmid. P(Lac) IQ fused with ribosome binding site (RBS) was synthesized and flanked by AciI and NcoI restriction sites on 5′ and 3′ ends, respectively, and the above cassette was designated as PRBSaceAK(Table S1). PRBSaceAK cassette and plasmid pGLY-1 were digested by AciI and NcoI, and the larger piece of pGLY-1 was isolated from agarose gel. The digested PRBSaceAK and the above larger piece were mixed and ligated by T4 ligase, and then were transformed to *E. coli* DH5α strain for screening by electroporation. The insertion of PRBSaceAK on the upstream of *aceA-aceK* of pGLY-1 was verified by PCR using primers aceAKF and aceAKR (Table S1) and the plasmid with the right structure was designated as pGLY-PRBSaceAK.

Second, in order to insert P(Lac) IQ and RBS on the upstream of *ycdW* on pGLY-PRBSaceAK, a DNA cassette PRBSycdW containing P(Lac) IQ fused with RBS was synthesized, and there were BsrGI and NdeI restriction sits at 5′ and 3′ ends of this cassette. The rest of the steps were the same as insertion of PRBSaceAK cassette described above. The insertion of PRBSaceAK on the upstream of *aceA-aceK* was verified by PCR using primers ycdWF and ycdWR (Table S1) and the resulting plasmid was designated as pGLY-PRBS.

Third, in order to attach Kanamycin resistant gene region as well as FRT sequences to the end of *ycdW* on pGLY-PRBS, pGLY-PRBS was digested by XhoI and XmnI. An FRTterminator cassette including XhoI restriction site, terminator, FRT sequence and XmnI restriction site was synthesized. FRTterminator cassette was digested by XhoI and XmnI. The digested FRTterminator and pGLY-PRBS was ligated by T4 ligase to form pGLY-PRBS-FRT plasmid. The insertion of FRTterminator was analyzed by PCR using primers FRTTF and FRTTR (Table S1). To add FRT sequence to the other end of Kanamycin resistant gene, pGLY-PRBS-FRT was digested by NheI. A FRT sequence flanked by two 39-nt sequences homologous to 5′ and 3' ends of the digested plasmid pGLY-PRBS-FRT was synthesized (FRTKan cassette). The FRTKan cassette was assembled with linear pGLY-PRBS-FRT plasmid using Gibson Assembly [12], forming pGLY-WAK plasmid. WAK cassette was amplified from pGLY-WAK plasmid by PCR using primers WAKF and WAKR, which