Metabolic Engineering ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

Activation of glyoxylate pathway without the activation of its related gene in succinate-producing engineered *Escherichia coli*

Li-Wen Zhu [a,1], Xiao-Hong Li [a,1], Lei Zhang [a], Hong-Mei Li [a], Jian-Hua Liu [b], Ya-Jie Tang [a,*]

[a] Key Laboratory of Fermentation Engineering (Ministry of Education), Hubei University of Technology, Wuhan 430068, China
[b] College of Life Science and Technology, Shanghai Jiao Tong University, Shanghai, China

ARTICLE INFO

Article history:
Received 23 September 2012
Received in revised form
5 April 2013
Accepted 11 July 2013

Keywords:
Escherichia coli
Succinate
Activated glyoxylate pathway
Acetate kinase and phosphotransacetylase (*ackA-pta*)
Metabolic flux analysis

ABSTRACT

For the first time, glyoxylate pathway in the biosynthesis of succinate was activated without the genetic manipulations of any gene related with glyoxylate pathway. Furthermore, the inactivation of succinate biosynthesis by-products genes encoding acetate kinase (*ackA*) and phosphotransacetylase (*pta*) was proven to be the key factor to activate glyoxylate pathway in the metabolically engineered *Escherichia coli* under anaerobic conditions. In order to enhance the succinate biosynthesis specifically, the genes (i.e., *ldhA*, *ptsG*, *ackA-pta*, *focA-pflB*, *adhE*) that disrupt by-products biosynthesis pathways were combinatorially deleted, while the *E. coli* malate dehydrogenase (MDH) was overexpression. The highest succinate production of 150.78 mM was obtained with YJ003 (Δ*ldhA*, *ptsG*, *ackA-pta*), which were 5-folds higher than that obtained with wild type control strain DY329 (25.13 mM). For further understand the metabolic response as a result of several genetic manipulations, an anaerobic stoichiometric model that takes into account the glyoxylate pathway have successfully been implemented to estimate the intracellular fluxes in various recombinant *E. coli*. The fraction to the glyoxylate pathway from OAA in DY329 was 0 and 31% in YJ003, which indicated that even without the absence of the *iclR* mutation; the glyoxylate pathway was also activated by deleting the by-products biosynthetic genes, and to be responsible for the higher succinate yields. For further strengthen glyoxylate pathway, a two-stage fed-batch fermentation process was developed by using a 600 g l^{-1} glucose feed to achieve a cell growth rate of 0.07 h^{-1} in aerobic fermentation, and using a 750 g l^{-1} glucose feed to maintain the residual glucose concentration around 40 g l^{-1} when its residual level decreased to 10 g l^{-1} in anaerobic fermentation. The best mutant strain YJ003/pTrc99A-*mdh* produces final succinate concentration of 274 mM by fed-batch culture, which was 10-folds higher than that obtained with wild type control strain DY329. This study discovered that glyoxylate pathway could be activated by deleting glyoxylate pathway irrelevant genes (i.e., genes encoding acetate and lactate) and consequently the succinate biosynthesis was effectively improved. This work provides useful information for the modification of metabolic pathway to improve succinate production.

1. Introduction

Succinate is a four-carbon dicarboxylic acid in the metabolic pathway of several anaerobic and facultative microorganisms and has a wide range of applications in the fields of green solvents and pharmaceutical intermediates (Song and Lee, 2006). Thus, succinic acid is classified as the most promising chemical of 12 bio-based chemicals by the US Department of Energy (Werpy and Petersen, 2004). Traditionally, it was produced via chemical synthesis from petrochemical feedstocks that are nonrenewable, and the chemical processes suffer from environment pollution (Bechthold et al., 2008). Therefore, the production of succinic acid by microbial fermentation has drawn much attention (Willke and Vorlop, 2004).

Succinic acid could be produced by naturally selected microorganisms (Samuelov et al., 1991; Lee et al., 2002; Van der Werf et al., 1997), and recombinant organisms (Vemuri et al., 2002). Several naturally selected microorganisms can produce succinate with a high yield and productivity, but require complex nutrients. Alternatively, *Escherichia coli* has the advantages of fast growth, simple nutrient requirement, and could be metabolically engineered to decrease by-products formation and improve succinic acid yield (Jantama et al., 2008; Singh et al., 2011; Lin et al., 2005a; Sánchez et al., 2006). Recently, several metabolic engineering strategies were employed to create recombinant *E. coli* strains that are able to catalyze a wide range of low-cost sugar feedstock to produce succinic acid, in order to develop a bio-based industrial production of succinic acid (Wang et al., 2011b; Blankschien et al., 2010).

* Corresponding author. Fax: +86 27 88015108.
E-mail address: yajietang@hotmail.com (Y.-J. Tang).
[1] With equal contribution to this work.

1096-7176/$ - see front matter © 2013 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ymben.2013.07.004

Under anaerobic condition, *E. coli* ferments glucose to a mixture of products consisting primarily of acetate, formate, and ethanol, as well as smaller amounts of lactate and succinate (Clark, 1989). However, succinate can be made as the only product from glucose and CO_2 in a balanced fermentation by blending rates from reductive arm of TCA with glyoxylate cycle (Sánchez et al., 2005). In this process, NADH generated during glycolysis is reoxidized through the reduction of organic intermediates derived from glucose. The fermentative pathway converting oxaloacetate (OAA) to succinate requires 2 mol of NADH per mole of succinate produced; however, 1 mol of glucose can provide only 2 mol of NADH through the glycolytic pathway. Therefore, the maximum theoretical yield of succinate from glucose is probably limited to the shortage of reducing force. Thus, many strategies were developed to overcome the absence of NADH. The glyoxylate pathway showed an experimental NADH requirement from glucose of about 1.25 mol of NADH per mole of succinate (Sánchez et al., 2005). The pathway design with activated glyoxylate pathway was developed and examined in silico (Cox et al., 2006; Yang et al., 2011) and implemented in vivo by deactivating the IclR transcriptional repressor (encoded by the gene *iclR*), which regulates the expression of the aceBAK operon involved in the induction of the glyoxylate pathway in genetically engineered *E. coli* (Sánchez et al., 2005; Lin et al., 2005b). The results proved that the activation of glyoxylate pathway could be promotive on succinic acid synthesis.

In this study, the activated glyoxylate pathway was discovered without the genetic manipulations of glyoxylate pathway related gene, and the inactivation of genes encoding acetate kinase (*ackA*) and phosphotransacetylase (*pta*) was proven to be the key factor to activate glyoxylate pathway. Firstly, major pathways leading to the biosynthesis of lactate, pyruvate, acetate, formate and ethanol were eliminated by deleting genes encoding D-lactate dehydrogenase (*ldhA*), glucose phophotransferase (*ptsG*), acetate kinase (*ackA*), phosphotransacetylase (*pta*), formate transporter (*focA*), pyruvate formate-lyase (*pflB*), and alcohol dehydrogenase (*adhE*) to construct *E. coli* mutation strains. Secondly, metabolic flux analysis (MFA) under batch cultivation condition was used to estimate intracellular fluxes and determine branch point flux split ratios at principal nodes. Finally, to strengthen the flux of glyoxylate pathway, the fed-batch strategy by controlling specific growth rate under glucose-limited in aerobic fermentation was developed. This study provides useful information for the activation of glyoxylate pathway to improve succinate production.

2. Materials and methods

2.1. Strains and plasmids

Plasmids and primers used in this study were summarized in Table 1.

2.2. Gene inactivation

Inactivation of genes was performed using two-step homologous recombination as described previously (Liang and Liu, 2008). With this method, no antibiotic genes or scar sequences remain on the chromosome after gene deletion. All mutations were made in *E. coli* DY329 (Lam-, IN (rrnD-rrnE) 1, rph-1 Δ*lacU169 nadA::Tn10 gal490 λcI857*Δ (*cro-bioA*) (Yu et al., 2000), which harboring temperature-dependent repressor tightly controls prophage expression, and thus, recombination functions can be transiently supplied by shifting cultures to 42 °C for 15 min. The efficient prophage recombination system does not require host RecA function and depends primarily on Exo, Beta, and Gam functions expressed from the defective λ prophage.

For the first recombination, a DNA cassette containing a tetracycline resistance gene and a levansucrase gene (*sacB-tet*) with flanking homologous regions was amplified using pEX18Tc (Hoang et al., 1998) as template employing the ST- primer (Table 1). Subsequently, the purified fragments were transformed into host *E. coli* DY329 to replace the target gene. After electroporation of the fragment into DY329 and 2 h of incubation at 32 °C to allow expression and segregation, recombinants were selected for tetracycline (10 mg l^{-1}) resistance on plates (32 °C, 24 h). The positive clones were chosen, grown in Luria–Bertani (LB) broth with tetracycline and prepared for electroporation. For the second recombination, after electroporation with 1 kb native sequences (after target gene) to omit the region of deletion, cells were incubated at 32 °C for 4 h and transferred into LB agar plate containing 10% sucrose. After overnight incubation (32 °C), clones were selected on LB plates containing 10% sucrose and tetracycline plates containing 10 mg l^{-1} of tetracycline. Clones were selected for loss of tetracycline and sucrose resistance. The resulting clones were verified and further confirmed using the primers designed to bind 300 bp upstream and downstream respectively of the target gene.

2.3. Plasmids construction procedure

The malate dehydrogenase (*mdh*) amplified from *E. coli* genome and digested with *EcoRI* and *HindIII* and then ligated into the plasmid pTrc99A. The forward and reverse primer is *mdh*-F and *mdh*-R, respectively (Table 1). The final vector is designated pTrc99A-*mdh* and confers ampicillin (Amp) resistance.

2.4. Batch culture

During strain construction, cultures were grown aerobically at 32 °C in LB medium (10 g l^{-1} tryptone, 5 g l^{-1} yeast extract, and 5 g l^{-1} NaCl) with 10 g l^{-1} glucose. Solid media for plates contained 15 g l^{-1} agar. Preculture and fermentation medium consisted of the following components (g l^{-1}): glucose 40, tryptone 20, yeast extract 10, $K_2HPO_4 \cdot 3\ H_2O$ 0.9, KH_2PO_4 1.14, $CaCl_2 \cdot 3\ H_2O$ 0.25, $(NH4)_2SO_4$ 3.0, $MgSO_4\ 7H_2O$ 0.5. For the first preculture, 50 ml of medium was prepared in a 250-ml flask, and a colony from a plate culture was inoculated and then incubated for 12 h at 32 °C on a rotary shaker at 250 rpm. For the second preculture, 50 ml of medium was prepared in a 250-ml rotary shaker, inoculated with 2.5 ml of the first preculture broth and incubated for 12 h at 32 °C on a rotary shaker at 250 rpm.

Dual-phase fermentation (Vemuri et al., 2002) was conducted in the 5.0-l (working volume) BioFlo 115 New Brunswick Scientific (NJ, USA) agitated bioreactor. The bioreactor was equipped with pH (Mettler-Toledo GmbH, Switzerland), DO (Mettler-Toledo GmbH, Switzerland), temperature and foam probes. Fermentation was conducted at 32 °C and inoculated with 5% (v/v) of the second preculture broth. In the aerobic fermentation stage, DO was set above 90% and controlled by adjusting agitation speed, aeration rate for 10 h. Then the anaerobic fermentation was started with 200 rpm of rotation speed and 0.1 vvm external CO_2 gas. The pH was maintained 6.8 by using 40 g l^{-1} $MgCO_3$ and 10 M NaOH.

Six cultures were carried out simultaneously in the stirred-tank bioreactors with different engineered strains under same experimental culture conditions. The identical and well-controlled process conditions made it possible to perform accurate head-to-head comparisons. The results presented here were confirmed to be reproducible in another experiment (data not shown).

Please cite this article as: Zhu, L.-W., et al., Activation of glyoxylate pathway without the activation of its related gene in succinate-producing engineered *Escherichia coli*. Metab. Eng. (2013), http://dx.doi.org/10.1016/j.ymben.2013.07.004