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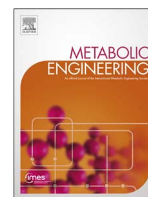

Improvement of succinate production by release of end-product inhibition in *Corynebacterium glutamicum*

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ABSTRACT

Succinate is a renewable-based platform chemical that may be used to produce a wide range of chemicals including 1,4-butanediol, tetrahydrofurane, and γ-butyrolactone. However, industrial fermentation of organic acids is often subject to end-product inhibition, which significantly retards cell growth and limits metabolic activities and final productivity. In this study, we report the development of metabolically engineered *Corynebacterium glutamicum* for high production of succinate by release of end-product inhibition coupled with an increase of key metabolic flux. It was found that the rates of glucose consumption and succinate production were significantly reduced by extracellular succinate in an engineered strain, S003. To understand the mechanism underlying the inhibition by succinate, comparative transcriptome analysis was performed. Among the downregulated genes, overexpression of the *NCgl0275* gene was found to suppress the inhibition of glucose consumption and succinate production, resulting in a 37.7% increase in succinate production up to 55.4 g/L in fed-batch fermentation. Further improvement was achieved by increasing the metabolic flux from PEP to OAA. The final engineered strain was able to produce 152.2 g/L succinate, the highest production reported to date, with a yield of 1.1 g/g glucose under anaerobic condition. These results suggest that the release of end-product inhibition coupled with an increase in key metabolic flux is a promising strategy for enhancing production of succinate.

1. Introduction

In 2004, the U.S. Department of Energy identified the top 12 building block chemicals that could be produced from biomass. Succinate is a C_4-dicarboxylic acid with interesting characteristics for a sustainable chemical industry because it can serve as a precursor for production of a great variety of important bulk chemicals, such as 1,4-butanediol, tetrahydrofurane, or γ-butyrolactone, which are currently produced petrochemically (Hermann et al., 2007; McKinlay et al., 2007; Werpy and Petersen, 2004; Zeikus et al., 1999). Thus, succinate is valued as one of the industrial building block compounds.

Succinate can be produced via microbial fermentation by a natural producer, such as *Actinobacillus succinogenes* (Guettler et al., 1999), *Anaerobiospirillum succiniciproducens* (Samuelov et al., 1991), and *Mannheimia succiniciproducens* (Lee et al., 2002b) as well as metabolically engineered *Escherichia coli* (Sanchez et al., 2005; Zhang et al., 2009) and *Corynebacterium glutamicum* (Litsanov et al., 2012). Furthermore, yeast species have been widely used and extensively engineered to produce succinate because of the advantages such as high tolerance to organic acids and to low pH (Yan et al., 2014; Yuzbashev et al., 2010). A variety of metabolic engineering strategies have been studied and applied to different hosts to improve succinate production. These include eliminating the pathway of byproduct accumulation (Lee et al., 2006; Litsanov et al., 2012; Sanchez et al., 2005; Zhang et al., 2009) to enhance succinate production, increasing the anaplerotic pathways to improve the metabolic flux to oxaloacetate (Gokarn et al., 1998; Lin et al., 2005; Millard et al., 1996; Sanchez et al., 2005), and increasing the redox potential to obtain the highest succinate yield for glucose (Litsanov et al., 2012). The combination of oxidative and reductive tricarboxylic acid (TCA) cycle (Sanchez et al., 2005; Vemuri et al., 2002b), nonphosphotransferase system (non-PTS) glucose uptake (Zhang et al., 2009), and evolutionary genome engineering strategies (Jantama et al., 2008) have also been beneficial for improvement of succinate production and yield. Furthermore, optimization of production conditions and of the fermentation process led to the advances in succinate production, yield, and productivity (Vemuri et al., 2002a).

Recently, some researchers reported the inhibitory effect of succinate. Lin et al. (2008) showed that the cell growth of *A. succinogenes* ATCC 55,618 is completely inhibited at 45.6 g/L succinate. A similar

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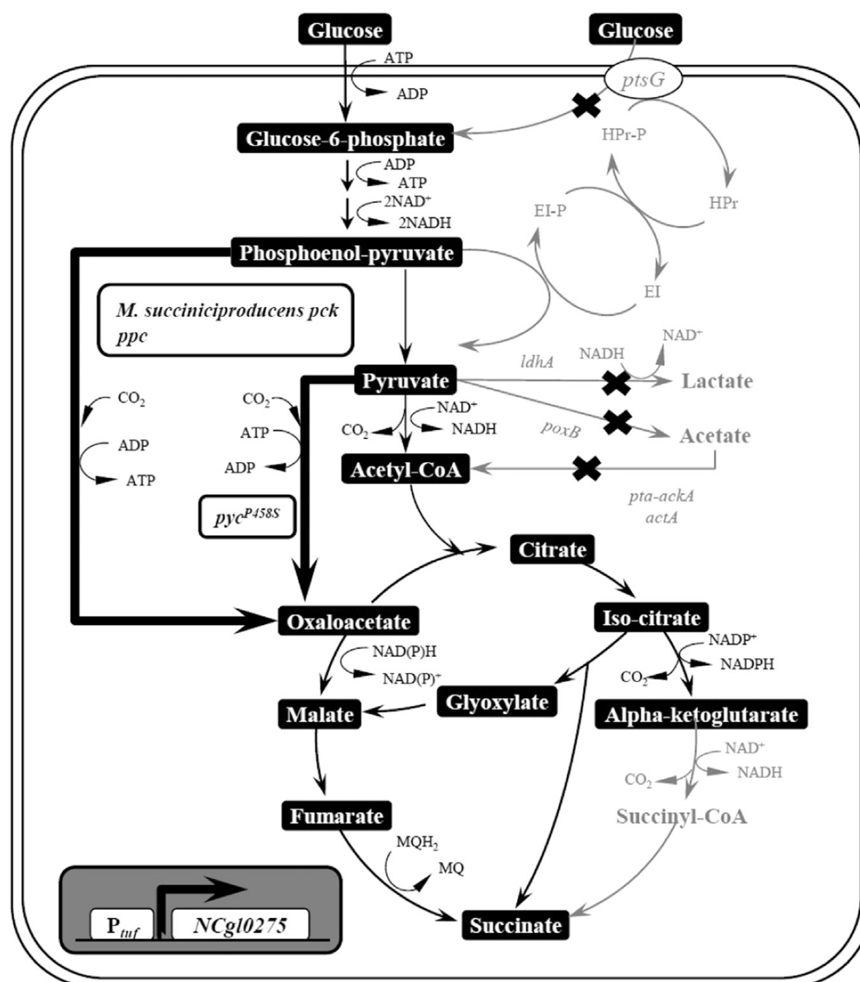

Fig. 1. Schematic representation of the metabolic pathways of succinate production in *C. glutamicum*. The bold black arrows indicate metabolic flux increased by overexpression of the corresponding genes under control of the constitutive *tuf* promoter or after point mutations. The gray arrows indicate the reactions leading to a byproduct or presumably irrelevant reactions. The gray box means that a reaction is not directly involved in central metabolic pathways but is important for increased succinate production and yield. The X sign indicates that reactions were removed by deletion of the corresponding genes.

phenomenon was also observed in strains *E. coli* AFP184, NZN111, AFP111, and *A. succinogenes* 130Z (Andersson et al., 2009; Li et al., 2010). Especially, *A. succinogenes* 130Z shows that glucose consumption is delayed, and the succinate yield for glucose is decreased from 1.1 to 0.49 g/g at the succinate concentration of 20 g/L (Li et al., 2010). It is known that the succinate productivity in most organisms is initially high but dramatically declines during anaerobic fermentation (Lee et al., 2006; Sanchez et al., 2005; Vemuri et al., 2002a, 2002b).

These observations suggest that release of end-product inhibition would be useful for increasing succinate production. However, there have been no reports on the metabolic engineering for the release of end-product inhibition. In this study, we successfully released the end-product inhibition by succinate using novel knowledge obtained by transcriptome analysis of *C. glutamicum*. The final engineered strain (Fig. 1) was able to produce 152.2 g/L succinate: the highest ever reported fermentative succinate production.

2. Materials and methods

2.1. Culture media and conditions

C. glutamicum strains were usually grown in the BHI medium containing 37 g/L brain heart infusion (Difco Laboratories, Detroit, MI). For preparation of competent cells, BHI broth containing 91 g/L sorbitol was used (van der Rest et al., 1999). The CGXII medium (Litsanov et al., 2012), which contained 20 g/L $(NH_4)_2SO_4$, 5 g/L urea, 1 g/L KH_2PO_4, 1 g/L K_2HPO_4, 0.25 g/L $MgSO_4{\cdot}7H_2O$, 10 mg/L $CaCl_2$, 10 mg/L $FeSO_4{\cdot}7H_2O$, 0.1 mg/L $MnSO_4{\cdot}H_2O$, 1 mg/L $ZnSO_4{\cdot}7H_2O$, 0.2 mg/L $CuSO_4{\cdot}5H_2O$, 20 mg/L $NiCl_2{\cdot}6H_2O$, 0.2 mg/L D-Biotin, 42 g/L 3-(N-morpholino)propanesulfonic acid, and 40 g/L glucose, was used to evaluate succinate production and the inhibitory effect of succinate. The pH level of the CGXII medium was adjusted to 7.0 with KOH. Succinate was dissolved in water to 1 M, adjusted to pH 7.0 with KOH, and sterilized by filtration.

2.2. Construction of plasmids and the metabolically engineered strain

C. glutamicum strains and plasmids constructed and used in this study are listed in Table S1. The primers used for gene cloning and deletion are listed in Table S2. Detailed procedures for the construction of plasmids and *C. glutamicum* strains are shown in *SI Text*. All DNA manipulations were carried out by means of standard protocols (Sambrook and Russell, 2001).

2.3. Evaluation of the inhibitory effect of succinate

The *C. glutamicum* strains were streaked onto a BHIS plate (2% agar) and incubated at 30 °C for 2 days. A single colony was inoculated into 25 mL of BHIS medium and cultured at 30 °C for 16 h. The cultured cells were washed with the CGXII medium and resuspended in the same medium containing succinate at different initial concentrations to an optical density at 600 nm (OD_{600}) of 30. The culture was

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