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Expression of global regulator IrrE for improved succinate production under high salt stress by *Escherichia coli*

Wenming Zhang[a,b], Junru Zhu[a], Xinggui Zhu[a], Meng Song[a], Ting Zhang[a], Fengxue Xin[a], Weiliang Dong[a], Jiangfeng Ma[a], Min Jiang[a,b,*]

[a] State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211800, PR China
[b] Jiangsu National Synergetic Innovation Center for Advanced Materials (SICAM), Nanjing Tech University, Nanjing 211800, PR China

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ABSTRACT

Poor high salt stress resistance remained as a main hurdle limiting the efficient bio-based succinic acid production. In this study, the metabolically engineered *E. coli* not only showed improvement of high salt stress tolerance through expression of a global regulator IrrE, but also could use seawater for succinic acid fermentation. The recombinant strain showed an increased 1.20-fold of cell growth rate and 1.24-fold of succinic acid production. Expression levels of genes related glucose uptake and succinic acid synthesis were up-regulated, and more glycerol and trehalose were accumulated. Moreover, no significant differences were observed in cell growth even when tap water was replaced by 60% artificial seawater. In the fermentation using Yellow Sea seawater, 24.5 g/L succinic acid was achieved with a yield of 0.88 g/g. This strategy set up a platform for improving abiotic stress tolerances and provide a possible approach for fermentation processes with low cost.

1. Introduction

Industrial biotechnology aims to produce fuels and chemicals on a large scale from biorenewable resources, such as lignocellulose et al. (Vazana et al., 2013). By partially replacing petrol-based industry, a renewable-based industry is becoming increasingly important and contribute to sustainable development of human society (da Silva et al., 2014). Succinic acid, a four-carbon dicarboxylic acid, has been identified as one of the top 12 chemical building blocks that can be produced commercially through biological conversion, according to the US Department of Energy (McKinlay et al., 2007). Succinic acid has a wide range of applications in the food, chemical, and pharmaceutical industries (Song and Lee, 2006). Moreover, CO_2 is fixated during succinic acid fermentation which can be considered as an environmental advantage (Cheng et al., 2012). However, high salt stress is one of the main stress during bio-based succinic acid production. To maintain the optimal neutral pH for cell growth and metabolism, large amounts of alkali were added, which brought in abundant positive ions simultaneously, such as Na^+, K^+ or NH_4^+ (Liu et al., 2007). As a result, high salt pressure in fermentation broth will increase due to the accumulation of these ions, which would impose a heavy burden on cells. Moreover, most microbial-based industrial biotechnology processes are not economically feasible in many cases, mainly due to the high cost of raw materials, energy-intensive sterilization processes, low products titers and yields, and heavy consumption of fresh water. Therefore, series of strategies were developed to reduce the production costs in various aspects. For example, to reduce the cost of feedstock, waste biomass, such as corn stover (Zheng et al., 2010), cane molasses (Liu et al., 2008) and sugarcane bagasse (Chen et al., 2016), and waste by-products, such as glycerol from biodiesel production (Li et al., 2016) appear to be promising based upon many successful attempts. Seawater, a widely available and inexpensive resource, can be a promising alternative to plain water in these processes with the possibility to potentially improve the overall economics of the process (Lin et al., 2011; Leema et al., 2010). Many products have been reported by a seawater-based fermentation, such as PHA (Yue et al., 2014), L-arginine (Oren 2002) and monapinone (Kawamoto et al., 2011). While the salinity of seawater is around 25–35 practical salinity units (PSU). Therefore, improving cell resistance against high salt stress is vital for realization of efficient bio-based succinate production.

During the past decades, series of non-rational and rational strategies were developed to endow cell with improved high salt stress resistance, including: 1) adaptive evolution (Wang et al., 2011; Wu et al., 2012); 2) exogenous addition or endogenous synthesis of osmoprotectants, e.g. glycine betaine, trehalose and glycerol (Metris et al., 2014; Yu et al., 2014); 3) efflux pump engineering (Zhang et al., 2016); 4) cell

* Corresponding author at: College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, No. 30 Puzhu South Road, Pukou District, Nanjing 211816, PR China.
E-mail address: bioengine@njtech.edu.cn (M. Jiang).

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membrane composition modification (Sheehan et al., 2006) and 5) transcriptional regulator engineering by using native, exogenous or artificial transcriptional regulators (Chen et al., 2012; Tan et al., 2016; Zhang et al., 2015). Compared to the former four methods, transcriptional regulator engineering has attracted more and more attention due to the genome-scale transcriptional regulation, which more fits in the cell response against abiotic stress. Since stress tolerance is a complex mechanism governed through multilayered regulatory networks, the use of regulators facilitates the modulation of the overall cellular processes of microbes, which cannot be achieved through the overexpression of a single functional gene. IrrE is a global regulator, which was firstly found in *Deinococcus radiodurans*. and is a vital transcriptional factor assisting *D. radiodurans* survive under extreme radiation conditions (Earl et al., 2002). Further investigation of the underlying mechanism by protein file analysis showed that five major protein groups including stress responsive proteins, protein kinases, glycerol-degrading enzymes, detoxification proteins and metabolism or growth-related proteins were regulated to improve cell resistance against salt in the IrrE-expressing strain. However, the effect of IrrE to improve cell resistance against high salt stress and carboxylic acids production under anaerobic growth was rarely investigated.

E. coli Suc260 is a promising succinate-producing strain with strong anaerobic growth capability. However, it shows poor resistance to high salt concentration stress, which constricts its large-scale production for succinate. To further increase the cell resistance to high stress, the *irrE* gene, from *D. radiodurans*, was introduced in *E. coli* Suc260 and the effect of *irrE* on cell growth, glucose consumption and succinic acid production was investigated. To elucidate the mechanism underlying the increased high salt tolerance, the transcriptional level of key genes involved in osmoprotectants synthesis and main metabolic pathway were evaluated. Additionally, a novel approach was investigated to use seawater instead of the fresh water as a water source in succinic acid fermentations process, which will reduce the production cost in industrial biotechnology.

2. Materials and method

2.1. Strains, media, and growth conditions

Suc260 (a derivate of *E. coli* BER208, CCTCC NO: M 2012351) was used as the host for succinic acid production (Wu et al., 2017). The medium for strain construction was conducted using Luria Bertani (LB) medium. For the fermentation experiments, the strains were grown in chemically defined (CD) medium: 3 g/L citric acid, 4 g/L $Na_2HPO_4{\cdot}12\ H_2O$, 8 g/L KH_2PO_4, 8 g/L $(NH_4)_2HPO_4$, 0.2 g/L NH_4Cl, 0.75 g/L $(NH_4)_2SO_4$, 1 g/L $MgSO_4{\cdot}7\ H_2O$, 10.0 mg L^{-1} $CaCl_2{\cdot}2\ H_2O$, 0.5 g/L $ZnSO_4{\cdot}7\ H_2O$, 0.25 mg L^{-1} $CuCl_2{\cdot}2\ H_2O$, 2.5 mg L^{-1} $MnSO_4{\cdot}H_2O$, 1.75 mg L^{-1} $CoCl_2{\cdot}6\ H_2O$, 0.12 mg L^{-1} H_3BO_3, 1.77 mg L^{-1} $Al_2(SO_4)_3$, 0.5 mg L^{-1} $Na_2MoO_4{\cdot}2\ H_2O$, 16.1 mg L^{-1} ferric citrate, 20.0 mg L^{-1} thiamine, and 2.0 mg L^{-1} biotin. Glucose was added separately after sterilization at a concentration of 30 g/L for seed culture and 30–200 g/L for fermentation. When required, the medium was supplemented with 100 mg L^{-1} ampicillin. The strains were grown at 37 °C, 200 rpm.

2.2. Expression of the irrE gene in E. Coli Suc260

The *groE* promoter and *irrE* gene was amplified from *D. radiodurans* R1 genomic DNA by PCR with primers GroE-F/R (GGATACCCCCATTCCCCGTCC/
GACGTTGGCACTGGGCACGTGGGGTCCTCCTGTGAGTG) and IrrE-F/R (CACTCACAGGAGGACCCCACGTGCCCAGTGCCAACGTC/
GTTCACTGTGCAGCGTCCTG) (Meima et al., 2001). The promoter was ligated into pMD-19T, generating the control plasmid TG. The products of *groE* promoter and *irrE* gene were fused by over-lap PCR. Then the fused fragment was ligated into pMD19-T, obtained the recombinant plasmid TGE. The recombinants strains, designated BE061 and BE062, were obtained by transformation of *E. coli* Suc260 with TG and TGE, respectively. The BE061 strain was used as the control strain.

2.3. Anaerobic fermentation and analytical method

A seed inoculum of 1 mL from an overnight 5 mL CD culture was added to a 500 mL flask containing 50 mL CD medium for aerobic growth at 37 °C and 200 rpm. After incubating for 8 h, a 10% inoculum was used to start the anaerobic culture. The anaerobic fermentation was conducted at 200 rpm and 37 °C for 48 h in 100 mL sealed bottles containing 30 mL CD medium supplemented with 30g/L glucose and 16g/L magnesium carbonate hydroxide to maintain the pH at 6.8. The medium was aerated pure CO_2 for at least 2 min to remove oxygen.

Anaerobic fermentation was also carried out in a 5-L bioreactor (Bioflo110, USA) containing 1.5 L CD medium. During fermentations, a seed inoculum of 1 mL from an overnight 5 mL CD culture was added to a 1000 mL flask containing 150 mL CD medium for aerobic growth at 37 °C and 200 rpm. After incubating for 8 h, a 10% inoculum was used to start the anaerobic culture. The anaerobic cultures were supplemented with 100g/L glucose. Anaerobic conditions were established by sparging the culture with CO_2 at a flow rate of 0.2 L/min. The pH was kept at 6.80 by 20% (w/v) Na_2CO_3. The temperature and agitation were maintained at 37 °C and 200 rpm, respectively.

Cell mass was estimated from the OD_{600}. Organic acids were measured by high-performance liquid chromatography (HPLC, UitiMate 3000 HPLC system, Dionex, USA). A UVD 170U and an ion exchange chromatographic column (Aminex HPX-87H, 7.8 mm × 300 mm, Bio-Rad, USA) were used at a wavelength of 215 nm, and the mobile phase was 5 mM H_2SO_4 with the flow rate of 0.6 mL/min at 55 °C. Glucose concentration in the fermentation broth was detected by a chronoamperometry method. In this detection process, a calibration line of current response vs. glucose concentration was first fitted by the continuous additions of a standard glucose concentration into a buffer solution. Then the target sample was added to calculate its glucose concentration by using the calibration line (Jiang et al., 2016; Yang et al., 2017).

2.4. Quantitative RT-PCR analysis

To study the effect of the IrrE during the natural fermentation, the level of expression of various genes in transformed *E. coli* cells at the fermentation time of 41.5 h was measured by real-time PCR. Total RNA was isolated using the TaKaRa RNAiso Plus (Takara), and then treated with RNase-free DNase I (TaKaRa) to eliminate genomic DNA. The RNA was reverse transcribed using a cDNA synthesis kit (Takara) according to the manufacturer's protocol. Appropriately diluted cDNA was used with a SYBRs Premix Ex TaqTM kit (Takara) according to the manufacturer's protocol. The expression levels of the selected genes were determined using the ABI 7500 Real-Time PCR system (Applied Biosystems). The 16S rRNA gene was used to standardize the mRNA levels. The Cycles Threshold (CT) values were used to calculate the mean fold change of the reactions via the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.5. Estimation of intercellular trehalose and glycerol content

For the analysis of the intracellular products, the whole cells were collected and immediately centrifuged at 10,000 × *g* at 4 °C for 5 min. The harvested cells were washed with cold distilled water and treated in a microwave oven at 700 W with five repeated treatments of 60 s each with a 30 s interval between each treatment (Liu et al., 2006). The trehalose and glycerol were then extracted with 1 mL of distilled water at room temperature for 1 h. The samples were centrifuged at 20,000 × *g* for 10 min, and the trehalose and glycerol in the supernatant were analyzed by HPLC. The resulting supernatants were filtered through a cellulose acetate filter (0.45 mm) and stored at −20 °C until