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Construction of reductive pathway in *Saccharomyces cerevisiae* for effective succinic acid fermentation at low pH value

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HIGHLIGHTS

- Metabolic engineering was developed to improve succinate production in yeast.
- Reductive pathway was efficient for improving succinate yield.
- Succinate production was improved by the deletion of *GPD1*.
- Succinate yield was regulated by urea and biotin levels.
- Succinic acid could be effectively produced at pH 3.8 in batch bioreactor.

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ABSTRACT

Succinic acid is an important precursor for the synthesis of high-value-added products. *Saccharomyces cerevisiae* is a suitable platform for succinic acid production because of its high tolerance towards acidity. In this study, a modified pathway for succinate production was established and investigated in *S. cerevisiae*. The engineered strain could produce up to 6.17 ± 0.34 g/L of succinate through the constructed pathway. The succinate titer was further improved to 8.09 ± 0.28 g/L by the deletion of *GPD1* and even higher to 9.98 ± 0.23 g/L with a yield of 0.32 mol/mol glucose through regulation of biotin and urea levels. Under optimal supplemental CO_2 conditions in a bioreactor, the engineered strain produced 12.97 ± 0.42 g/L succinate with a yield of 0.21 mol/mol glucose at pH 3.8. These results demonstrated that the proposed engineering strategy was efficient for succinic acid production at low pH value.

1. Introduction

Succinic acid, a 1,4-dicarboxylic acid, is widely used in the food, pharmaceutical and chemical industries (Zeikus et al., 1999). It serves as an important precursor for the synthesis of high-value-added products with a potential global market of over 2 billion USD annually (McKinlay et al., 2007). Nowadays, succinic acid is primarily derived through petroleum-based processes, which leads to high prices and environmental problems. On the other hand, microbial fermentation is considered a green process that can produce succinate from renewable resources. It has seen great developments in recent years due to increases in petroleum prices and environmental concerns (Liu et al., 2012, 2013; Liang et al., 2013).

Saccharomyces cerevisiae is a robust and important industrial microorganism with a thoroughly researched genetic background. Well-established manipulation tools are also available for this organism. Recently, the use of *S. cerevisiae* in production of biochemicals besides the traditional ethanol has been intensively investigated (Zelle et al., 2008; Kim et al., 2013). Current production practices for succinic acid primarily utilize *Actinobacillus succinogenes* and genetically engineered *Escherichia coli* (Liu et al., 2013; Song and Lee, 2006). However, in these cases, large quantities of neutralizing compounds need to be added during the fermentation process to maintain a neutral environment. Compared with prokaryotes, *S. cerevisiae* is highly tolerant of low pH values, making it superior for succinic acid production. Fermentation at low pH values markedly reduces the demand for alkaline neutralizers, prevents bacterial pollution and facilitates the downstream process (Li et al., 2010).

Since succinate is not normally produced at high levels in *S. cerevisiae*, metabolic engineering strategy provides an important approach to improve succinate production. All studies to date focused on methods utilizing the TCA cycle in the oxidative direction or glyoxylate shunt to produce succinate (Raab et al., 2010;

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Otero et al., 2013 and Agren et al., 2013). At present, the highest level achieved for succinate production is 0.11 mol/mol glucose with a productivity of 0.022 g/L/h in *S. cerevisiae* (Raab et al., 2010). However, if a reductive TCA pathway is used, the maximum theoretical yield of succinate is 1.714 mol of per mol of glucose, superior to values achieved by either the oxidative TCA cycle or glyoxylate shunt (Li et al., 2013). Moreover, this process results in net CO_2 fixation instead of release which has benefits for reducing global warming. The reductive TCA pathway for succinate production contains four steps: pyruvate carboxylation, oxaloacetate reduction, reversible hydrated translation of malate and fumarate, and fumarate reduction (Zeikus et al., 1999). However, there are some obstacles in succinate production via this pathway in *S. cerevisiae*. First, yeast fumarase (FUM) FUM1p exhibits characteristically irreversible catalysis of conversion of fumarate to malate, which represents a major obstacle for production via the reductive pathway (Pines et al., 1996). Second, the fumarate reductase (FRD) genes *FRDS1* and *OSM1* are only expressed under anaerobic conditions (Muratsubaki and Enomoto, 1998). In addition, production of 1 mol of succinic acid consumes 2 mol of NADH via the reductive pathway, while production of malic acid and fumaric acid needs less. Due to the limited NADH supply in the cytosol, the production of succinic acid is more difficult than that of malic acid and fumaric acid through the reductive TCA pathway in *S. cerevisiae*.

This study aims to improve succinate production by using metabolic engineering and to achieve effective fermentation of succinate at low pH values. The TAM strain, a pyruvate decarboxylase (*pdc*)-deficient *S. cerevisiae* strain that produces substantial amounts of pyruvate, was used as the parent strain (Maris et al., 2004). The effects of reductive pathway through overexpression of pyruvate carboxylase PYC2p, cytosolic retargeted MDH3p, FRDS1p and *E. coli* FumCp on succinate production were investigated. And the resultant effectiveness of the altered pathway on succinate production was evaluated. Furthermore, *GPD1* was deleted to induce accumulations of carbon reserves and reduction potential. The metabolic engineering strategy was shown in Fig. 1. Besides, the levels of neutralizing agent, nitrogen source and biotin were optimized to further improve succinic acid production. Finally, fermentation by the engineered strain at low pH in a bioreactor setting was explored.

2. Methods

2.1. Strains, media, and growth conditions

Yeast strains used in this study are derived from the evolved *pdc*-deficient *S. cerevisiae* strain TAM (Maris et al., 2004). All strains and plasmids used in this study were listed in Table 1. During yeast strain construction, cultures were grown aerobically at 30 °C, 200 rpm in SD media consisting of 20 g/L glucose, demineralized water, 6.7 g/L YNB (yeast nitrogen base without amino acids, Difco). After the solution was sterilized for 20 min at 115 °C, 0.1 g/L amino acids (uracil or/and histidine) was added. *E. coli* DH5α was used as a vector host and cultivated in Luria–Bertani medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) at 37 °C.

2.2. Construction of plasmids for overexpressing genes

For limited restriction sites, fusion PCR was used to construct the expression cassettes of target genes (Fig. S1). All primers used in this study were listed in Table S1. *PYC2* expression cassette was digested with *Xba* I, *Sal* I and ligated to YIPmcherry, resulting in YIP-PYC2. The same strategy was adopted to introduce the *MDH3R* (retargeted *MDH3* which lacking of the last 9 base pairs) expression cassette into YIP-PYC2. The resulting plasmid was designated as YIP-PYC2MDH3R. The expression cassettes of Ec*FumC*, and *FRDS1* were generated and introduced into pRS313 by the same strategies described above. Ec*FumC* were cloned from *E. coli* DH5α genome. *FRDS1* was cloned from TAM genome. Promoter, terminator, *PYC2*, *MDH3R*, and *FRDS1* were cloned from the genome of TAM. Restriction endonucleases (Takara, Japan) and DNA polymerase (KOD-Plus-Neo, Toyobo, Japan) were used according to the instructions. The plasmids were electroporated into yeast cells using a Gene Pulser Xcell Electroporation System (Bio-Rad, USA) and selected on solid SD auxotrophic plates with 1.5% agar powder.

2.3. PCR-mediated seamless gene deletion method for gene knockout

To allow for the introduction of additional plasmid, *HIS3* was deleted as a second auxotrophic selection marker. *HIS3* was deleted according to the PCR-mediated seamless gene deletion and marker recycling method (Akada et al., 2006). The single clone was checked on SD plate with or without histidine. *FUM1* and *GPD1* were deleted using the same strategy. The resulting strain was checked by PCR for losing of the gene locus.

2.4. Cell extracts and enzyme activity assays

Yeast cell cultures were collected at 24 h after inoculation. Cell extracts were carried out according to the method used by Remize et al. (2000). Activities of pyruvate carboxylase, malate dehydrogenase, isocitrate lyase and malate synthase were assayed based on the method described previously (Zelle et al., 2008). The method used for determining fumarase activity (conversion of L-malic acid to fumaric acid) was identical to the method of Kanarek and Hill (1964). The fumarase activity using fumaric acid as the substrate was assayed according to the method described by Xu et al. (2012). The fumarate reductase assay condition differed from malate dehydrogenase assay in that 1 mmol/L fumarate was added as the substrate. BSA was used as a standard to determine the total protein concentrations of the crude extract (Lowry et al. 1951). The results of enzyme activity analyses were carried out in triplicate.

2.5. Shake flask fermentation conditions

Synthetic media contained 3 g/L KH_2PO_4, 6.6 g/L K_2SO_4, 0.25 g/L $MgSO_4$, 15 mg/L EDTA, 4.5 mg/L $ZnSO_4{\cdot}7H_2O$, 0.3 mg/L $CoCl_2{\cdot}6H_2O$, 1 mg/L $MnCl_2{\cdot}4H_2O$, 0.3 mg/L $CuSO_4{\cdot}4H_2O$, 4.5 mg/L $CaC1_2{\cdot}H_2O$, 3 mg/L $FeSO_4{\cdot}7H_2O$, 0.4 mg/L $NaMoO_4{\cdot}2H_2O$, 1 mg/L H_3BO_4, 0.1 mg/L KI. Vitamin solution and indicated urea (both were filter-sterilized) were added to the media after sterilization. Final vitamin concentrations per liter were: biotin, 0.05 mg; calcium pantothenate, 1 mg; nicotinic acid, 1 mg; inositol, 25 mg; vitamin B1, 1 mg; vitamin B6, 1 mg and para-aminobenzoic acid, 0.2 mg. Glucose was separately sterilized and added to the media. Accurate weight of $CaCO_3$ was sterilized by heating the solid in glass tube at 160 °C and 6 h.

Single clone was inoculated to SD media and cultured for 48 h in a 100 mL flask containing 20 mL medium. Then, the yeast cells were collected by centrifugation, and resuspended with fresh synthetic medium. The cells were further inoculated to a 500 mL flask containing 100 mL medium with an initial OD_{660} at 0.7 ± 0.05 and addition of indicated $CaCO_3$. Fermentation was carried out at 30 °C and 200 rpm in a rotary shaker. Fermentation samples were collected every 6 or 12 h. At the last few hours of fermentation, samples were taken out every 2 h. All shake flask fermentation results were performed by three independent experiments.