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Production of pyruvate in *Saccharomyces cerevisiae* through adaptive evolution and rational cofactor metabolic engineering

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ABSTRACT

Pyruvate-decarboxylase (Pdc)-negative *Saccharomyces cerevisiae* has been proven as a suitable metabolic engineering platform to produce organic acids. *S. cerevisiae* BY5419 Pdc$^-$ strain cannot grow in batch cultures on synthetic medium with glucose as the sole carbon source, yet grows well on synthetic medium with ethanol or acetate. In this study, by combining adaptive evolution and cofactor engineering, we obtained a series of engineered yeasts that can produce pyruvate using glucose as sole carbon source. Differential expression of *noxE*, encoding a water-forming NADH oxidase from *Lactococcus lactis*, and *udhA*, encoding a soluble pyridine nucleotide transhydrogenase from *Escherichia coli*, was investigated. Of all the constructed recombinant strains, G2U1-A_0 was able to produce 75.1 g l^{-1} pyruvate, increased 21% compared with the original strain A_0. The production yield of this strain reached 0.63 g of pyruvate g of glucose^{-1}. This study demonstrates that the fine regulation of intracellular NADH/NAD$^+$ ratio is critical for cell metabolism and pyruvate production. Combining the adaptive evolution and fine regulation of intracellular NADH/NAD$^+$ ratio provides a new strategy for improving the Pdc$^-$ strain engineering platform.

1. Introduction

Saccharomyces cerevisiae is extensively known for its molecular genetics, physiology and genomics, allowing excellent accessibility for genetic modification and making it an excellent candidate as a biocatalyst for metabolic engineering [1]. The collection of compounds that are produced using *S. cerevisiae* has expanded to include organic acids and even secondary metabolites [2].

Pyruvate, a product of glycolysis, is located at an important branching point in the metabolism of carbohydrates in *S. cerevisiae*. Apart from its significance in metabolism, pyruvate serves as an effective starting material for the synthesis of many drugs and agrochemicals and is presently in the food industry as a fat burning supplement. It is also a valuable substrate for the enzymatic production of amino acids such as L-tryptophan, L-tyrosine, and L-dihydroxyphenylalanine (L-DOPA) [3]. Even under fully aerobic conditions, high glycolytic fluxes in wild-type *S. cerevisiae* strains are intrinsically linked to alcoholic fermentation [4]. Therefore, to avoid reduced product yields as a result of ethanol co-production, the metabolic engineering strategy for high-yield production of pyruvate and other organic acids with *S. cerevisiae* must focus on the reduction or elimination of ethanol formation. One approach for eliminating ethanol formation was to block pyruvate decarboxylase activity [5]. Pyruvate decarboxylase is critical for switching sugar catabolism from respiratory to fermentative. Pyruvate decarboxylase negative *S. cerevisiae* (Pdc$^-$), in which three structural genes (*PDC1*, *PDC5* and *PDC6*) [6] that encode active pyruvate decarboxylase isoenzymes were knocked out, could excrete significant amounts of pyruvate into the medium when limited amount of glucose was supplied. However, this strain was unable to grow in batch culture under normal growth conditions, and the growth of this strain in synthetic media with limited glucose required the supply of C_2 compound (acetate or ethanol) [7]. Over-expression of relative enzymes producing acetaldehyde could not completely restore the growth of Pdc$^-$ strains [8]. The requirement for C_2-compounds and high glucose sensitivity presented obstacles in the use of Pdc$^-$ strains for production of pyruvate and other organic acids. Glucose sensitivity has been observed in Pdc$^-$ strain constructed *S. cerevisiae* with genetically different backgrounds [7,8]. However, its molecular basis remains unknown.

Adaptive evolution, which does not require *a priori* knowledge on the molecular basis of cellular tolerance, provides a

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global regulation strategy using irrational metabolic engineering, which is especially useful for metabolite resistance or sugar utilization [9]. During evolution, microbial populations or single-celled microorganisms are driven to adapt to environmental changes. The response to environmental changes includes physiological and genetic changes, which ranges from alternations of DNA/RNA sequences to major variation of genome structure [9,10]. This evolutionary process could be replicated in the laboratory or in industrial environments for improving strain phenotype or biotechnological processes, thus is named evolutionary engineering [11].

Maintenance of redox balance is extremely important for metabolism and cell growth. The redox couples NADH/NAD^+ and NADPH/$NADP^+$ cannot pass the mitochondrial membrane [12]. Thus, the surplus of cytosolic NADH has to be re-oxidized in the compartment where they are produced. Usually, the maintenance of cytosolic redox balance is limited in the presence of oxygen at high glucose concentration, in which glycerol formation and external NADH dehydrogenase take effect [13]. Production of pyruvate from glucose generates two mole NADH per mole glucose in the cytoplasm through the catalyzation of glyceraldehyde phosphate dehydrogenase. The surplus of NADH in the cytoplasm may inhibit the glucose consumption and cell growth if it is not immediately consumed during the subsequent metabolism. Since the emergence of metabolic engineering, it has achieved many successful applications in optimization of cellular processes by manipulating the throughput of certain pathways [14,15]. In this view, cofactor engineering by alternation of intracellular NADH pool or NADH/NAD^+ ratio might be helpful to alleviate redox unbalance burden *in vivo*. *NoxE*, encoding a water-forming NADH oxidase from *Lactococcus lactis* [16], could potently decrease the intracellular NADH concentration and NADH/NAD^- ratio, causing a large redistribution of metabolic flux. Another candidate gene, *UdhA*, encoding a soluble pyridine nucleotide transhydrogenase from *Escherichia coli*, could catalyze the reversible transfer between NADH and NADPH [17].

In this study, we first performed the adaptive evolution of the Pdc^- yeast BY5419, aiming at metabolic utilization of glucose as sole carbon sources in batch culture. Then, *noxE* and *udhA* were expressed in the evolved strain at different levels using various promoter strengths and plasmid copy number to investigate the regulation of NADH/NAD^+ on cell metabolism and pyruvate production. Pyruvate decarboxylase-negative (Pdc^-) strain has been proven as a suitable metabolic engineering platform to produce organic acids. Our present study provides a new strategy to improve this strain as an engineering platform.

2. Materials and methods

2.1. Strains and cultivation conditions

E. coli DH5α was used for molecular manipulation, and was cultivated in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C. *S. cerevisiae* BY5419 (*pdc1*Δ::LEU2 *pdc5*Δ::URA3 *pdc6*Δ::TRP1 leu2-3,112 ura3-52 trp1-92 GAL) was obtained from the Japanese Yeast Genetic Resource Center (NBRP), and was cultivated in YPE-3% medium (1% yeast extract, 2% tryptone, 3% ethanol). YPD medium (1% yeast extract, 2% tryptone, 2% glucose) or SD minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) was applied for normal cultivation. Geneticin (200 μg ml^{-1}) was added to the culture medium when necessary.

Fermentation of the evolved strain A_0 or corresponding recombinant strains were performed aerobically in a flask at pH 5.0. Cells were first pre-cultivated overnight in YPD liquid medium at 30 °C and was inoculated into fresh YPD liquid medium supplied with indicated amount of glucose. Cell growth was monitored by measuring OD_{600} at regular time points.

2.2. Evolutionary adaptation

S. cerevisiae BY5419 taken from stock stored at −80 °C was spread onto YPE-3% plates. A single colony from the plate was inoculated into 50 ml YPE-3% medium and was incubated at 30 °C for two days. After cultivation, the flask was removed from agitation and set still for 2 h. After decanting the supernatant, part of the culture slurry was fed with fresh SD medium adding 3% ethanol and 0.5% glucose for further cultivation. By the same operation, the media was replaced periodically (about 2–5 days) with a stepwise increase in glucose concentration and decreased ethanol concentration (YNB medium with 3% ethanol; 3% ethanol and 0.5% glucose; 0.5% glucose and 2% ethanol; 0.5% glucose and 1% ethanol; 0.5% glucose and 0.5% ethanol; 0.5% glucose; 1% glucose; 2% glucose; 3% glucose; till 10% glucose).

After the 135-day adaptive cultivation process, 5 ml of culture was transferred into new 500-ml shake flasks with 100 ml of SD-10% medium (0.67% yeast nitrogen base without amino acids, 10% glucose) for growth-phenotype analysis. A single colony was obtained by plate screening. The evolved single colony, named as BY5419-A_0 (A_0 for short), was verified with respect to its growth phenotype and acid accumulation.

2.3. Analytical procedures

Optical density (OD) was measured at 600 nm with a spectrophotometer. The culture was diluted to the linear range with 0.15 M NaCl. For analyzing glucose, glycerol, pyruvate and ethanol, 1 ml of culture was centrifuged (12,000 × *g* for 2 min at 4 °C) and the supernatant was then filtered through a 0.22 μm syringe filter for analysis. The HPLC system was equipped with a cation exchange column (HPX-87H, BioRad Labs) with a differential refractive index (RI) detector (Shimadzu RID-10A). The column was maintained at 65 °C with a mobile phase of 5 mM sulfuric acid (in Milli-Q water) and flow rate of 0.6 ml min^{-1}.

18srDNA sequence analysis was done by Beijing Genomics Institute.

2.4. Genetic materials and methods

All the primers used in this study are listed in Table 1. Plasmids pRS41K and pRS42K were used as cloning vectors [18]. The GPD promoter was obtained from plasmid p426-GPD [19], GPD2 promoter and ADH1 promoter from *S. cerevisiae* CENPK 113-7D, TEF promoter from plasmid pUG6 [20].

pRS41K-GPD was generated by inserting the GPD-CYC1TT fragment, which was digested by *SacI* and *KpnI* from p426-GPD, into pRS41K. pRS41K-GPD2 was generated by inserting the GPD2 promoter fragment into pRS41K-GPD, in which both the GPD2 promoter fragment and pRS41K-GPD were digested by *SacI* and *SpeI*. The GPD2 promoter fragment was amplified by PCR from total DNA isolated from *S. cerevisiae* CENPK 113-7D, using the primers GPD2-pF and GPD2-pR. For the pRS41K-ADH1 and pRS41K-TEF, ADH1 promoter fragment or TEF promoter fragment was inserted into pRS41K-GPD separately, which was digested by *SacI* and *SpeI*. Both promoter fragments were amplified from total DNA isolated from *S. cerevisiae* CENPK 113-7D or pUG6 by PCR using corresponding primers.

The *noxE* gene, amplified from genome DNA of *L. lactis* NZ9000, was inserted into pRS41K-GPD, pRS41K-GPD2, pRS41K-ADH1 and pRS41K-TEF using *BamHI* and *SalI* site. The resulting plasmids were transformed into evolved Pdc^- strain A_0, resulting in the corresponding recombinant strains (Table 2). The plasmid pRS42K