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Modular optimization of multi-gene pathways for fumarate production

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ABSTRACT

Microbial fumarate production from renewable feedstock is a promising and sustainable alternative to petroleum-based chemical synthesis. Here, we report a modular engineering approach that systematically removed metabolic pathway bottlenecks and led to significant titer improvements in a multi-gene fumarate metabolic pathway. On the basis of central pathway architecture, yeast fumarate biosynthesis was re-cast into three modules: reduction module, oxidation module, and byproduct module. We targeted reduction module and oxidation module to the cytoplasm and the mitochondria, respectively. Combinatorially tuning pathway efficiency by constructing protein fusions RoMDH-P160A and KGD2-SUCLG2 and optimizing metabolic balance by controlling genes *RoPYC*, *RoMDH-P160A*, *KGD2-SUCLG2* and *SDH1* expression strengths led to significantly improved fumarate production (20.46 g/L). In byproduct module, synthetizing DNA-guided scaffolds and designing sRNA switchs enabled further production improvement up to 33.13 g/L. These results suggest that modular pathway engineering can systematically optimize biosynthesis pathways to enable an efficient production of fumarate.

1. Introduction

Fumarate has been designated as one of the top 12 biomass building block chemicals that has a great potential to replace traditionally used fossil fuels and promises a significantly higher market application (Werpy and Petersen, 2004). Although numerous natural producers, such as *Rhizopus formosa*, *Rhizopus arrhizus* and *Rhizopus oryzae*, have been investigated into the development of fermentation processes for fumarate production, this process unfortunately remains less efficient than other commercial organic acid production due to the fact that their morphology and pathogenic properties can strongly affect production characteristics and product safety, respectively (Xu et al., 2012c). Given these defects, considerable interest has been shown in the fermentative production of fumarate by the engineered microorganisms.

Six routes have been investigated for fumarate production, and they relate to four microorganisms, *R. oryzae*, *Candida glabrata*, *Saccharomyces cerevisiae*, and *Escherichia coli* (Fig. 1). Route I is the reductive reactions of the TCA cycle. The carbon flux towards oxaloacetate is increased through overexpression of endogenous pyruvate carboxylase and exogenous phosphoenolpyruvate carboxylase in *R. oryzae*. The resultant *R. oryzae* strain is capable of producing about 25 g/L fumarate (Zhang et al., 2012). Route II is the oxidation of citrate via the TCA cycle. Fumarate production in the final engineered *C. glabrata* strain T.G-$KS_{(H)}$-$S_{(M)}$-A-2S is increased to 15.76 g/L by mitochondrial engineering of the TCA cycle starting from α-ketoglutarate catalyzed by α-ketoglutarate dehydrogenase complex, succinyl-CoA synthetase, and succinate dehydrogenase. Route III is combination of reductive with oxidative TCA cycle. Under the addition of 32 μg/L biotin and the optimal carbon/nitrogen ratio, the engineered *S. cerevisiae* strain, in which the genes *THI2* and *FUM1* were deleted and the exogenous genes *RoPYC*, *RoMDH* and *RoFUM1* were overexpressed, can produce up to 5.64 g/L fumarate (Xu et al., 2013a). Route IV is the noncyclic glyoxylate cycle. The evolved mutant *E. coli* E2 developed for succinate production from glycerol is engineered for fumarate production (up to 41.5 g/L) through deletion of three fumarases, overexpression of phosphoenolpyruvate carboxylase gene *ppc* and the glyoxylate shunt operon *aceBA* (Li et al., 2014). Route V is the urea cycle and the purine nucleotide cycle. The highest fumarate titer (up to 8.83 g/L) was obtained with strain T.G-$ASL_{(H)}$-$ADSL_{(L)}$-SpMAE1 by controlling the strength of arginino-succinate lyase at a high level and adenylosuccinate lyase at a low level and overexpressing of the C_4-dicarboxylic acids transporter SpMAE1 (Chen et al., 2015). Route VI is simultaneous use of oxidative TCA cycle, reductive TCA cycle and glyoxylate cycle. The highest level achieved for fumarate production is 28.2 g/L with the engineered *E. coli* strain CWF812 by combining deletion of the *iclR*, *fumA*, *fumB* and *fumC* genes to redirect the carbon flux through the glyoxylate shunt, overexpression of the native *ppc*

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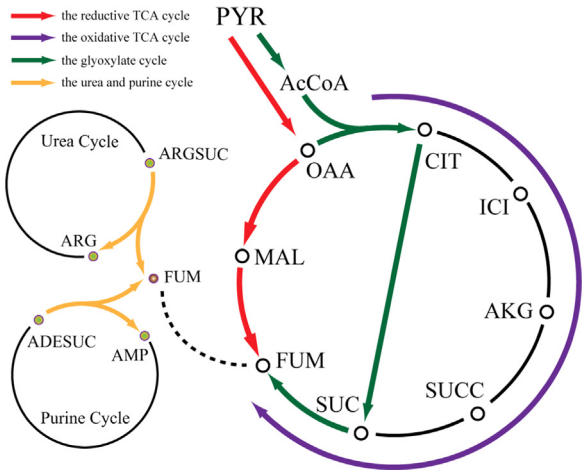

Fig. 1. The reported metabolic pathways leading to the formation of fumarate. PYR, pyruvate; AcCoA, acetyl-coenzyme A; OAA, oxaloacetate; CIT, Citrate; ICI, Isocitrate; AKG, α-ketoglutrarate; SUCC, Succinyl-CoA; SUC, Succinate; FUM, Fumarate; MAL, Malate; FUM, fumarate; ARGSUC, argininosuccinate; ARG, arginine; ADESUC, adenylosuccinate; AMP, adenosine monophosphate.

gene under the strong *tac* promoter to enhance the reductive TCA cycle flux and deletion of the *arcA* and *ptsG* genes to reinforce the oxidative TCA cycle flux (Song et al., 2013).

All these results make us clear that the challenge in metabolic engineering of *S. cerevisiae* for the production of fumarate involves at least three aspects: (i) engineering fast and efficient metabolic or synthetic pathways, (ii) enhancing intermediate metabolites transmission efficiency in metabolic or synthetic pathways, (iii) reducing carbon flux loss by repressing byproducts production. As an attempt to address intermediate metabolites transmission efficiency, mitochondrial engineering offers a frugal way to direct metabolic flux toward target product, and decreases the chances of off-target effects such as the possibility of overproducing side products (Avalos et al., 2013). In addition, protein scaffolds capturing different stoichiometric number of enzymes in proximity have been used in preventing intermediates loss (Dueber et al., 2009). Further, protein fusions can be used to engineer the active sites into close proximity for enhanced metabolic flux channeling to metabolite biosynthesis, and thus offer tremendous opportunities for customized optimization of multi-gene pathways (Zhou et al., 2012). The production of byproducts can be well repressed, and thus the pathway efficiency can be significantly improved when the sRNA switchs are used (Liu et al., 2014; Na et al., 2013).

In the present study, we described modular pathway engineering strategies to optimize a multi-gene pathway for fumarate production. By re-casting central carbon metabolic network, a total of 10 essential genes were selected and arranged into three modules to partition the complete fumarate metabolic pathway into reduction module (PMFM module), oxidation module (KSSS module) and byproduct module (RPSF module) (Fig. 2). These modules were optimized systematically to enable an efficient microbial production of fumarate. Under controlled culture conditions, the engineered strains TGFA091-16 produced up to 33.13 g/L fumarate.

2. Materials and methods

2.1. Strains and maintenance

All *S. cerevisiae* strains used in this study were obtained from the Laboratory of Food Microbial-Manufacturing Engineering at Jiangnan

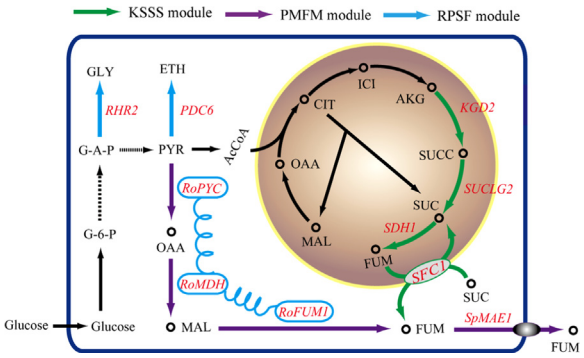

Fig. 2. The fumarate biosynthetic pathway. The engineered targets are shown in green, violet and blue, and its related target genes in red. *RoPYC*, pyruvate carboxylase; *RoMDH*, malate dehydrogenase; *RoFUM1*, fumarase; *SpMAE1*, C_4-dicarboxylic acids transporter; *KGD2*, α-ketoglutarate dehydrogenase complex E2 component; *SUCLG2*, succinyl-CoA synthetase *β* subunit; *SDH1*, succinate dehydrogenase flavoprotein subunit; *SFC1*, succinate-fumarate transporter; *RHR2*, glycerol-1-phosphate phosphohydrolase 1; *PDC6*, pyruvate decarboxylase isozyme 3; G-6-P, glucose-6-phosphate; G-A-P, glyceraldehyde-3-phosphate; GLY, glycerol; ETH, ethanol; PYR, pyruvate; AcCoA, acetyl-CoA; OAA, oxaloacetate; ICI, isocitrate; AKG, α-ketoglutarate; SUCC, succinyl-CoA; SUC, succinate; FUM, fumaric acid; MAL, malate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

University (Table S1). The yeasts were stored at −80 °C in sterile vials with 25% glycerol and 75% yeast culture. *E. coli* JM109 was used as a host for recombinant DNA manipulation and plasmid maintenance. *E. coli* BL21(DE3) was used to express and purify the fumarase.

2.2. Isolation of the fumarate biosynthesis genes

The pyruvate carboxylase (*RoPYC*), malate dehydrogenase (*RoMDH*) and cytosolic fumarase (*RoFUM1*) were amplified by PCR using the *c*DNA of *R. oryzae* NRRL1526 as template. The C_4-dicarboxylic acids transporter (*SpMAE1*) was amplified by PCR from the chromosomal DNA of *Schizosaccharomyces pombe*. The α-ketoglutarate dehydrogenase complex E2 component (*KGD2*), succinyl-CoA synthetase *β* subunit (*SUCLG2*), succinate dehydrogenase flavoprotein subunit (*SDH1*) and succinate-fumarate transporter (*SFC1*) genes were amplified by PCR using the chromosomal DNA of *S. cerevisiae* CEN.PK2-1C as template. The sequences of synthetic small regulatory RNAs were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Bayer and Smolke, 2005). Switch sRNA-RHR2 was used to regulate glycerol-1-phosphate phosphohydrolase 1 (*RHR2* also called *GPP1*), whereas sRNA-PDC6 was able to regulate pyruvate decarboxylase (*PDC1/5/6*) via homologous sequence "aaaATGTCTGAAAT".

2.3. Pathway construction and transformation

Pathway construction and DNA manipulation were performed following standard molecular cloning protocols. All plasmids and primers used in this work were listed in Tables S1 and S2, respectively. The fusion enzymes encoding genes *RoMDH* and *P160A*, *P160A* and *RoMDH*, *KGD2* and *SUCLG2*, *KGD2* and *SDH1*, *SUCLG2* and *SDH1* were constructed by inserting a widely used GGGS linker encoding sequence "GGT GGT GGT TCT" between the two corresponding genes (Zhou et al., 2012). The KGD2, SUCLG2, and SDH1 were targeted to mitochondria using the N-terminal mitochondrial localization signal. The encoding sequences of zinc finger proteins ADB2 and ADB3 were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) (Lee et al., 2013). ADB2 and RoPYC were fused with fusion PCR linked with a widely used GGGGS linker to obtain the encoding sequence of fusion protein ADB2-RoPYC. The encoding sequence of fusion protein ADB3-RoMDH-P160A was attained in the same manner.