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Design and construction of acetyl-CoA overproducing *Saccharomyces cerevisiae* strains

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ABSTRACT

Saccharomyces cerevisiae has increasingly been engineered as a cell factory for efficient and economic production of fuels and chemicals from renewable resources. Notably, a wide variety of industrially important products are derived from the same precursor metabolite, acetyl-CoA. However, the limited supply of acetyl-CoA in the cytosol, where biosynthesis generally happens, often leads to low titer and yield of the desired products in yeast. In the present work, combined strategies of disrupting competing pathways and introducing heterologous biosynthetic pathways were carried out to increase acetyl-CoA levels by using the CoA-dependent *n*-butanol production as a reporter. By inactivating ADH1 and ADH4 for ethanol formation and GPD1 and GPD2 for glycerol production, the glycolytic flux was redirected towards acetyl-CoA, resulting in 4-fold improvement in *n*-butanol production. Subsequent introduction of heterologous acetyl-CoA biosynthetic pathways, including pyruvate dehydrogenase (PDH), ATP-dependent citrate lyase (ACL), and PDH-bypass, further increased *n*-butanol production. Recombinant PDHs localized in the cytosol (cytoPDHs) were found to be the most efficient, which increased *n*-butanol production by additional 3 fold. In total, *n*-butanol titer and acetyl-CoA concentration were increased more than 12 fold and 3 fold, respectively. By combining the most effective and complementary acetyl-CoA pathways, more than 100 mg/L *n*-butanol could be produced using high cell density fermentation, which represents the highest titer ever reported in yeast using the clostridial CoA-dependent pathway.

1. Introduction

As a central metabolite, acetyl-CoA plays important roles in a series of cellular functions involved in enzymatic acetyl transfer reactions. Acetyl-CoA is the starting compound of the tricarboxylic acid (TCA) cycle, and a key precursor in the biosynthesis of sterols, fatty acids and lipids, amino acids, and polyketides (Tai and Stephanopoulos, 2013; Vorapreeda et al., 2012; Xu et al., 2013, 2011; Zaidi et al., 2012; Zha et al., 2009). In addition, acetyl-CoA functions as the donor of acetyl group for post-translational acetylation reactions of histone and non-histone proteins. To accommodate the cellular requirement, nature has evolved a variety of routes for acetyl-CoA synthesis (Fig. S1), such as the oxidative decarboxylation of pyruvate (Guest et al., 1989), the oxidation of long-chain fatty acids (Trotter, 2001), and the oxidative degradation of certain amino acids (Vorapreeda et al., 2012). In terms of the bulk synthesis of acetyl-CoA, the most common mechanism is the direct conversion from pyruvate, either by pyruvate dehydrogenase (PDH) under aerobic conditions (Guest et al., 1989), or by pyruvate ferredoxin oxidoreductase (PFO) (Ragsdale, 2003), pyruvate $NADP^+$ oxidoreductase (PNO) (Inui et al., 1987), or pyruvate formate lyase (PFL) (Knappe and Sawers, 1990) under anaerobic conditions. Due to the compartmentalization of acetyl-CoA metabolism in eukaryotes, direct conversion of pyruvate to acetyl-CoA only happens in some organelles, such as mitochondria and chloroplasts. In the cytosol, two ATP consuming mechanisms are used to synthesize acetyl-CoA. One is PDH-bypass (Shiba et al., 2007), i.e., from pyruvate

Abbreviations: PDH, pyruvate dehydrogenase; ACL, ATP-dependent citrate lyase; ACS, acetyl-CoA synthetase; PFO, pyruvate ferredoxin oxidoreductase; PNO, pyruvate $NADP^+$ oxidoreductase; PFL, pyruvate formate lyase; ADH, alcohol dehydrogenase; GPD, glycerol-3-phosphate dehydrogenase; PDC, pyruvate decarboxylase; CIT, citrate synthase; MLS, malate synthase; Thl, thiolase; Hbd, β-hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Ter, *trans*-2-enoyl-CoA reductase; Bad, butyraldehyde dehydrogenase; Bdh, butanol dehydrogenase; AdhE/Aad, bifunctional aldehyde/alcohol dehydrogenase; PHB, polyhydroxybutyrate; FAEE, fatty acid ethyl ester; TCA, tricarboxylic acid; MTS, mitochondrial targeting sequence

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to acetaldehyde and then to acetate, which is then activated to acetyl-CoA by the acetyl-CoA synthetase (ACS) at the cost of two ATP molecules (Starai and Escalante-Semerena, 2004). PDH-bypass is widely distributed in ethanogenic species, such as yeasts, filamentous fungi, and plants. The other is ATP-dependent citrate lyase (ACL), using a TCA cycle intermediate citrate as the substrate, which is enzymatically converted to acetyl-CoA and oxaloacetate at the cost of one ATP molecule (Zaidi et al., 2012). ACL is found in both ethanogenic and non-ethanogenic species such as fungi, plants, and animals. Notably, comparative genomic studies reveal that ACL is present in oleaginous yeasts such as *Yarrowia lipolytica*, but not in non-oleaginous yeasts such as *Saccharomyces cerevisiae* (Vorapreeda et al., 2012), indicating the significance of ACL in the supply of precursor metabolites to the biosynthesis of fatty acids and lipids. The phosphoketolase pathway is also reported to contribute to cytosolic acetyl-CoA generation in some fungal species (Kocharin et al., 2013).

In addition to its significant roles as a building block for cellular metabolism, acetyl-CoA is also the key precursor for biological synthesis of a variety of fuel and chemical molecules, such as *n*-butanol, fatty acid ethyl esters (FAEEs), alkanes, polyhydroxybutyrate (PHB), and isoprenoid-derived drugs (Chen et al., 2013; Kocharin et al., 2012, 2013; Krivoruchko et al., 2013; Shiba et al., 2007). Because of the concerns over sustainability and energy security, biological production of these molecules in industrially friendly hosts such as *Escherichia coli* and *S. cerevisiae* has attracted increasing attention (Du et al., 2011). Compared with *E. coli*, *S. cerevisiae* has the advantages of high tolerance to harsh industrial conditions and resistance to phage infection (Chen et al., 2013; Hong and Nielsen, 2012). However, the production of acetyl-CoA derived molecules in *S. cerevisiae* is far behind that in *E. coli*, in terms of the titer of the desired products. One possible reason is the difference in acetyl-CoA metabolism. In *E. coli*, acetyl-CoA is steadily synthesized from pyruvate by either PDH under aerobic conditions (Guest et al., 1989) or PFL under anaerobic conditions (Knappe and Sawers, 1990). In *S. cerevisiae*, the metabolism of acetyl-CoA is separated into several compartments, including the mitochondria, peroxisomes, nucleus, and cytosol (Strijbis and Distel, 2010). Acetyl-CoA is mainly generated in the mitochondria; however, *S. cerevisiae* lacks the machinery to export the mitochondrial acetyl-CoA to the cytosol where the synthesis of desired products generally occurs (Strijbis and Distel, 2010). In the cytosol, acetyl-CoA is generated via PDH-bypass, and the activation of acetate is the rate-limiting step, resulting from the low activity and high energy input requirement of ACS (Shiba et al., 2007). Several metabolic engineering strategies have been carried out to boost the availability of acetyl-CoA in yeast, such as the use of an ACS mutant from *Salmonella enterica* ($Se Acs^{L641P}$) with increased activity (Chen et al., 2013; Kocharin et al., 2012; Krivoruchko et al., 2013; Shiba et al., 2007) or the introduction of heterologous acetyl-CoA biosynthetic pathways with lower energy input requirement (Kocharin et al., 2013; Tang et al., 2013). Notably, these strategies have been applied to improve the production of isoprenoids (Chen et al., 2013; Shiba et al., 2007), PHB (Kocharin et al., 2012, 2013), *n*-butanol (Krivoruchko et al., 2013), and fatty acids (Tang et al., 2013) in *S. cerevisiae*.

Nevertheless, most of the recent efforts on acetyl-CoA pool engineering in yeast were mainly focused on the introduction of heterologous pathways, especially the PDH-bypass pathway with the ACS mutant. However, host engineering to redirect the metabolic flux to acetyl-CoA can be as important as the introduction of heterologous acetyl-CoA biosynthetic pathways. As shown in Fig. 1, acetaldehyde is the branch point to control the flux to ethanol and acetyl-CoA, and during glucose fermentation most of the metabolic flux goes to ethanol known as the Crabtree effect. Thus, the inactivation of alcohol dehydrogenases (ADHs) would direct the flux to acetyl-CoA. However, previous reports indicated that decreased ADH activity led to increased production of glycerol (de Smidt et al., 2012). Therefore, simultaneous disruption of the glycerol pathway and ethanol pathway would be necessary to increase the acetyl-CoA levels. In *S. cerevisiae*, the transport of acetyl-CoA between different compartments is carried out by the glyoxylate shunt (Chen et al., 2012; Strijbis and Distel, 2010), with several organic acids as the intermediates, such as citrate, malate, succinate, and oxaloacetate. The disruption of the glyoxylate shunt by knocking out *CIT*2, encoding a peroxisomal citrate synthase, or *MLS*1, encoding a cytosolic malate synthase, could increase the levels of acetyl-CoA in the cytosol (Chen et al., 2013). Therefore, the combination of "push" and "pull" strategies was carried out to increase the cytosolic acetyl-CoA levels in yeast.

In the present study, various metabolic engineering strategies on enhancing acetyl-CoA availability were carried out, including the disruption of competing pathways and the introduction of heterologous biosynthetic pathways with higher catalytic efficiency and lower energy input requirement. By inactivating the glycerol pathway and major ADHs involved in ethanol production, the metabolic flux was redirected to acetyl-CoA and the production of *n*-butanol was increased by 4 fold. Subsequent introduction of heterologous acetyl-CoA biosynthetic pathways individually or in combination, including the PDH-bypass pathways, cytosolic localized PDHs (cytoPDHs), and ACLs, into the engineered host further increased the production of *n*-butanol. Among several acetyl-CoA pathways tested, the cytoPDHs worked the best, which increased the production of *n*-butanol by an additional 3 fold. By combining the most effective acetyl-CoA pathways, an *n*-butanol titer as high as 120 mg/L was achieved under high cell density fermentation, which is the highest *n*-butanol titer ever reported in *S. cerevisiae*. To our knowledge, this is the first report of the functional expression of PDHs in the cytosol of yeast, as confirmed by three independent observations: increased acetyl-CoA levels, increased *n*-butanol production, and growth complementation of the *pdc*− strain. The acetyl-CoA overproducing strains constructed in this work would be useful for efficient production of a wide range of acetyl-CoA derived products of industrial interest.

2. Materials and methods

2.1. Strains, media, and cultivation conditions

All engineered strains used in this study are based on *S. cerevisiae* CEN.PK2-1C strain. *E. coli* strain DH5α was used to maintain and amplify plasmids, and recombinant strains were cultured at 37 °C in Luria-Bertani (LB) broth containing 100 μg/mL ampicillin. Yeast strains were cultivated in complex medium consisting of 2% peptone and 1% yeast extract supplemented with 2% glucose (YPD) or galactose (YPG). Recombinant strains were grown on synthetic complete medium consisting of 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and the appropriate amino acid drop out mix, supplemented with 2% glucose (SCD) or galactose (SCG). The *adh*1–*adh*4− yeast strains were pre-cultured in galactose medium under aerobic conditions (30 °C and 250 rpm) and inoculated to glucose medium for *n*-butanol fermentation under oxygen-limited conditions (30 °C and 100 rpm). All restriction enzymes, Q5 polymerase, and the *E. coli–S. cerevisiae* shuttle vectors were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. DNA manipulation

The yeast homologous recombination based DNA assembler method was used to construct the recombinant plasmids (Shao et al., 2009). Briefly, DNA fragments sharing homologous regions to adjacent DNA fragments were co-transformed into *S. cerevisiae*