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

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*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.

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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 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
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 (Stara 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 (Varapreda 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 (Kocharın 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 isopropanoid-derived drugs (Chen et al., 2013; Kocharin et al., 2012, 2013; Krivoruchko et al., 2013; Shibà 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 increased 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 titers 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 (Knapp 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 (Shibà 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 (SeAcs164ATP) with increased activity (Chen et al., 2013; Kocharin et al., 2012; Krivoruchko et al., 2013; Shibà et al., 2007) or the 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 adh1–adh4– 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.
along with the linearized backbone to assemble several elements in a single step. Oligonucleotides used in this study are listed in Table S1, and the recombinant plasmids constructed in this study are listed in Table 1. Wizard Genomic DNA Purification Kit (Promega, Madison, WI) was used to extract the genomic DNA from both bacteria and yeasts, according to the manufacturer’s protocol. To construct ScPDH, the mitochondrial targeting sequences (MTSs) were predicted using the MITOPROT online tool (http://ihg.gsf.de/ihg/mitoprot.html) (Claros and Vincens, 1996) and were excluded for subsequent cloning. All acetyl-CoA biosynthetic pathways were first cloned into pRS424. In order to combine the overexpression of complementary acetyl-CoA pathways, PDH-bypass pathways were subcloned into pRS425, and ACLs were subcloned into pRS423. To confirm the correct clones, yeast plasmids were isolated using a Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research, Irvine, CA) and amplified in E. coli for verification by both restriction digestion and DNA sequencing.

2.3. Strain construction

All the strains used in this study are listed in Table 2. For the construction of gpd1−, gpd2−, adh1−, adh4−, cit2−, and/or mls1− strains, the loxP-KanMX-loxP method (Hegemann et al., 2006) was used for successive deletion of these genes. The pdc− strain was constructed in our previous work (Lian et al., 2014). The deletion of each gene was verified by diagnostic PCR. Yeast strains were transformed using the LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007), and transformants were selected on the appropriate SCD or SCG plates.

2.4. n-Butanol fermentation and detection

A single colony from the newly transformed plate was inoculated into 3 mL of the appropriate SCD or SCG medium, and cultured under aerobic conditions for 36 h. Then 200 μL of seed culture was transferred into 10 mL of the corresponding SCD medium in a 50 mL baffled flask at an initial OD600 of about 0.05, and cultured under oxygen-limited conditions. Samples were taken every 24 h after inoculation until no further increase in n-butanol production was observed, and the highest titer detected was used to compare the effects of different metabolic engineering strategies on n-butanol production.

Samples were centrifuged at 14,000 rpm for 10 min and the resulting supernatant was analyzed using a Shimadzu GCMS-QP2010 Plus GC–MS equipped with an AOC-20i+ s autosampler and a DB-Wax column with a 0.25 μm film thickness, 0.25 mm diameter, and 30 m length (Agilent Inc., Palo Alto, CA). Injection port and interface temperature was set at 250 °C, and the ion source set to 230 °C. The helium carrier gas was set at a constant flow rate of 2 mL/min. The oven temperature program was set as
the following: (a) 3 min isothermal heating at 50 °C, (b) increase at the rate of 15 °C/min to 120 °C, (c) increase at the rate of 50 °C/min to 230 °C, (d) and then isothermal heating at 230 °C for an additional 2.5 min. The mass spectrometer was operated with a solvent cut time of 1.5 min, an event time of 0.2 s, and a scan speed of 2500 from the range of 30–500 mass to charge (m/z) ratio. Concentrations were determined using standard curves, with 50 mg/L methanol as the internal standard. Each data point of the n-butanol titer represents the average of at least duplicates.

2.5. Acetyl-CoA assay

Yeast cells were pre-cultured, inoculated, and cultured under the same conditions as n-butanol fermentation. After growing to the mid-log phase, cell metabolism was quenched by adding 8 mL cells into 40 mL pre-chilled (–80 °C) methanol, and cells were harvested by centrifugation at 4000g for 5 min. Then 2 mL boiling ethanol was added to the cell pellets, followed by boiling for an additional 15 min to release intracellular metabolites. After centrifugation, the supernatant was vacuum dried and resuspended into 200 μL ddH2O. The resultant solution containing acetyl-CoA was analyzed by the acetyl-Coenzyme A Assay Kit (Sigma-Aldrich). The concentration of acetyl-CoA was the average of biological duplicates and normalized by the dry cell weight.

3. Results

3.1. Construction of an n-butanol pathway as a reporter of cytosolic acetyl-CoA levels

Although several protocols for the detection of CoAs in biological systems have been developed, it is still rather tedious and
labor intensive to carry out the extractions and assays (Park et al., 2007; Steen et al., 2008). Thus, the acetyl-CoA dependent n-butanol pathway (Fig. S2) was chosen as a model to study the effects of various metabolic engineering strategies on the acetyl-CoA pool in the cytosol of yeast. An artificial n-butanol biosynthetic pathway (named HZ1982) consisting of the Clostridium acetobutylicum thiolase (CaThl), C. acetobutyricum β-hydroxybutyryl-CoA dehydrogenase (CaHbd), Clostridium beijerinckii crotonase (CBChr), Treponema denticola trans-2-enoyl-CoA reductase (TdTer), C. beijerinckii butyraldehyde dehydrogenase (CbBad), and C. acetobutyricum butanol dehydrogenase B (CaBdhB) was constructed. Unfortunately, no n-butanol production could be detected (Fig. S3). Enzyme activity assay indicated the lack of Bad activity, while all other activities including Thl, Hbd, Crt, Ter, and Bdh were detected (Fig. S2). To obtain a functional n-butanol pathway, candidate genes with either putative Bad activity or bi-functional aldehyde/alcohol dehydrogenase (AdhE/Aad) activity were cloned from various organisms. Indeed, complementing HZ1982 with a functional Bad, such as MhpF, EutE, and AdhE from E. coli and Aad from C. beijerinckii, allowed the production of a small amount of n-butanol in yeast (Fig. S3). CbBad and AdhE from C. acetobutyricum (CaAdhE2), which have been widely used for efficient n-butanol production in various hosts (Atsumi et al., 2008; Lan and Liao, 2011; Nielsen et al., 2009), were nonfunctional for n-butanol production in yeast in our present study. Western-blot analysis indicated that CbBad was only expressed as the insoluble form and no expression of CaAdhE2 was observed (Fig. S4). Among all the Bad or AdhE/Aad candidates tested, EutE from E. coli gave the highest n-butanol production, which was cloned to replace the nonfunctional CbBad in HZ1982. The resultant n-butanol pathway (BuPa28) was used as a reporter of the cytosolic acetyl-CoA levels. In the wild-type yeast strain with BuPa28 (JL0511), 2–3 mg/L n-butanol could be produced, a titer similar to previous reports using the Clostridia CoA-dependent pathway (Chen et al., 2013; Steen et al., 2008). Recently, the ketoacid pathways were proposed and determined to enable the production of n-butanol at a titer of 94 mg/L (with glycine as the precursor) or 284 mg/L (with threonine as the precursor) in S. cerevisiae (Branduardi et al., 2013; Si et al., 2014). However, such high n-butanol production could only be achieved with the supplementation of glycine (Branduardi et al., 2013) or in rich medium (Si et al., 2014). In addition, n-butanol was not directly synthesized from acetyl-CoA in these novel pathways. Therefore, these two works were not included for comparison in the present study.

3.2. Host engineering to direct metabolic flux to acetyl-CoA

To redirect the glycolytic flux to acetyl-CoA, ethanol and glycerol biosynthetic pathways were partially or completely inactivated in S. cerevisiae. Glyceraldehyde-3-phosphate dehydrogenases are the rate-limiting enzymes for glyceral production (Fig. 1). Thus both of the structural genes, GPD1 and GPD2, were deleted to eliminate glyceral production. As for the ethanol production, several ADHs, including but not limited to ADH1, ADH2, ADH3, ADH4, ADH5, ADH6, and ADH7, catalyze the NAD(P)H dependent reduction of acetaldehyde to ethanol (de Smidt et al., 2012), which directly competes with acetyl-CoA biosynthesis in the cytosol. ADH2 is glucose repressed at the transcription level, ADH3 and ADH5 are mainly localized in the mitochondrion, and ADH6 and ADH7 were determined to be specific for medium chain and branched chain substrates (de Smidt et al., 2012). As a result, ADH1 and ADH4 were chosen as the targets to decrease ethanol production and redirect the flux to acetyl-CoA when cultured on glucose. As shown in Fig. S5, the growth and sugar utilization were dramatically decreased when the gpd1–gpd2–adh1–adh4 strain was cultured on glucose, although no significant difference was observed for galactose fermentation. In addition, both acetaldehyde and acetate, the precursors for cytosolic acetyl-CoA, were accumulated to high levels after the inactivation of ADH1 and ADH4 (Fig. S6). Accordingly, the n-butanol titer in strain JL0516 was increased by more than 4 fold, compared with that in the wild-type strain JL0511 (Fig. 2 and Fig. S7). The productivity of n-butanol was only increased about 2 fold (Fig. S7), probably because of the impaired growth and poor sugar utilization.

Disruption of the cytosolic acetyl-CoA consuming pathways, such as the glyoxylate shunt, was also carried out to enhance the availability of acetyl-CoA. As shown in Fig. 2, n-butanol titer was increased about 2 fold by inactivating CIT2 or MLS1 in the wild-type strain. However, further deletion of CIT2 or MLS1 in the gpd1–gpd2–adh1–adh4 strain (JL0111) led to decreased n-butanol production, probably caused by the accumulation of acetate, a metabolite that shows cytotoxicity at high levels (Fig. S6). Therefore, JL0111 was chosen as the host for subsequent introduction of acetyl-CoA biosynthetic pathways.

3.3. Introduction of various acetyl-CoA biosynthetic pathways into the engineered host

Considering the low efficiency of the endogenous PDH-bypass pathway, heterologous acetyl-CoA biosynthetic pathways with higher efficiency and/or lower energy input requirement would be desirable. The most straightforward strategy was to overexpress the PDH-bypass pathway containing the ACS mutant with increased activity. However, overexpression of the whole PDH-bypass pathway (HZ1983 and pYCO8), which was demonstrated to improve the production of a series of acetyl-CoA derived molecules in wild-type yeast, nearly blocked the production of n-butanol in the engineered host (Fig. 3). Later it was found that low n-butanol production was accompanied with the accumulation of a large amount of acetate (Fig. S6). To figure out the reason for low n-butanol production, additional recombinant plasmids containing ALD6–SeAcsL641F (HZ2000) or only SeAcsL641F (ACS*) were constructed. Interestingly, the production of n-butanol was increased, with the highest titer produced by the engineered strain containing only ACS*. After codon optimization of this mutant, the titer of n-butanol was further improved to about 20 mg/L (Figs. 3 and S8). In contrast with the overexpression of the SeAcs mutant, the overexpression of the endogenous ACS1 and ACS2 did not improve the production of n-butanol (Fig. 3), probably due to low activity or post-translational deactivation (Shiba et al., 2007).

Although PDH is only found in the mitochondria in eukaryotes, expression of the structural genes without MTs will relocate the PDH enzymes to the cytosol. If they are functionally reconstituted in this new compartment, a new route to generate acetyl-CoA directly from pyruvate can be created in the cytosol of yeast (cytoPDH). A similar strategy is to use the bacterial counterparts, which do not possess MTs. In the present work, cytoPDHs were constructed by cloning the MTS-free PDH structural genes from S. cerevisiae (cytoPDH1–cytoPDH1–cytoPDH1–cytoPDH1–cytoPDH1–ScPDH) and PDH genes from E. coli (EclPdA–EcAceE–EcAceF, EcPDH). As shown in Fig. 3 and Fig. S8, after the introduction of cytoPDHs into the engineered host, n-butanol titer was improved by an additional 3 fold, resulting in a total of more than 12-fold increase compared with the wild-type strain.

The last strategy tested in the present study was ACL. Comparative genomic analysis reveals that one of the biggest differences between oleaginous yeasts and non-oleaginous yeasts are the presence of ACL (Vorapreeda et al., 2012), a route to generate cytosolic acetyl-CoA from citrate, an intermediate of the TCA cycle. Thus, ACL genes (ACL1 and ACL2) from an oleaginous yeast Y. lipolytica (YIACL) and the model plant Arabidopsis thaliana (AtACL) were cloned and introduced into the engineered S. cerevisiae strain. As shown in Fig. 3, the introduction of ACL from Y. lipolytica
Host engineering to increase cytosolic acetyl-CoA levels in *S. cerevisiae*. GPD1 and GPD2 responsible for glycerol formation and ADH1 and ADH4 involved in ethanol production were inactivated to redirect the glycolytic flux to acetyl-CoA. CIT2 and MLS1 were deleted to disrupt the glyoxylate shunt contributing to the consumption of acetyl-CoA in the cytosol of yeast.

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Fig. 2. Host engineering to increase cytosolic acetyl-CoA levels in *S. cerevisiae*. GPD1 and GPD2 responsible for glycerol formation and ADH1 and ADH4 involved in ethanol production were inactivated to redirect the glycolytic flux to acetyl-CoA. CIT2 and MLS1 were deleted to disrupt the glyoxylate shunt contributing to the consumption of acetyl-CoA in the cytosol of yeast.

Increased n-butanol production by overexpression of various acetyl-CoA biosynthetic pathways in the *gpd1–gpd2–adh1–adh4*–*S. cerevisiae* strain. Three series of complementary pathways, including the PDH-bypass, cytoPDH, and ACL, were introduced into the engineered yeast host.

Fig. 3. Increased n-butanol production by overexpression of various acetyl-CoA biosynthetic pathways in the *gpd1–gpd2–adh1–adh4*–*S. cerevisiae* strain. Three series of complementary pathways, including the PDH-bypass, cytoPDH, and ACL, were introduced into the engineered yeast host.
could increase the n-butanol titer by approximately 2 fold in the engineered host, while ACL from *A. thaliana* had no effect on the production of n-butanol. Since previous reports showed that AtACL could be functionally expressed in *S. cerevisiae* (Fatland et al., 2002), the inconsistent results might be caused by different cell culture conditions. Oxygen-limited condition was used for n-butanol fermentation in this study, while the previous work was carried out under aerobic conditions, which would supply more precursor molecules (citrate) for ACL. Considering the amount of fatty acids synthesized in oleaginous yeast and plant and the corresponding role of ACL, it was reasonable to assume that YlACL might have higher affinity and/or higher activity than the plant counterpart. To test these assumptions, citrate supplementation was carried out and tested for ACL activity and thus n-butanol production. As shown in Fig. S9, the supplementation of citrate to the medium increased the production of n-butanol by JLO533 (AtACL) to a similar level as that of JLO532 (YlACL). However, the production of n-butanol by JLO532 was not affected by citrate supplementation. These results demonstrated that the activity of AtACL was limited by the supply of citrate under oxygen-limited conditions, which on the contrary was sufficient for a more kinetically favorable ACL (YlACL).

### 3.4. Heterologous pathways in synthesizing cytosolic acetyl-CoA

To verify that the introduced acetyl-CoA pathways were functional and the increased n-butanol production was resulted from increased acetyl-CoA levels, the acetyl-CoA concentrations in various strains were determined using an acetyl-CoA Assay Kit. As shown in Fig. 4A, the deletion of *GPD1, GPD2, ADH1*, and *ADH4* increased the acetyl-CoA level by approximately 2 fold, and the introduction of heterologous acetyl-CoA pathways could further increase the acetyl-CoA level in *S. cerevisiae*. Although acetyl-CoA concentration was similar for the engineered strain with different acetyl-CoA pathways, the amount of n-butanol produced was higher in the strains with cytoPDHs than those with ACL or ACS (Fig. 4A and Fig. S10). Since the activities of ACL and ACS are coupled to ATP consumption and cytoPDHs are ATP-independent, the increased n-butanol production may result from the energetic benefits of cytoPDHs, which was represented by the higher growth rate, sugar consumption rate, n-butanol productivity, and n-butanol yield (Fig. S8 and Table 3). In other words, the increased acetyl-CoA level and reduced energy input requirement lead to the highest n-butanol titer achieved in the cytoPDH harboring strains in the present study. Another explanation for this observation lies on the fact that acetyl-CoA concentration in *S. cerevisiae* may be limited by the total CoA pool as well.

Previously it was reported that the *pdc*—strain could not grow on glucose as a sole carbon source and required the supplementation of C4 compound to generate cytosolic acetyl-CoA (Flikweert et al., 1999; Lian et al., 2014; van Maris et al., 2004). Therefore, the *pdc*—strain could be an ideal selection/screening platform to test the activity of the cytosolic acetyl-CoA pathways. As expected, the introduction of alternative acetyl-CoA pathways rescued the growth of the *pdc*—strain on glucose as the sole carbon source (Fig. 4B). Interestingly, the growth rate of these strains agreed well with the titer of n-butanol produced (indicating the activity of these biosynthetic pathways). Together with the increased n-butanol titer and increased acetyl-CoA level, it was concluded that EcPDH, ScPDH, and YlACL were all functional in synthesizing cytosolic acetyl-CoA in *S. cerevisiae*.

### 3.5. Combining complementary acetyl-CoA biosynthetic routes

After verifying the activity of the acetyl-CoA biosynthetic pathways, we hypothesized that it would be beneficial to combine the most effective and complementary pathways to achieve maximal acetyl-CoA availability in the cytosol. As shown in Figs. S11 and S12, EcPDH with ACS*Opt* was the best combination, and could increase the titer of n-butanol by approximately 6 fold compared with the engineered host with empty vectors. However, the total n-butanol titer was not significantly improved compared with JLO530, probably due to the burden of maintaining several plasmids, which was confirmed by the decreased n-butanol titer in the control strain (JLO521). Interestingly, the combination of ACS and ACL failed to increase the production of n-butanol, which might result from the energy input requirement for both pathways (Table 3). Thus, the combination of cytoPDH, ACL, and ACS was not further tested for n-butanol fermentation. To eliminate the plasmid burden issue, high cell density fermentation was carried out for n-butanol production. As shown in Fig. 5, the highest n-butanol titer over 100 mg/L could be achieved, which represented the highest n-butanol titer ever reported in yeast using the CoA-dependent pathway.

### 4. Discussion

There is a growing interest in developing *S. cerevisiae* as a cell factory for the production of biofuels and chemicals, owing to its high tolerance to harsh industrial conditions (Chen et al., 2013; Runguphan and Keasling, 2014). Besides its essential roles in numerous metabolic pathways and cellular functions, acetyl-CoA is the building block for the biosynthesis of many products of industrial interest. However, acetyl-CoA metabolism in yeast is rather complex and highly regulated. In addition, this precursor molecule is synthesized in various compartments and cannot be directly transported between these compartments. In this study, acetyl-CoA metabolism was engineered in *S. cerevisiae* to enhance the availability of this precursor metabolite in the cytosol.

Since most of the glycolytic flux goes to ethanol formation even under respiration conditions (the Crabtree effect), the flux towards acetyl-CoA is very limited. To redirect the metabolic flux towards acetyl-CoA, the synthesis of ethanol was decreased by deleting the two major ADHS (*ADH1* and *ADH4*) in the cytosol, which was confirmed in part by the accumulation of acetaldehyde in the yeast cells. However, ethanol was still the major product, indicating the presence of other genes encoding enzymes with ADH activity in the yeast genome. Further disruption of an acetyl-CoA consuming pathway (the glyoxylate shunt) by the inactivation of *CIT2* or *MLS1* led to decreased n-butanol production, which was inconsistent with the results obtained in the wild-type yeast strain (Fig. 2). This discrepancy might result from the effect of *ADH* deletion on acetate metabolism. In the wild-type strain, only a small amount of acetate was produced (Fig. S5), which was considered as one of the rate-limiting factors for acetyl-CoA biosynthesis (Shiba et al., 2007). Thus the disruption of the glyoxylate shunt would result in more acetate in the cytosol for acetyl-CoA biosynthesis (Fig. 1). On the contrary, acetate was already accumulated to relative high levels in the *adhl1–adhl4*—strain (Figs. S5 and S6), and the activity of ACS or the supply of ATP was rate-limiting for acetyl-CoA biosynthesis. Further disruption of the glyoxylate shunt in the *adhl1–adhl4*—strain resulted in the accumulation of acetate to cytotoxic levels. The difference in acetate metabolism could also explain the observation that overexpression of the whole PDH-bypass pathway led to increased acetyl-CoA level in the wild-type strain while impaired the synthesis of acetyl-CoA derived products in the *adhl1–adhl4*—strain.

Due to the lack of ATP-dependent citrate lyase (ACL) and the carnitine shuttle when cultured in the minimal medium (Chen et al., 2013), the activation of acetate is the only route to generate cytosolic acetyl-CoA in *S. cerevisiae*. This PDH-bypass route suffered from low enzyme activity and high energy input.
requirement. Therefore, heterologous synthetic pathways with higher efficiency and lower energy input requirement were introduced to enhance the availability of acetyl-CoA in the cytosol. Among all acetyl-CoA pathways tested, cytoPDHs from both *E. coli* and *S. cerevisiae* and ACL from *Y. lipolytica* worked the best to increase the production of *n*-butanol. Notably, all these three pathways required oxygen supply for optimal activity, whereas anaerobic fermentation is preferred for industrial fermentation.

![Graph A](image1.png)

**Fig. 4.** Verification of the function of the acetyl-CoA biosynthetic pathways. Besides increased *n*-butanol production, the function of these pathways for cytosolic acetyl-CoA generation was confirmed by increased acetyl-CoA concentration (A) and the ability to complement the growth of the pdc− strain on glucose as the sole carbon source (B). The pdc− strains were pre-cultured on ethanol and inoculated to the SC medium supplemented with 5 g/L glucose. The introduction of *MTH1* or *MTH1T* (*MTH1* with internal truncation) into the pdc− strain was reported to enable the growth on glucose as the sole carbon source. The resulting strain was used as a positive control in this study.

![Graph B](image2.png)
Thus future engineering work will be focused on anaerobic acetyl-CoA biosynthetic pathways, such as PFL, PNO, PFO, or the PDH mutant with oxygen independent activity (Kim et al., 2008). In addition, although n-butanol titer was different, the acetyl-CoA concentration in strains JL0539, JL0540, JL0541, and JL0542 was nearly the same, indicating that the synthesis of acetyl-CoA may also be limited by the total CoA pool. Engineering of CoA biosynthesis (Vadali et al., 2004a, 2004b), especially the rate-limiting step catalyzed by pantothenate kinase, would further improve the concentration of acetyl-CoA in yeast.

Considering the accumulation of acetaldehyde and acetate in the adh-C0 strain, the inactivation of the upstream enzymes, pyruvate decarboxylases (PDCs), is proposed to be a better strategy for efficient production of fuels and chemicals (Lian et al., 2014; Oud et al., 2012; van Maris et al., 2004). However, the pdc-C0 strain is notorious for its inability to grow with glucose as the sole carbon source and requirement of C2 supplementation to provide cytosolic acetyl-CoA (Flikweert et al., 1999). As an alternative to C2 supplementation, introduction of acetyl-CoA biosynthetic pathways, such as cytoPDHs and ACL, was found to be able to rescue the growth on low concentrations of glucose (Fig. 4B). Similarly, a recent work (Kozak et al., 2013) showed that alternative acetyl-CoA biosynthetic pathways were able to complement the growth of a S. cerevisiae strain without ACS activity (acs1–acs2–). In their work, the authors found that bacterial acetylating acetaldehyde dehydrogenases and PFLs could functionally replace ACS in S. cerevisiae for cytosolic acetyl-CoA biosynthesis. Although the growth was still rather poor, protein engineering and pathway engineering strategies (Du et al., 2012; Eriksen et al., 2014) could be carried out to increase the activity of these pathways. Since the growth rate of the pdc-C0 strain was the direct readout of acetyl-CoA levels in the cytosol, a high throughput selection/screening platform could be readily developed. As ethanol production was completely eliminated and pyruvate was accumulated to high levels, the pdc-C0 strain could be

<table>
<thead>
<tr>
<th>Theoretical value/glucose</th>
<th>Glucose consumption rate (g/L h)</th>
<th>n-Butanol productivity (mg/L h)</th>
<th>n-Butanol Yield (mg/g glucose)</th>
<th>n-Butanol specific production (mg/g dry cell weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATPa NADHa NADPHb</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JL0520 (Endogenous)</td>
<td>0 2 2</td>
<td>0.23 ± 0.01</td>
<td>0.11 ± 0.01</td>
<td>0.51 ± 0.02</td>
</tr>
<tr>
<td>JL0529 (ACS*Opt)</td>
<td>0 2 2</td>
<td>0.19 ± 0.02</td>
<td>0.18 ± 0.01</td>
<td>0.85 ± 0.08</td>
</tr>
<tr>
<td>JL0530 (EcPDH)</td>
<td>2 4 0</td>
<td>0.22 ± 0.01</td>
<td>0.42 ± 0.02</td>
<td>1.58 ± 0.09</td>
</tr>
<tr>
<td>JL0531 (ScPDH)</td>
<td>2 4 0</td>
<td>0.21 ± 0.00</td>
<td>0.35 ± 0.07</td>
<td>1.32 ± 0.09</td>
</tr>
<tr>
<td>JL0532 (YlACL)</td>
<td>1 4 0</td>
<td>0.20 ± 0.02</td>
<td>0.26 ± 0.04</td>
<td>1.23 ± 0.18</td>
</tr>
</tbody>
</table>

* The numbers are the theoretical values for the heterologous acetyl-CoA biosynthetic pathways generated from one glucose molecule. A combination of the endogenous system and the heterologous system was used in the n-butanol fermentation process.

Fig. 5. High cell density fermentation for n-butanol production with the combined acetyl-CoA pathways. The combination of EcPDH and ACS*Opt was found to be the most effective to boost acetyl-CoA levels in the engineered yeast strain, with more than 100 mg/L n-butanol produced in approximately 48 h.
used as a good start point for acetyl-CoA pool engineering, especially for the cytopDHs.

In terms of the compartmentalization of acetyl-CoA metabolism, the whole n-butanol pathway can be relocated to the same compartment as the precursor metabolite. Besides the enhanced availability of the precursor molecule, compartmentalization engineering was also found to have higher efficiency, as a result of increased local concentration and fewer competing pathways (Avalos et al., 2013; Farhi et al., 2011). A similar strategy is to reconstruct the carnitine shuttle exporting mitochondrial acetyl-CoA to the cytosol in S. cerevisiae, as occurring in higher organisms (Strijbis and Distel, 2010). Interestingly, all components of the carnitine shuttle, such as the carnitine acetyltransferase and acetyl-carnitine translocase, exist in S. cerevisiae except for the de novo carnitine biosynthetic pathway (Franken et al., 2008). Due to the lack of carnitine in synthetic medium and its high cost, introducing a heterologous de novo carnitine biosynthetic pathway would be a viable option for industrial production of acetyl-CoA derived fuels and chemicals.

In summary, combined strategies of disrupting competing pathways to redirect the flux towards acetyl-CoA and introducing heterologous acetyl-CoA pathways with higher activity and lower energy input requirement were carried out to enhance the availability of acetyl-CoA in yeast. The yeast host was engineered to eliminate or decrease the formation of glycerol and ethanol and redirect the glycolytic flux to acetyl-CoA biosynthesis. Subsequent introduction of three complementary acetyl-CoA biosynthetic pathways, with acetate (PDH-bypass), pyruvate (cytoPDH), and citrate (ACL) as the substrate, respectively, into the host increased the concentration of acetyl-CoA by 3 fold. To our knowledge, this is the first report of the functional reconstitution of the PDH complex in the cytosol of yeast, whose activity was confirmed by increased acetyl-CoA concentration, improved n-butanol production, and the ability to complement the growth of the pdh2Δ strain on glucose. Using high cell density fermentation, n-butanol titers as high as 120 mg/L could be achieved in the optimized strain, representing the highest n-butanol titer ever reported in yeast using the CoA-dependent pathway.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jmb.2014.05.010.

References


