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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY

Improving biobutanol production in engineered *Saccharomyces cerevisiae* by manipulation of acetyl-CoA metabolism

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Abstract Recently, butanols (1-butanol, 2-butanol and iso-butanol) have generated attention as alternative gasoline additives. Butanols have several properties favorable in comparison to ethanol, and strong interest therefore exists in the reconstruction of the 1-butanol pathway in commonly used industrial microorganisms. In the present study, the biosynthetic pathway for 1-butanol production was reconstructed in the yeast *Saccharomyces cerevisiae*. In addition to introducing heterologous enzymes for butanol production, we engineered yeast to have increased flux toward cytosolic acetyl-CoA, the precursor metabolite for 1-butanol biosynthesis. This was done through introduction of a plasmid-containing genes for alcohol dehydrogenase (*ADH2*), acetaldehyde dehydrogenase (*ALD6*), acetyl-CoA synthetase (ACS), and acetyl-CoA acetyltransferase (*ERG10*), as well as the use of strains containing deletions in the malate synthase (*MLS1*) or citrate synthase (*CIT2*) genes. Our results show a trend to increased butanol production in strains engineered for increased cytosolic acetyl-CoA levels, with the best-producing strains having maximal butanol titers of 16.3 mg/l. This represents a 6.5-fold improvement in butanol titers compared to previous values reported for yeast and demonstrates the importance of an improved cytosolic acetyl-CoA supply for heterologous butanol production by this organism.

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Introduction

In recent years, biobutanol has generated attention as a potential gasoline additive. Butanol (1-butanol, 2-butanol, and isobutanol) is sufficiently similar to gasoline to be blended with it at any ratio. It is compatible with existing pipeline infrastructure and is superior to ethanol as a fuel because of its higher energy content and lower volatility, hygroscopicity, and corrosiveness [1, 8]. The biological pathway for 1-butanol production is through acetone–butanol–ethanol (ABE) fermentation in the *Clostridia* species of bacteria. However, difficulties associated with clostridial fermentation, such as the formation of by-products and requirement for strictly anaerobic conditions, have driven various research efforts to reconstruct the butanol production pathway in more commonly used industrial microorganisms. This includes engineering of various bacteria for butanol production either by introduction of the clostridial butanol pathway [14, 18, 22, 23] or intermediate genes of amino acid pathways [17, 23]. Despite high titers obtained from some of these studies, several major drawbacks exist with the use of bacteria for industrial biofuel production. These include a complex separation process from the fermentation media, narrow and neutral pH growth rate [9, 12], and susceptibility to phage infections when grown on a large scale [13].

The use of the yeast *Saccharomyces cerevisiae* as a cell factory for biofuel production could overcome these limitations. *S. cerevisiae* is a robust industrial organism that can grow under various industrial conditions, including low

pH and less stringent nutritional requirements [11]. In addition, the larger size (as well as higher mass) of *S. cerevisiae* makes it easier to separate it from the fermentation media than bacteria, reducing process costs. Furthermore, *S. cerevisiae* is very well characterized, with a wide variety of tools and resources available for its genetic manipulation [16, 19]. Much information is available on *S. cerevisiae*, including a complete genome sequence, as well as characterization of its metabolic pathways [7, 20]. A previous attempt to engineer *S. cerevisiae* for 1-butanol production involved the introduction of butanol-pathway genes together with overexpression of the native thiolase gene to obtain butanol titers of 2.5 mg/l [25]. Therefore, significant improvement is required to further facilitate the use of *S. cerevisiae* for butanol production.

A possible limiting factor to butanol production by *S. cerevisiae* is the availability of the precursor acetyl-CoA. Acetyl-CoA metabolism is highly compartmentalized in yeast and occurs in the cytosol, mitochondria, peroxisomes, and the nucleus. Cytosolic acetyl-CoA is produced via the pyruvate dehydrogenase (PDH) bypass and is derived from acetaldehyde, which is formed by the decarboxylation of pyruvate. However, during growth on glucose, the majority of the glycolytic flux is directed toward ethanol because of the Crabtree effect [27], limiting the availability of acetyl-CoA in the cytosol. Previous studies have shown that engineering the PDH bypass in *S. cerevisiae* enhanced the cytosolic acetyl-CoA supply, resulting in increased production of acetyl-CoA derived products such as the isoprenoids amorphadiene [24] and α-santalene [4], as well as the polymer polyhydroxybutyrate [15]. Therefore, a similar strategy could potentially be applied for butanol production.

In the present study, we aimed to increase biobutanol production by *S. cerevisiae* by increasing the pool of available cytosolic acetyl-CoA and by evaluating alternative enzymes of the 1-butanol pathway. This strategy resulted in increased butanol titers in *S. cerevisiae*, suggesting the availability of cytosolic acetyl-CoA to be rate-limiting in butanol production.

Materials and methods

Strains and media

Escherichia coli DH5α was used for general cloning procedures in this study. Lysogeny broth (LB) medium was used for routine culturing with 80 mg/l ampicillin added when needed.

S. cerevisiae strain CEN.PK113-11C (*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 his3-Δ1,* provided by P. Kötter, University of Frankfurt, Germany) was used as the background strain for evaluation of butanol-producing yeast strains. All yeast strains used in this study are summarized in Table 1. Engineered yeast strains were selected on synthetic dextrose (SD) medium with uracil and histidine omitted where appropriate.

Table 1 Yeast strains used in this study and relevant genotypes

CEN.PK113-11C	*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 his3-Δ1*
SCIYC32 [6]	*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 his3-Δ1 cit2*Δ
SCIYC33 [6]	*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 his3-Δ1 mls1*Δ
AKY0	CEN.PK113-11C pAK01-pIYC04
AKY1	CEN.PK113-11C pAK01-pIYC08
AKY2	SCIYC33 pAK01-pIYC08
AKY3	SCIYC32 pAK01-pIYC08
AKY4	CEN.PK113-11C pAK01-pCS01

All restriction enzymes used in this study were from Thermo Fisher Scientific (Waltham, MA, USA).

Plasmid construction

The plasmid pAK01 was used to express four heterologous genes for butanol production. This plasmid was based on the pSP-GM1 vector [5] with constitutive promoters P_{TEF1} and P_{PGK1} (see Table 2 for the summary of plasmids used in this study).

Butyraldehyde dehydrogenase/butanol dehydrogenase (*adhE2*), 3-hydroxybutyryl-CoA dehydrogenase (*hbd*) and crotonase (*crt*) sequences were from *Clostridium beijerinckii*. The *trans*-enoyl-CoA reductase (*ter*) sequence was from *Treponema denticola*. All these genes were codon-optimized for high levels of expression in yeast and synthesized by DNA 2.0.

Adhe2 was cloned into pSP-GM1 using *Not*I and *Pac*I under the control of a *TEF1* promoter. *Ter* was then cloned into the same plasmid under the control of a *PGK1* promoter using *BamH*I and *Nhe*I. This resulted in the plasmid pAK0.

Crt was cloned into pSP-GM1 using *Not*I and *Pac*I under the control of a *TEF1* promoter, and *hbd* was then cloned into the same plasmid under the control of a *PGK1* promoter using *BamH*I and *Nhe*I. A cassette containing both of these genes and their promoters was then amplified

Table 2 Description of plasmids used in this study

Name	Gene expressed	Marker
pIYC04		*HIS3*
pIYC08	P_{TEF1}-*acs*^{L641P} P_{PGK1}-*ALD6* P_{TEF1}-*ERG10* P_{HXT7}-*ADH2*	*HIS3*
pAK01	P_{TEF1}-*adhE2* P_{PGK1}-*ter* P_{TEF1}-*crt* P_{PGK1}-*hbd*	*URA3*
pCS01	P_{TEF1}-*ERG10*	*HIS3*

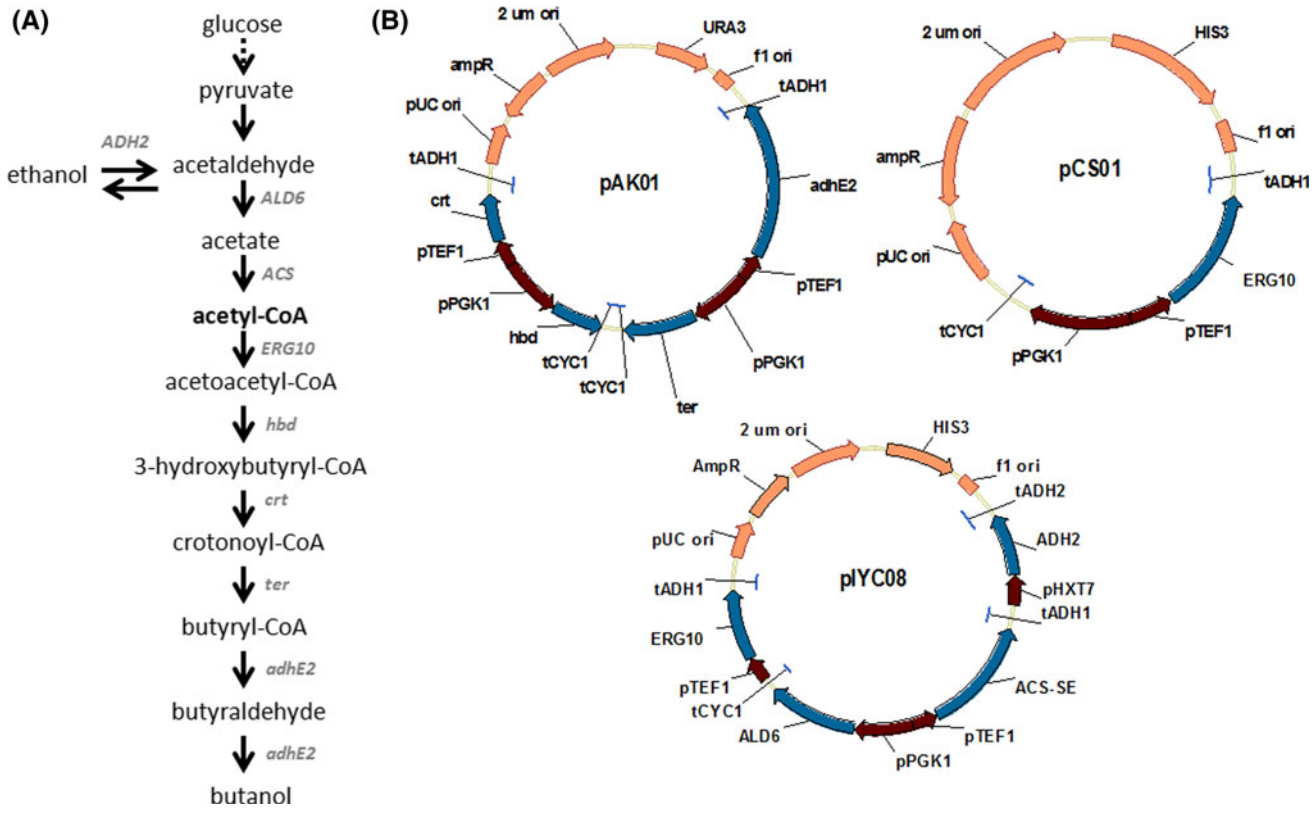

Fig. 1 Engineering *S. cerevisiae* for butanol production. **a** The biosynthetic pathway for butanol production. **b** The major plasmids used in this study. pAK01 encodes heterologous enzymes for butanol production. pCS01 contains *ERG10*. pIYC08 encodes enzymes used to direct flux toward acetyl-CoA production. All plasmids contain the 2 μ origin of replication and either the *URA3* or *HIS3* marker

from this plasmid using the primers Kpn-TCYC (GTTGTT**TCCGGA**TGTTACATGCGTACACGCGTC) and Mre-TADH (GAAGAA**CGCCGGCG**GAGCGACC TCATGCTATACCTG), which contained *Kpn*2I and *Mre*I restriction sites. This cassette was then cloned into pAK0, yielding pAK01.

The plasmid pCS01 was constructed by cloning the native *ERG10* gene into pIYC04 using the enzymes *Spe*I and *Sac*I. This gene was cloned under the control of a *TEF1* promoter.

The detailed construction of pIYC04 as a background plasmid and pIYC08 as acetyl-CoA plasmid was previously described [6]. The metabolic pathway and relevant plasmid maps are shown in Fig. 1.

Engineered yeast strain generation and characterization

Strains used in this study are listed in Table 1. All yeast strain transformations were performed by the standard lithium acetate method [10].

Strains AKY1, AKY2, and AKY3 were constructed by co-transforming CEN.PK113-11C, SCIYC33 and SCIYC32 (from Chen et al. [6]) with pIYC08 and pAK01. Strain AKY0 was constructed by transforming CEN.PK113-11C with pIYC04 and pAK01. Strain AKY4 was constructed by co-transforming CEN.PK113-11C with pCS01 and pAK01. Strains were selected on SD-URA-HIS plates.

Shake flask cultivation and analysis of butanol production

To test for butanol production from different strains, 20-ml cultures were started in 100-ml unbaffled flasks by inoculating an amount of pre-culture that resulted in a final optical density of 0.02 at 600 nm (OD_{600}). The strains were grown at 30 °C with 180 rpm orbital shaking in defined minimal medium with the following composition: 7.5 g/l $(NH_4)_2SO_4$; 14.4 g/l KH_2PO_4; 0.5 g/l $MgSO_4{\cdot}7H_2O$; 2 ml/l trace metal solution [per liter, pH 4.0: EDTA (sodium salt), 15.0 g; $ZnSO_4{\cdot}7H_2O$, 4.5 g; $MnCl_2{\cdot}2H_2O$, 0.84 g; $CoCl_2{\cdot}6H_2O$, 0.3 g; $CuSO_4{\cdot}5H_2O$, 0.3 g; $Na_2MoO_4{\cdot}2H_2O$, 0.4 g; $CaCl_2{\cdot}2H_2O$, 4.5 g; $FeSO_4{\cdot}7H_2O$, 3 g; H_3BO_3, 1 g and KI, 0.1 g]. The pH of the mineral medium was adjusted to 6.5 by adding 2 M NaOH and autoclaved separately from the carbon source solution. Glucose was added at a

concentration of 20 g/l. Vitamin solution (per liter, pH 6.5: biotin, 0.05 g; *p*-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g) was filter sterilized and aseptically added to the medium after autoclaving at a concentration of 1 ml/l. To prepare the pre-culture, culture tubes containing 5 ml of defined medium (as described above) were inoculated with a single colony of strains of interest. These inocula were cultured at 30 °C with 200 rpm orbital shaking to an OD_{600} between 1 and 2.

To quantify 1-butanol levels, samples at different time points were collected, centrifuged, and filtered. Samples were then analyzed by high-pressure liquid chromatography (Dionex-HPLC, Sunnyvale, CA) equipped with an Aminex HPX-87H ion exclusion column (300 × 7.8 mm; Bio-Rad, Hercules, CA) and RI detector. Commercially available 1-butanol (Sigma-Aldrich, St, Louis, MO) was used as a standard. The HPLC was operated at 45 °C and a flow rate of 0.6 ml/min of 5 mM H_2SO_4.

Results and discussion

Due to its favorable properties, 1-butanol has been considered as a possible gasoline replacement, and several metabolic engineering efforts have been geared toward optimization of the 1-butanol production pathway in industrial hosts. However, while *S. cerevisiae* is an ideal organism for biofuel production, only one study has reported the engineering of yeast for production of 1-butanol, with maximal titers of only 2.5 mg/l [25]. We originally tested the functionality of the 1-butanol pathway using the same enzyme combination reported in the highest producer in that study. This included using a 3-hydroxybutyryl-CoA dehydrogenase (Hbd) that utilizes NADH as a cofactor, a crotonase (Crt), a bifunctional butyraldehyde, and butanol dehydrogenase (AdhE2) from *Clostridium beijerinckii* and a crotonyl-CoA reductase (Ccr) that utilizes NADH from *Streptomyces collinus* [25]. However, we were not able to detect any product formation using these enzymes with our vector system (data not shown). We therefore replaced the crotonyl-CoA reductase with an NADH-dependent crotonyl-CoA-specific *trans*-enoyl-CoA reductase (Ter) from *Treponema denticola* [3]. This enzyme does not catalyze the reverse oxidation of butyryl-CoA to crotonoyl-CoA [26], and the replacement of Ccr with this enzyme has been previously shown to result in a 3.5-fold increase in butanol production in *E. coli* [3]. Codon-optimized genes encoding Ter, as well as Hbd, Crt, and AdhE2, were cloned into one episomal plasmid, yielding 1-butanol production plasmid pAK01. To check for the functionality of this pathway in producing butanol, this plasmid was co-expressed in *S. cerevisiae* along with an empty *HIS3* plasmid (strain AKY0), and the cultures expressing these genes were grown in minimal media with 2 % glucose. Maximal titers of 2.1 mg/l 1-butanol were observed (Fig. 2) when all sugar had been consumed (see Table 3 for ethanol values), an amount that is comparable to that previously reported for *S. cerevisiae* [25]. The improved functionality of this pathway in our system compared to the Ccr-containing pathway suggests Ter to be an important contributor to the forward pathway flux.

pAK01 does not contain a thiolase gene. This gene is necessary for the conversion of acetyl-CoA to acetoacetyl-CoA and represents the first step in the butanol pathway. We therefore co-transformed pAK01 with pCS01, a plasmid that encodes the native thiolase gene, *ERG10*. The resulting strain (AKY4) produced 6.6 mg/l of butanol, representing a 3.1-fold increase over the strain containing the heterologous genes only, suggesting levels of acetoacetyl-CoA to be limiting in AKY0.

We then further engineered *S. cerevisiae* for increased levels of cytosolic acetyl-CoA, which serves as a precursor for butanol production. This involved the use of plasmid pIYC08, which ensures overexpression of endogenous *ADH2* encoding alcohol dehydrogenase, *ALD6* encoding NADP-dependent aldehyde dehydrogenase, and a codon-optimized *acs* variant (L641P) from *Salmonella enterica* (acs^{L641P}), encoding acetyl-CoA synthetase. While the native ACS enzymes Acs1 and Acs2 are subject to regulation via acetylation, acs^{L641P} contains a point mutation that prevents the enzyme from being inhibited by acetylation, bypassing this regulation. Furthermore, the use of this variant was previously demonstrated to successfully redirect flux from acetaldehyde to acetyl-CoA in the cytosol to increase production of isoprenoids in yeast [24]. In addition, this plasmid also leads to overexpression of *ERG10*. Co-expression of the butanol pathway with plasmid pIYC08 (strain AKY1) resulted in butanol titers of

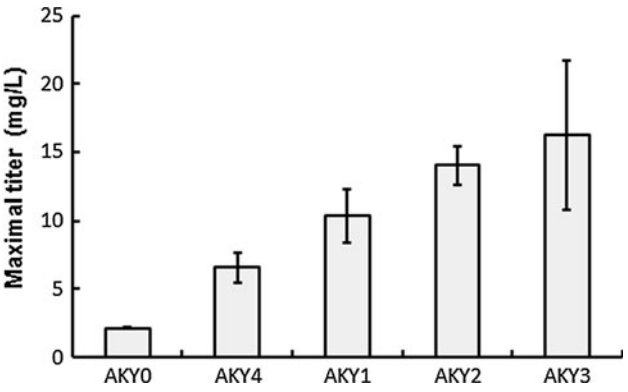

Fig. 2 Butanol production by engineered *S. cerevisiae*. Cultures were grown in 100-ml shake flasks containing 20 ml defined minimal medium with 20 g/l glucose as the carbon source. 1-Butanol levels were quantified by HPLC

Table 3 Maximal ethanol titers observed for the strains at the end of the glucose phase

Strain	Ethanol titers
AKY0	6.2 ± 0.2
AKY1	6 ± 0.1
AKY2	5.5 ± 0.2
AKY3	5.8 ± 0.1
AKY4	6.4 ± 0.1

Table 4 Clonal variation in butanol production

Strain	Transformant	Max. butanol titers
AKY0	1	2.3
	2	2.1
	3	2.0
AKY1	1	10.3
	2	7.1
	3	17.8
	4	7.0
	5	9.5
AKY2	1	16.2
	2	11.4
	3	14.5
AKY3	1	5.5
	2	20.8
	3	22.5
AKY4	1	5.7
	2	5.3
	3	8.7

10.3 mg/l, which represents a 4.9-fold increase in 1-butanol titers compared to AKY0 and a 1.6-fold increase compared to AKY4 (Fig. 2). These results demonstrate the importance of an adequate acetyl-CoA supply for 1-butanol production.

We then used strains carrying deletions in the glyoxylate cycle *CIT2* and *MLS1* genes to reduce the drainage of acetyl-CoA through the glyoxylate pathway. *CIT2* encodes peroxisomal citrate synthase, while *MLS1* encodes cytosolic malate synthase. While this pathway has been shown to be mostly active during growth on C_2 compounds, activity of this pathway has also been shown during growth on glucose [21]. Therefore, deletion of these enzymes should contribute to increasing the cytosolic acetyl-CoA supply. Co-expression of the butanol pathway with pIYC08 in the *MLS1* and *CIT2* deletion strains (strains AKY2 and AKY3) resulted in a further increase in butanol production to 14.0 and 16.3 mg/l, respectively. However, our strains displayed a large clone-to-clone variation with some clones producing very low levels of 1-butanol and others producing very high levels (see Table 4). Repeating the experiment several times yielded similar results. Therefore, we cannot conclude with certainty that the increase in production in strains AKY2 and AKY3 is significant in comparison to AKY1. However, since all three strains where a strategy toward increased acetyl-CoA levels has been employed produced more butanol than AKY0 or AKY4, our results demonstrate the importance of manipulating acetyl-CoA metabolism to increase butanol production in yeast. The clonal variation observed in our system is most likely due to the use of episomal plasmids. Both pAK01 and pIYC08 are large (approximately 15 kb each), and each contains four genes cloned under control of strong constitutive promoters. Such a system might be unstable, leading to loss of genes and/or plasmids during the cultivations. To avoid such problems in future efforts to optimize butanol production by *S. cerevisiae*, it might be advisable to integrate the genes involved directly into the genome.

The maximal titers obtained in the present study represent a 6.5-fold improvement in butanol production over previous values reported in yeast [25], and this therefore is an important proof of principle. However, further optimization is necessary to make yeast a commercially competitive host for butanol production. Steen et al. [25] have pointed to the step catalyzed by AdhE2 as a potential bottleneck. Further engineering efforts might therefore benefit from testing different AdhE2 variants that might have improved activity/solubility in the yeast cytosol. Another approach that has benefited isobutanol production in yeast involved the targeting of pathways to the mitochondria [2], and this approach might also be beneficial for 1-butanol production. Finally, another strategy that could be of potential interest is the deletion of *ADH1* in butanol-producing strains to increase carbon flux toward butanol and away from ethanol production.

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