



Utilizing an endogenous pathway for 1-butanol production in *Saccharomyces cerevisiae*

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ABSTRACT

Microbial production of higher alcohols from renewable feedstock has attracted intensive attention thanks to its potential as a source for next-generation gasoline substitutes. Here we report the discovery, characterization and engineering of an endogenous 1-butanol pathway in *Saccharomyces cerevisiae*. Upon introduction of a single gene deletion *adh1Δ*, *S. cerevisiae* was able to accumulate more than 120 mg/L 1-butanol from glucose in rich medium. Precursor feeding, ¹³C-isotope labeling and gene deletion experiments demonstrated that the endogenous 1-butanol production was dependent on catabolism of threonine in a manner similar to fusel alcohol production by the Ehrlich pathway. Specifically, the leucine biosynthesis pathway was engaged in the conversion of key 2-keto acid intermediates. Overexpression of the pathway enzymes and elimination of competing pathways achieved the highest reported 1-butanol titer in *S. cerevisiae* (242.8 mg/L).

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1. Introduction

Microbial cell factory, which offers sustainable solutions to global warming and energy crisis, has emerged as a promising alternative to the traditional petrochemical industry (Du et al., 2011; Lee et al., 2012). Among the most prominent examples of microbial cell factory is the yeast *Saccharomyces cerevisiae*, which is currently used to produce ethanol as an alternative fuel (Hong and Nielsen, 2012). However, due to their superior fuel characteristics compared to ethanol, advanced biofuels have attracted a growing interest (Buijs et al., 2013; Peralta-Yahya et al., 2012). In particular, 1-butanol is considered a substantially better gasoline substitute than ethanol (Jin et al., 2011). The energy density of 1-butanol (29.2 MJ/L) is comparable to that of gasoline (32.5 MJ/L), and much higher than that of ethanol (21.2 MJ/L) (Dürre, 2007). While ethanol can only be blended with gasoline to a final volume percentage of 85%, 1-butanol can be used in pure form or blended in gasoline at any ratio (Dürre, 2007). Furthermore, 1-butanol is more hydrophobic and less corrosive than ethanol, and therefore can be transported through the existing pipeline infrastructure (Dürre, 2007).

Traditionally, biological synthesis of 1-butanol is performed by *Clostridium* species through the acetone–butanol–ethanol (ABE)

fermentation process (Lee et al., 2008). However, inherent challenges of *Clostridium* manipulation and fermentation, such as a lack of genetic tools, unavoidable byproducts and intolerance to 1-butanol and oxygen, have hindered further improvement of the ABE fermentation. Therefore, production of 1-butanol in industrially friendly organisms, such as *Escherichia coli* and *S. cerevisiae*, has been the focus of recent efforts (Peralta-Yahya et al., 2012). Two metabolic routes have been explored to produce 1-butanol. The first route is the heterologous expression of the *Clostridium* 1-butanol pathway (Atsumi et al., 2008a; Inui et al., 2008), which is essentially the same as the reversed β-oxidation pathway for 1-butanol production (Dellomonaco et al., 2011). This CoA-dependent pathway enables an impressive production titer (30 g/L) in *E. coli* (Shen et al., 2011), but its performance in *S. cerevisiae* is far less promising (2.5 mg/L (Steen et al., 2008) and 16.3 mg/L (Krivoruchko et al., 2013)). The second route takes advantage of the amino-acid metabolic pathways (Atsumi et al., 2008b), where 1-butanol is produced via keto-acid intermediates in *E. coli*. The drawback of this strategy is the unavoidable co-production of 1-propanol, the synthesis of which shares a common intermediate (2-ketobutyrate) with the 1-butanol pathway (Shen and Liao, 2008).

Although the current production level of 1-butanol in *S. cerevisiae* is far behind *Clostridium* and *E. coli* (Krivoruchko et al., 2013), there are advantages to utilize *S. cerevisiae* as a 1-butanol producer. *S. cerevisiae* is very robust toward inhibitors and low pH condition, and there is no phage contamination issues with yeast compared to bacterial hosts (Hong and Nielsen, 2012). *S. cerevisiae*

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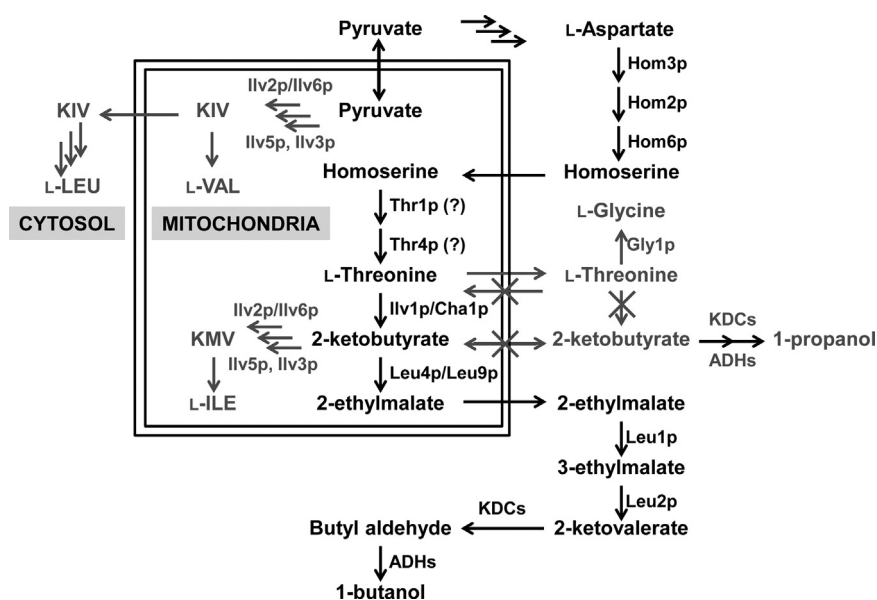


Fig. 1. Proposed endogenous 1-butanol pathway in *S. cerevisiae*. Co-enzymes and co-factors are omitted for simplicity. Pathways that are not directly involved in 1-butanol production are shown in gray, and the metabolites in these pathways are written in short forms. The information of biochemical pathways and enzyme locations is from literature (Kohlhaw, 2003; Strathern et al., 1982). The localization of Thr1p and Thr4p are in question, but our results suggest they are mitochondrial enzymes (see Section 4 and Table S4 for details). KIV: α -ketoisovalerate; KMV: α -keto- β -methylvalerate; LEU: leucine; VAL: valine; ILE: isoleucine; KDCs: 2-keto-acid decarboxylases; ADHs: alcohol dehydrogenases.

has also been shown as one of the most tolerant organisms toward 1-butanol (Gonzalez-Ramos et al., 2013; Knoshaug and Zhang, 2009). Additionally, with the widest application in biorefinery, *S. cerevisiae* can serve as a “drop-in” cell factory with compatibility to current industrial infrastructure (Williams et al., 2013). Low concentrations of 1-butanol have been found in fusel oil isolated from yeast wine fermentation (Webb et al., 1952), and the origin of 1-butanol is believed to be from plant-derived 2-keto acid precursors (Romagnoli et al., 2012) or bacterial contamination (Ingraham et al., 1961). Early studies showed 1-butanol accumulation in mutated isoleucine- and valine-requiring *S. cerevisiae* strains (Ingraham et al., 1961), implying a native 1-butanol pathway may exist in *S. cerevisiae* strains with certain genetic background. However, it is not clear whether endogenous 1-butanol production is a universal capacity of *S. cerevisiae*.

In this study, we report characterization and engineering of an endogenous 1-butanol pathway in *S. cerevisiae*. Adh1p was found as a switch for the endogenous 1-butanol production, and an amino-acid dependent pathway was proposed which is similar to the metabolic route engineered in *E. coli* (Fig. 1) (Shen and Liao, 2008). The involvement of the proposed pathway in 1-butanol production was characterized by precursor feeding, ^{13}C -isotope tracer experiments and genetic manipulations. A substantial 1-butanol production level (242.8 mg/L) was achieved in our metabolically engineered yeast strains, which is the highest 1-butanol titer reported in *S. cerevisiae* from glucose (Krivoruchko et al., 2013).

2. Materials and methods

2.1. Media and cultivation conditions

Citric acid/phosphate buffer was used to prepare culture media with controlled pH (<http://www.sigmaaldrich.com/life-science/core-bioreagents/biological-buffers/>). *S. cerevisiae* strains were cultivated in either synthetic dropout medium (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate and 0.083% amino acid drop out mix, 0.01%

adenine hemisulfate and 2% glucose) or YPAD medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate and 2% glucose). For 1-butanol production in precursor feeding and gene knockout experiments, only 2% glucose and indicated precursors were added into citric acid/phosphate buffer. Media pH was adjusted to 5 after addition of precursors by 12 M HCl or 10 M NaOH. To select and maintain strains carrying the KanMX marker, G418 (200 mg/L) was supplemented in YPAD medium. *S. cerevisiae* strains were cultured at 30 °C with 250 rpm agitation in baffled shake-flasks for aerobic growth. For micro-anaerobic fermentation, 4 mL cultures were grown at 30 °C and 250 rpm in Bellco 18 × 150 mm anaerobic glass tubes sealed with rubber stoppers and aluminum crimps (Chemglass, Vineland, NJ). Vacuum was applied through a syringe needle for 20 min, and sterile nitrogen was then added to create micro-aerobic conditions. *E. coli* strains were cultured at 37 °C and 250 rpm in Luria broth (LB) medium (Fisher Scientific, Pittsburgh, PA) with the supplement of 100 $\mu\text{g}/\text{mL}$ ampicillin. All chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

2.2. DNA manipulation

Plasmid cloning was performed by In-fusion HD cloning Kit (Clontech Laboratories, Mountain View, CA) following the manufacturer's instructions or by the DNA assembler method (Shao et al., 2009). The complete list of plasmids in this study is summarized in Table S1. For DNA manipulations, yeast plasmids were isolated using a Zymoprep II yeast plasmid isolation kit (Zymo Research, Irvine, CA) and transferred into *E. coli* for amplification. QIAprep Spin Plasmid Mini-prep Kits (Qiagen, Valencia, CA) were employed to prepare plasmid DNA from *E. coli*. Yeast genomic DNA was isolated by Wizard Genomic DNA Purification Kit (Promega, Madison, WI). All enzymes used for recombinant DNA cloning were from New England Biolabs (Ipswich, MA) unless otherwise noted. The products of PCR, digestion and ligation reactions were purified by QIAquick PCR Purification and Gel Extraction Kits (Qiagen, Valencia, CA).

2.3. Strain constructions

The complete list of strains in this study was summarized in Table S2. *S. cerevisiae* strain YSG50 *ura3Δ* (*MATα ura3Δ ade2-1 his3-11,15 leu2-3,112 can1-100 trp1-1*) was designated as the wild type (WT) strain (Nair and Zhao, 2009). Zymo 5α Z-competent *E. coli* (Zymo Research, Irvine, CA) was used for yeast plasmid amplification. To perform gene deletions, the open reading frames (ORF) of target genes were disrupted by inserting the *LoxP-ura3-LoxP* or *LoxP-KanMX-LoxP* cassettes which were PCR-amplified from plasmid pUG72 or pUG6, respectively (Guedener et al., 2002). Between consecutive gene deletions, the selection marker was recycled through the Cre-*LoxP* recombination mechanism by pSH47 or pSH63 (Guedener et al., 2002). Pathway integration into the delta sites in the yeast genome was achieved by a home-made integration plasmid. Following *PmeI* digestion, the recombinant plasmid would release a linear DNA fragment in which the pathway to integrate and the KanMX marker were flanked by delta sequences.

2.4. Metabolite detection

Alcohol and aldehyde compounds were quantified by an Agilent 7890 gas chromatograph equipped with an Agilent 5975 mass selective detector (Agilent Inc., Palo Alto, CA) at the Roy J. Carver Metabolomics Center (University of Illinois, Urbana, IL). Filtered culture supernatant was stored at -80°C prior to analysis to minimize evaporation. Samples (1 μL) were injected in split mode (10:1), and analyzed on a 15 m Zebron ZB-FFAP column with 0.25 mm I.D. and 0.25 μm film thickness (Phenomenex, Torrance, 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 1.6 mL/min. The oven temperature program was set as the following: (a) 2 min isothermal heating at 60°C , (b) increase at a rate of $10^{\circ}\text{C min}^{-1}$ to 80°C for 0 min, (c) and then $40^{\circ}\text{C min}^{-1}$ to 200°C for the final 4 min. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy with an m/z 25–300 scan range. All chromatogram spectra were analyzed with the HP Chemstation program (Agilent, Palo Alto, CA, USA).

3. Results

3.1. *ADH1* as a switch for 1-butanol production

We noted that the wild-type *S. cerevisiae* strain can consume 1-butanol in YPAD medium (Fig. 2A), which contains ~ 200 mg/L

1-butanol after filtration and ~ 70 mg/L 1-butanol after autoclave sterilization. We speculate that alcohol dehydrogenases (ADHs) are responsible for 1-butanol oxidation. To test our hypothesis, the *ADH1* gene, which encoded the most abundant ADH in *S. cerevisiae* (Leskovac et al., 2002), was deleted. *Adh1p* catalyzes the reversible redox reaction between acetaldehyde and ethanol in vitro, and the normal function of *Adh1p* is to reduce acetaldehyde to ethanol employing NADH as a cofactor (Leskovac et al., 2002). In the *adh1Δ* strain, reduced ethanol production (Fig. 2B and Fig. S1) and acetaldehyde accumulation (Fig. S6B) were observed. The *adh1Δ* mutant strain grew poorly compared to the wild type strain (Fig. S1), probably resulting from acetaldehyde toxicity and disrupted cofactor balance of $\text{NAD}^{+}/\text{NADH}$ (Drewke et al., 1990). Unexpectedly, the *adh1Δ* strain also accumulated 1-butanol under both micro-anaerobic (Fig. 2A) and aerobic (Fig. S1) conditions in YPAD medium. Without glucose or the *adh1Δ* strain, no 1-butanol production was observed in the rich medium alone (Fig. S7). The production of 1-butanol at much lower concentrations by the *adh1Δ* strain was also observed in synthetic dropout medium and citric/phosphate buffer supplemented with only glucose (Fig. S2). The *adh1Δ* strain with *Adh1p* over-expression restored 1-butanol consumption at a reduced rate than the wild-type strain (Fig. 2A), implying that there were other ADHs involved in 1-butanol oxidation. Another reason why the *adh1Δ* deletion can activate 1-butanol production is that when ethanol production is reduced, metabolic flux through the 1-butanol pathway is increased.

3.2. Precursor feeding studies in the proposed 1-butanol pathway

Based on similarity to the Ehrlich pathway which produces other fusel alcohols (Hazelwood et al., 2008), we propose an endogenous 1-butanol pathway as follows (Fig. 1). First, 2-ketobutyrate (2-KB) is generated from L-threonine through a transamination reaction catalyzed by *Ilv1p/Cha1p*. Then 2-KB is converted to 2-ketovalerate (2-KV) via a keto-acid chain elongation process that is carried out by the leucine biosynthesis pathway enzymes *Leu4p/Leu9p*, *Leu2p* and *Leu1p* (Kohlhaw, 2003; Shen and Liao, 2008). Butyl aldehyde is produced from the decarboxylation of 2-KV by 2-keto acid decarboxylases (KDCs), and is further reduced to 1-butanol by ADHs (Romagnoli et al., 2012).

We fed the precursors in the proposed pathway and quantified 1-butanol production. In citric acid/phosphate buffer (pH=5) supplemented with 2% glucose, addition of L-threonine stimulated 1-butanol production in a dose-dependent manner (Fig. 3A and Fig. S3). To further elucidate the pathway, ^{13}C -labeled glucose ($\text{D-Glucose-}^{13}\text{C}_6$) and threonine ($\text{L-Threonine-1,2-}^{13}\text{C}_2$) were used,

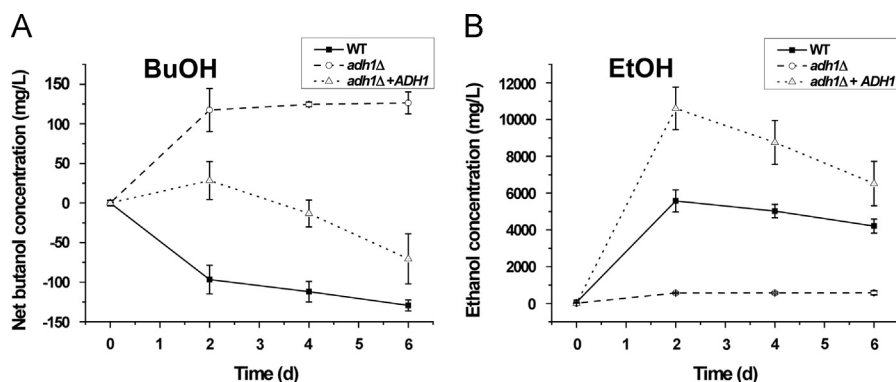


Fig. 2. Switching on 1-butanol production by *adh1* knockout. Time courses of 1-butanol (A) and ethanol (B) production in filtered YPAD media under micro-anaerobic condition are shown for the wild type strain (filled square and solid line), the *adh1Δ* strain (open circle and dashed line) and the *adh1Δ* strain with *adh1* overexpressed on pRS425 (open triangle and dotted line). The filtered YPAD medium contains ~ 200 mg/L 1-butanol at the beginning of fermentation, which is defined as 0 mg/L in the figure. The negative values indicate 1-butanol consumption. Error bars indicate standard deviation of three biological replicates.

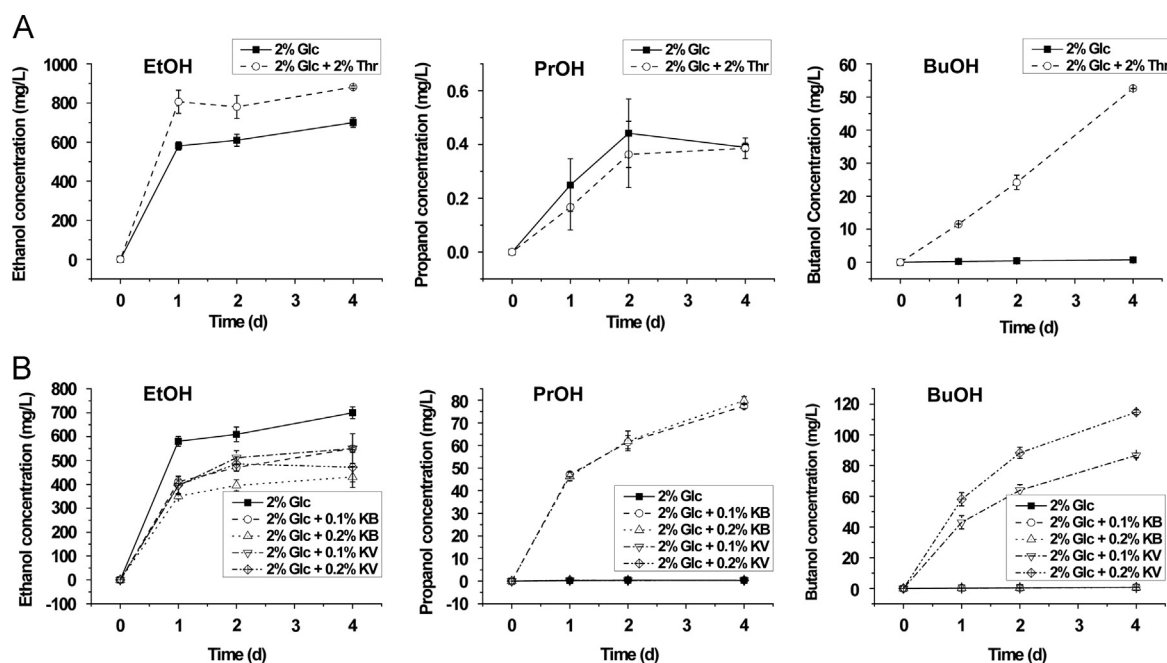


Fig. 3. Effects of precursor feeding on alcohol production by the *adh1Δ* strain. Fermentation was performed in citric acid/phosphate buffer with 2% glucose (pH=5) under micro-anaerobic condition with added L-threonine (A) or 2-keto acids (B). Glc: D-glucose; Thr: L-threonine; KB: 2-ketobutyrate; KV: 2-ketovalerate; EtOH: ethanol; PrOH: 1-propanol; BuOH: 1-butanol. Symbol—2% Glc (closed square), 2% Glc+2% Thr in (A) (open circle), 2% Glc+0.1% KB in (B) (open circle), 2% Glc+0.2% KB (open triangle), 2% Glc+0.1% KV (open inverse triangle) and 2% Glc+0.2% KV (open diamond). Error bars indicate standard deviation of three biological replicates.

and mass spectrometry (MS) fragmentation patterns of the 1-butanol product were analyzed (Fig. 4 and Fig. S4A). To our surprise, the carbon atoms from exogenous threonine were not incorporated into 1-butanol, and 1-butanol was solely synthesized from glucose (Fig. S4 and Table 1). Such findings implied that the increased accumulation of 1-butanol was not due to direct transformation of exogenous threonine. Given that threonine deaminase (*Ilv1p/Cha1p*) and α -isopropylmalate synthase (*Leu4p*), which catalyze conversion of L-threonine to 2-ethylmalate, are localized in the mitochondria (Fig. 1) (Kohlhew, 2003), we reason that exogenous threonine cannot be transported from the cytosol into the mitochondria to enter the proposed 1-butanol pathway. To rationalize how exogenous threonine stimulated 1-butanol production, we hypothesize that it can satisfy cytosolic requirement of threonine for protein expression and glycine synthesis. Thus threonine transport from mitochondria to cytosol is reduced and more threonine is available in the mitochondria for transformation into 1-butanol through the proposed pathway. To test this hypothesis, we also supplemented L-glycine to the medium, suspecting that this would also reduce the requirement for threonine efflux from the mitochondria (Fig. 1) (Monschau et al., 1997). We found that L-glycine supplementation indeed resulted in improved 1-butanol production, but to a lesser extent compared to threonine (Fig. 3A and Fig. S5). A ^{13}C -labeling study with L-Glycine-2- ^{13}C also indicated that exogenous glycine was not directly incorporated into 1-butanol, and all the carbon atoms of 1-butanol were from glucose (Fig. S4).

Addition of 2-KB had no observable effect on 1-butanol production, but resulted in significant 1-propanol production (Fig. 3B and Fig. S6A). These results indicate that supplemented 2-KB cannot enter the keto-acid chain elongation process catalyzed by the leucine biosynthesis enzymes. There might be two reasons: (1) 2-KB cannot be transported from the cytosol into the mitochondria, where it is converted to 2-ethylmalate by α -isopropylmalate synthase (Fig. 1); (2) KDCs have much higher affinity for 2-KB than α -isopropylmalate synthase, and therefore decarboxylation of 2-KB by KDCs prevents incorporation of 2-KB into the 1-butanol pathway. In addition, the

very low level of 1-propanol production from glucose with or without added threonine was observed (Fig. 3A). This phenomenon indicates that the concentration of cytosolic 2-KB is very low, probably resulting from the lack of cytosolic transamination activity toward threonine for 2-KB synthesis (Fig. 1).

Contrary to threonine and 2-KB, our results indicate that 2-KV can be directly converted to 1-butanol by *S. cerevisiae*. At pH=5, a condition under which most 2-KV ($\text{pK}_a=3.38$) is in the dissociated form that cannot freely diffuse across the plasma membrane, only moderate 1-butanol production was observed (0.08 g 1-butanol/g 2-KV, Fig. 3B). Without pH adjustment, addition of 2-KV greatly lowered the pH value of the medium, and 2-KV should mainly exist in the un-dissociated form. In this case, substantial conversion of 2-KV to 1-butanol and butyl aldehyde was observed (Fig. S6B, yield of 1-butanol plus butyl aldehyde from 2-KV: 0.36 g/g (0.1% KV), 0.44 g/g (0.2% KV), and 0.64 g/g (theoretical yield)).

3.3. Characterization of the 1-butanol pathway by gene deletion

We investigated how deletion of individual genes from the proposed 1-butanol pathway (Fig. 1) could affect 1-butanol production in the *adh1Δ* background. Due to the greatly reduced fitness of the *adh1Δ* strain, we were unable to obtain the following double-mutant strains: *adh1Δ/thr1Δ*, *adh1Δ/thr4Δ*, and *adh1Δ/cha1Δ*. For the constructed double mutants, *ilv1Δ* or *leu1Δ* led to substantial reduction of 1-butanol production from glucose in citric acid/phosphate buffer (pH=5), and *leu4Δ* nearly abolished 1-butanol accumulation (Fig. 5A). Deletion of the *LEU9* gene, which encodes an isoenzyme of *Leu4p* (Kohlhew, 2003), had no obvious effect on 1-butanol production (Fig. 5A), suggesting that *Leu9p* plays a minor role in the aldol addition of acetyl-CoA to 2-KB. We then explored how these mutants behaved with supplemented threonine. Production of 1-butanol was reduced for all double-deletion mutant strains compared to the *adh1Δ* single-deletion strain in citric acid/phosphate buffer with 2% glucose and 2% L-threonine (pH=5), and reduction was observed to a greater extent relative to the condition with no added threonine (Fig. 5B).

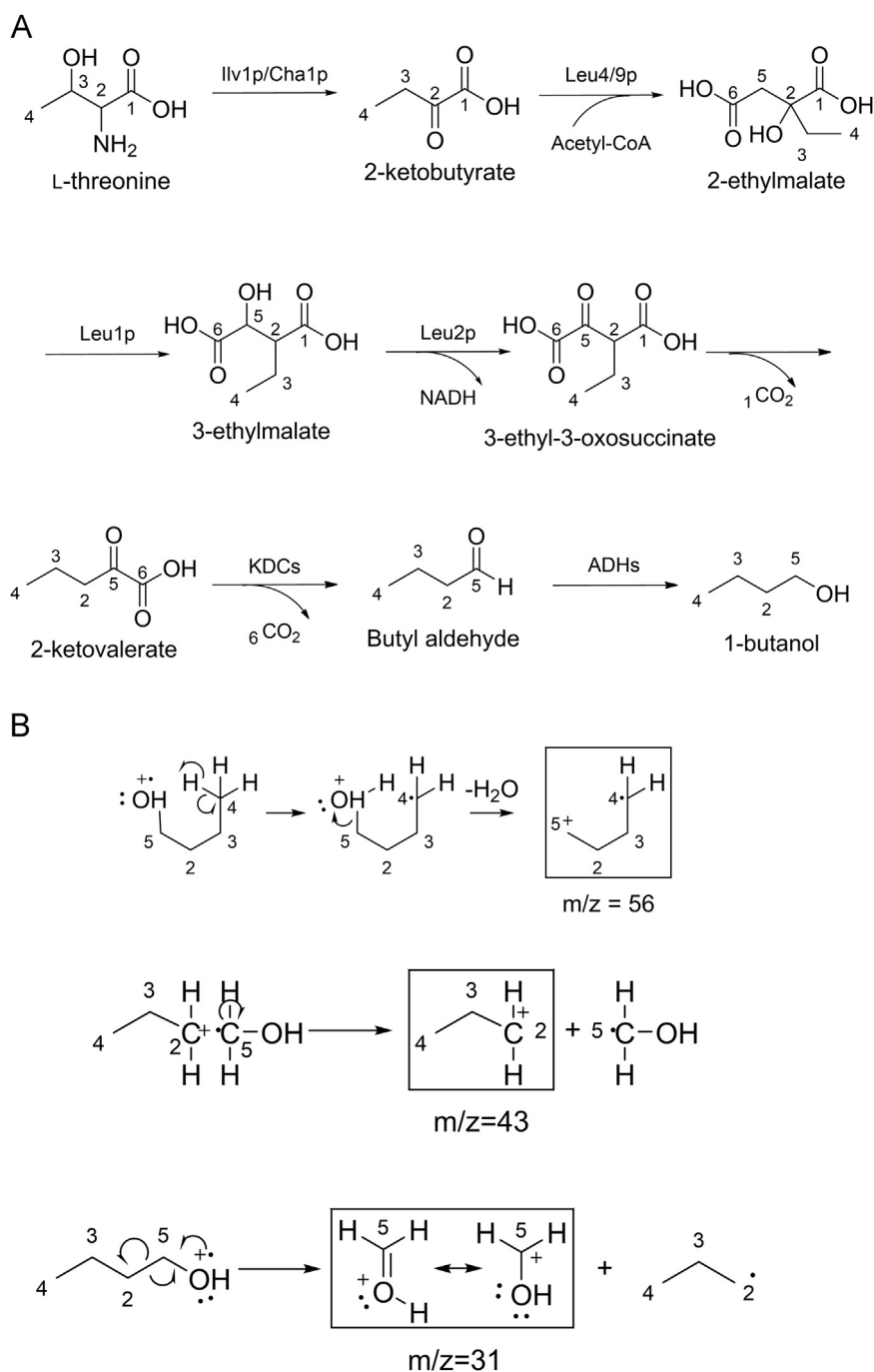


Fig. 4. Production of 1-butanol from ^{13}C -labeled substrates. (A) Biochemical transformation from L-threonine to 1-butanol. (B) Generation of fragment ions of 1-butanol in positive electron impact (EI) mode.

Table 1
Predicted and measured 1-butanol MS fragmentation patterns from ^{13}C -labeled substrates. Fermentation was performed in citric acid/phosphate buffer (pH=5) under micro-anaerobic condition. Measured 1-butanol ^{13}C -labeling patterns match the predicted patterns based on the assumption that 1-butanol is produced from endogenous threonine rather than exogenous threonine. See Fig. S4 for detailed analysis.

	D-Glc- $^{12}\text{C}_6$		D-Glc- $^{12}\text{C}_6$ L-Thr-1,2- $^{13}\text{C}_2$		D-Glc- $^{13}\text{C}_6$ L-Thr- $^{12}\text{C}_4$	
Labeled carbon# ^a in acetyl-CoA	None		None		5, 6	
Carbon source of threonine	Glc	Added	Glc	Added	Glc	Added
Labeled carbon# in threonine	None	None	None	1,2	1–4	None
Labeled carbon# in 1-butanol (predicted)	None	None	None	2	2–5	5
Labeled carbon# in 1-butanol (measured)	None		None ^b		2–5	

^a Carbon # is referred as in Fig. 4A and B.

^b Absence of ^{13}C -labeled 1-butanol was also confirmed by ^{13}C NMR (data not shown).

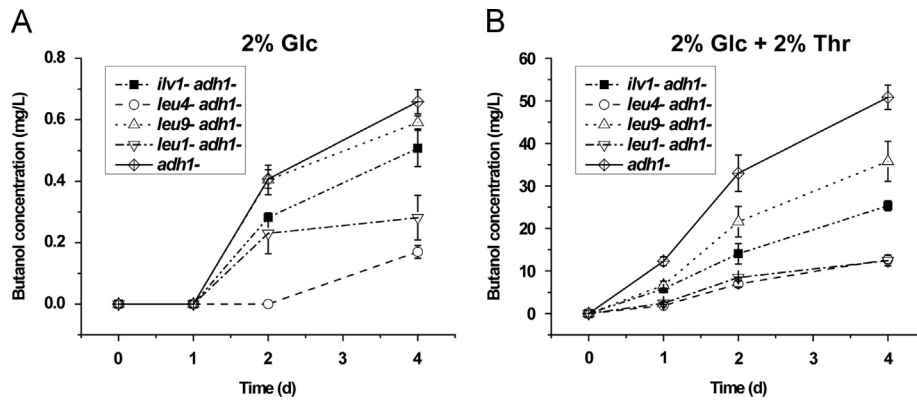


Fig. 5. Comparison of 1-butanol production in various gene knockout strains. A second gene deletion was introduced in the *adh1Δ* strain. Fermentation was performed in citric acid/phosphate buffer with 2% glucose (pH=5) under micro-anaerobic condition, with (A) or without (B) 2% L-threonine supplemented. Symbol—*ilv1Δ adh1Δ* (closed square), *leu4Δ adh1Δ* (open circle), *leu9Δ adh1Δ* (open triangle), *leu1Δ adh1Δ* (open inverse triangle) and *adh1Δ* (open diamond). Glc: D-glucose; Thr: L-threonine. Error bars indicate standard deviation of three biological replicates.

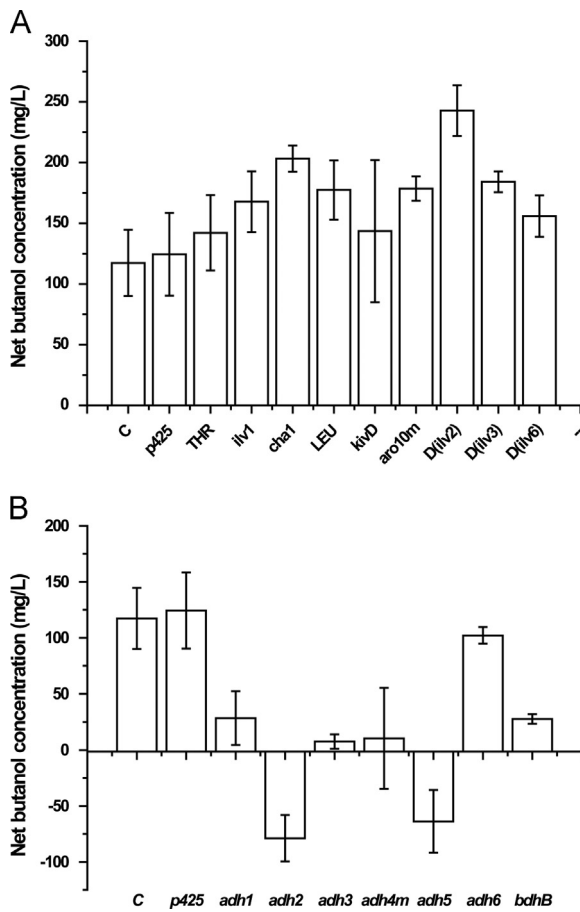


Fig. 6. 1-Butanol production by metabolically engineered strains. Recombinant strains were constructed in the *adh1Δ* strain (denoted as C). p425 is an empty plasmid harboring a *LEU2* expression cassette. For over-expression, backbone vectors are omitted for simplicity and can be found in Table S3. For double-mutant strains, only the second knockout in addition to *adh1Δ* is shown as D(gene name). Fermentation was performed in filtered YPAD media (pH=5) under micro-anaerobic condition. The filtered YPAD medium contains ~200 mg/L 1-butanol at the beginning of fermentation, which is defined as 0 mg/L in the figure. The net butanol production on Day 2 was reported, and the negative values indicate 1-butanol consumption. All metabolic engineering efforts are summarized in (A) except for over-expression of ADHs in (B). Error bars indicate standard deviation of three biological replicates.

The gene-knockout mutations of *ilv1Δ*, *leu4Δ* and *leu1Δ* also led to reduced 1-butanol production with 2% supplemented L-glycine relative to the *adh1Δ* single-deletion strain (Fig. S5). Together,

these results indicate that 1-butanol production from glucose is dependent on the proposed pathway, and the stimulated 1-butanol accumulation by exogenous L-threonine and L-glycine is also related to the proposed pathway.

3.4. Gene overexpression to improve 1-butanol production

To re-direct metabolic flux toward the endogenous 1-butanol pathway, the genes in the proposed pathway were over-expressed individually or in combination (Fig. 6). Resting cells were cultured under micro-aerobic condition in the filtered YPAD medium. Accumulation of 1-butanol decreased after reaching a maximum, probably due to activation of Adh2p which is known for its ability to oxidize alcohols (Leskovac et al., 2002). Thus, comparison of the 1-butanol production was performed with the titer data from day one and day two, when the butanol production peak had not yet been reached (Fig. 6 and Table S3). Unless indicated otherwise, pRS425, which harbors a *LEU2* expression cassette, was the backbone plasmid for most of the constructs (Table S3). Background α -isopropylmalate dehydrogenase activity has been reported in laboratory *S. cerevisiae* strains that lack a functional *LEU2* gene (Branduardi et al., 2013), and additional copies of the *LEU2* gene on the pRS425 plasmid resulted in only slightly increased 1-butanol production after day two (Fig. 6A).

To increase threonine biosynthesis, five genes encoding for enzymes that are responsible for converting aspartic acid to threonine (i.e., *hom3*, *hom2*, *hom6*, *thr1* and *thr4*) were over-expressed simultaneously on a multi-copy plasmid pRS426-THR (Maria-Jose Farfan, 2000). The strains harboring pRS426-THR accumulated $280.8 \pm 77.2\%$ more 1-butanol than the *adh1Δ* strain on the first day, but the 1-butanol concentration did not increase after the first day (Table S3). To increase 2-KB availability, additional copies of the *ILV1* and *CHA1* genes were introduced on the pRS425 plasmid. On day two, $42.9 \pm 21.4\%$ and $73.1 \pm 9.2\%$ improvement in 1-butanol production was detected with over-expression of *Ilv1p* and *Cha1p*, respectively (Fig. 6A). Enhanced conversion from 2-KB to 2-KV was achieved by over-expression of the *LEU4* and *LEU1* genes on pRS425, and the strain with the resultant plasmid pRS425-LEU reached a $51.2 \pm 20.8\%$ higher 1-butanol titer than the *adh1Δ* strain on day two (Fig. 6A).

Regarding KDCs, it has been previously reported that decarboxylation of linear-chain 2-keto acids, pyruvate, 2-KB and 2-KV, is catalyzed exclusively by Pdc1p, Pdc5p, and Pdc6p, and not by Aro10p or Thi3p (Romagnoli et al., 2012). Given the preference of these enzymes toward pyruvate over 2-KV (Romagnoli et al., 2012), we did not test over-expression of these KDCs, which we speculated would lead to increased production of ethanol rather

than 1-butanol. Two other KDCs, KivD from *Lactococcus lactis* (Atsumi et al., 2008b) and an *S. cerevisiae* Aro10p mutant Aro10p^{I355Y} (Kneen et al., 2011), were tested on the basis of their reported activity and preference toward 2-KV. Whereas KivD had no significant effect on 1-butanol production, the strain over-expressing the Aro10p^{I355Y} mutant accumulated $52.1 \pm 8.5\%$ more 1-butanol than the *adh1Δ* strain on day two (Fig. 6A).

For the last step, ADHs catalyze reduction of butyl aldehyde to 1-butanol. Six ADHs from *S. cerevisiae* (Adh1p–Adh6p) together with BdhB from *Clostridium acetobutylicum* were over-expressed in the *adh1Δ* background (Fig. 6B). The mitochondrial signal peptide of Adh4p was removed to create a cytosolic mutant, which increased 1-butanol production by $216.5 \pm 55.3\%$ on the first day in the *adh1Δ* background (Table S3). The Adh6p over-expression strain showed a similar 1-butanol production profile as the *adh1Δ* strain (Fig. 6B). Strains over-expressing other ADHs all exhibited no accumulation or even consumption of 1-butanol (Fig. 6B and Table S3).

3.5. Elimination of competing pathways

Competing reactions were eliminated to avoid loss of precursors in the proposed 1-butanol pathway (Fig. 1). The benefit of eliminating the acetolactate synthase activity (*ilv6Δ/ilv2Δ*) is threefold: (1) it prevents the conversion of 2-KB to α -isoleucine, and thus increases the availability of 2-KB for 1-butanol synthesis; (2) it raises the concentrations of two key intermediates (aspartic acid and acetyl-CoA) by conserving their common precursor, pyruvate, which is otherwise used for α -leucine and α -valine biosynthesis; (3) it reduces flux through the leucine biosynthesis pathway, which could then take a more active participation in 2-KV synthesis. On day two, a 1-butanol titer of 242.8 ± 21.0 mg/L was observed for the *ilv2Δ/adh1Δ* double mutant, which was $106.8 \pm 17.9\%$ higher than the *adh1Δ* strain (Fig. 6A). The *ilv6Δ* mutation had a less obvious impact on 1-butanol accumulation ($32.8 \pm 14.5\%$ improvement, Fig. 6A and Table S3). Knockout of *ilv3* was also performed to decrease the isoleucine, leucine and valine biosynthesis, which led to a 1-butanol titer that was $56.9 \pm 7.3\%$ more than the single mutant *adh1Δ* strain (Fig. 6A and Table S3).

4. Discussion

Previous efforts toward reconstituting the *Clostridium* 1-butanol pathway in *S. cerevisiae* met with many challenges, including difficulty in functional expression of bacterial proteins, limited availability of cytosolic acetyl-CoA, and sequestration of the CoA supply to synthesize acyl-CoA intermediates (Krivoruchko et al., 2013; Steen et al., 2008). On the contrary, employment of an endogenous amino-acid pathway for 1-butanol synthesis not only avoids issues related to introduction of a heterologous pathway, but also takes advantage of accumulated knowledge for amino-acid hyper-production. Though a similar pathway has been constructed and engineered in *E. coli* (Atsumi et al., 2008b; Shen and Liao, 2008), performance of this threonine-dependent pathway should be better in *S. cerevisiae* by avoiding 1-propanol co-production (Shen and Liao, 2008). The absence of 1-propanol as a byproduct is enabled by separation of 2-KB from cytosolic KDCs by the mitochondrial membrane (Fig. 1). In addition, computational metabolic simulations suggested that *S. cerevisiae* possesses greater potential in higher alcohol overproduction than *E. coli*, upon introduction of extra flexibility in the yeast central metabolism by incorporating metabolic shortcuts from *E. coli* (such as pyridine nucleotide transhydrogenase, the anaplerotic pathways, the Entner–Doudoroff pathway, and acetyl-CoA synthesis by

pyruvate formate-lyase) (Matsuda et al., 2011). Together with the advantages of yeast host over bacterial hosts for alcohol fermentation (see Section 1), we believe that the engineering of the proposed pathway for 1-butanol production in *S. cerevisiae* is promising.

We observed that exogenous α -threonine cannot enter mitochondria to be directly incorporated into 1-butanol synthesis. This observation suggests that some enzymes catalyzing the threonine biosynthesis reactions are located in the mitochondria (Fig. 1). Especially for the Thr1p and Thr4p enzymes that catalyze the last two steps of threonine synthesis, no direct biochemical evidence is available to determine their subcellular localization (Table S4). The green fluorescence protein (GFP) has been fused to the carboxyl terminal of Thr1p to assign its localization by imaging, but only ambiguous result was obtained (Huh et al., 2003). Though the GFP-fused Thr4p protein was localized to cytoplasm and nucleus (Huh et al., 2003), computational analysis of the Thr4p primary sequence suggested an 82% probability that Thr4p would be transported into mitochondria (Claros and Vincens, 1996) (Table S4). To make it more complicated, the C-terminal fusion of GFP has been found to cause mis-localization of a target protein (Huh et al., 2003). Together, no clear localization assignment of Thr1p and Thr4p can be made from literature, and our results imply that they are mitochondrial proteins. Further biochemical investigation by subcellular fractionation will be valuable to determine the Thr1p and Thr4p localization.

We found that exogenous α -glycine boosted 1-butanol accumulation but cannot be converted directly into 1-butanol (Fig. S4), and also noted that the stimulation of 1-butanol production by α -glycine was dependent on the proposed pathway (Fig. S5). These findings provides an alternative explanation to the observations in a recent report that suggested a metabolic route for 1-butanol production with α -glycine and butyl-CoA as substrates in *S. cerevisiae* (Branduardi et al., 2013). Based on that route, ^{13}C atom of α -Glycine-2- ^{13}C would be incorporated in 1-butanol, but it was not observed in this study (Fig. S4). Also, involvement of butyl-CoA in 1-butanol production is questionable, as butyl-CoA concentration is rather low in wild type *S. cerevisiae* (Steen et al., 2008). Therefore, we argue that the stimulation of 1-butanol production by α -glycine may be due to the reduced conversion of α -threonine to α -glycine in the presence of exogenous α -glycine, resulting in increased transformation of α -threonine to 1-butanol through our proposed pathway.

The reduced 1-butanol accumulation by gene knockout mutant strains suggested the involvement of the proposed pathway. However, 1-butanol production was not 100% abolished. There are two possibilities. First, the isoenzymes catalyzing the same reaction (Ilv1p/Cha1p or Leu4p/Leu9p) may account for the residual 1-butanol production. Second, there might also be other endogenous routes for 1-butanol production. Over-expression of the enzymes in the proposed pathway led to increased 1-butanol titer to various degrees. Moderate improvement by over-expression of the wild-type threonine biosynthesis pathway is consistent with the previous observation that threonine biosynthesis is tightly regulated (Maria-Jose Farfan, 2000). It is interesting to further investigate whether feedback-inhibition mutant enzymes (Maria-Jose Farfan, 2000) can enable deregulation and over-production of α -threonine and 1-butanol. Enhanced threonine transamination activity by Ilv1p/Cha1p over-expression led to substantial improvement in 1-butanol titer, indicating this is a key step to direct the metabolic flux toward the proposed pathway. The fact that Cha1p over-expression led to higher 1-butanol accumulation than that of Ilv1p was consistent with the results of the gene knockout experiment, where *ilv1Δ* resulted in only $23.0 \pm 9.0\%$ reduction of 1-butanol production (Fig. 5A). Cha1p might also play a role in the stimulated 1-butanol production by

exogenous L-threonine, as Cha1p is transcriptional induced by L-threonine (Bornaes et al., 1993; Pedersen et al., 1997). Over-expression of the leucine biosynthesis pathway resulted in only limited improvement in 1-butanol production, which might be due to the fact that conversion of 2-KB to 2-KV is not a natural biochemical transformation catalyzed by the leucine biosynthesis pathway (Atsumi et al., 2008b). The leucine pathway naturally takes 2-ketoisovalerate (KIV) as its substrate (Fig. 1), which possesses one more methyl group than 2-KB. It is therefore highly possible that the catalytic reactions with 2-KB as the starting precursor is not as efficient as the leucine synthesis. No specific wild-type KDCs or ADHs enzymes were found to catalyze transformation of 2-KV to butyl aldehyde and to 1-butanol. Engineered or heterologous KDCs and ADHs with preferences toward 2-KV and butyl aldehyde, respectively, are therefore highly desirable to selectively pull out the 2-keto acid intermediate to form 1-butanol. Elimination of the valine, leucine and isoleucine biosynthesis pathway by *ilv2Δ* resulted in the highest 1-butanol production in this study, which is consistent with the observation in *E. coli* (Shen and Liao, 2008). As ethanol was still the main fermentation product by the *adh1Δ* strain, metabolic engineering effort to eliminate ethanol production should greatly improve 1-butanol productivity. Possible strategies include disruption of all ADH genes (Ida et al., 2012) or creation of a KDC-negative *S. cerevisiae* strain (van Maris et al., 2004). Particularly, gene deletion of the *ADH2* gene is highly desirable as it oxidizes 1-butanol once being activated (Fig. S1 and Table S3).

In addition to 1-butanol, other short-chain alcohols have also been proposed as advance fuel alternatives (Buijs et al., 2013; Wen et al., 2013). These alcohols are naturally found as trace products in yeast fermentation and known as “fusel alcohols” (Hazelwood et al., 2008). The Erlich pathway is responsible for the biosynthesis of these alcohols, from 2-keto acids as the degradation intermediates of branched-chain amino acids (Hazelwood et al., 2008). Recently, introduction of a broad specificity KDC and the yeast *ADH2* has activated the Erlich pathway in *E. coli* and enabled the accumulation of isobutanol, 1-propanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol and 2-phenylethanol (Atsumi et al., 2008b). Among the fusel alcohols, the branched-chain isomer of 1-butanol, isobutanol, has been the focus of recent studies in both *E. coli* and yeast (Buijs et al., 2013; Wen et al., 2013). In *E. coli*, the enhanced conversion of pyruvate to 2-ketoisovalerate (KIV), together with the decreased competition of pyruvate, resulted in a titer of 22 g/L isobutanol (Atsumi et al., 2008b). In *S. cerevisiae*, the engineering has been complicated by the organelle compartmentation, where the synthesis of KIV from L-valine and the conversion of KIV to isobutanol are performed in the mitochondria and the cytosol, respectively (Buijs et al., 2013; Hazelwood et al., 2008). Therefore, by now the most successful strategy to over-produce isobutanol in yeast (0.63 g/L) involves cytosolic over-expression of *Ilv2p*, *Ilv5p*, *Ilv3p* mutants without mitochondria targeting signals, as well as inactivation of the endogenous mitochondrial pathway by deleting the first enzyme *Ilv2p* (Brat et al., 2012). In addition, highly specific KDC and ADH enzymes have also been screened to further improve isobutanol production (Brat et al., 2012; Kondo et al., 2012). These strategies may offer useful perspectives on the engineering of the endogenous 1-butanol pathway in the present study, given its similarity with the isobutanol pathway. Recent research on the isobutanol biosynthesis engineering in *S. cerevisiae* is summarized in an excellent review (Buijs et al., 2013).

In summary, we reported the endogenous 1-butanol production in *S. cerevisiae* from a renewable substrate, glucose, upon deletion of the *ADH1* gene. We proposed and characterized an endogenous 1-butanol pathway in *S. cerevisiae*, and showed potential for over-production of 1-butanol by metabolic

engineering. Combination of beneficial manipulations and systematic strain engineering are in progress to further improve the titer and productivity of 1-butanol fermentation. Given the accumulated knowledge about amino-acid biosynthesis and regulation, it is believed that high productivity of 1-butanol production can be achieved through this threonine-dependent pathway by further strain engineering.

Author contributions

T.S. designed and performed experiments with assistance from Y.L. and H.X. T.S. and H.Z. wrote the manuscript. H.Z. supervised all aspects of the study.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jmben.2014.01.002>.

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