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Effect of GPD2 and PDC6 Deletion on Isobutanol Titer in *Saccharomyces Cerevisiae*

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Abstract

Objectives: Isobutanol is regarded as a next-generation biofuel for its higher octane number and higher energy density than ethanol. However, during isobutanol biosynthesis, ethanol and glycerol are major unwanted byproducts. In order to improve isobutanol production in *Saccharomyces cerevisiae*, we used molecular biology and genetic recombination technologies to eliminate ethanol and glycerol titers.

Methods: In this study, GPD2 and PDC6 were deleted to increase isobutanol production in microaerobic fermentation of *Saccharomyces cerevisiae*. Engineered strain HZAL–13 (PGK1p–BAT2 gpd2Δ::RYUR) was constructed by overexpressing of BAT2 (which encodes a branched-chain amino-acid aminotransferase) and deleting GPD2 (which encodes glycerol-3-phosphate dehydrogenase). Engineered strain HZAL–14 (PGK1p–BAT2 pdc6Δ::R gpd2Δ::RYUR) was obtained by further deleting PDC6 (which encodes pyruvate decarboxylase) in HZAL–13 pILV2. Then we tested the fermentation performances of engineered strains and control strain. During microaerobic fermentation, cultures were performed at 30°C in the unbaffled shake flasks kept at constant stirring speed of 100 rev/min with 100 ml medium for 48 hours.

Results: The maximum isobutanol titers of control strain, HZAL–13 pILV2 and HZAL–14 pILV2 were 29.8 mg/l, 162.3 mg/l and 309.3 mg/l, respectively. These results demonstrate that decreasing glycerol formation and ethanol biosynthesis in combination through deletion of PDC6 and GPD2 could increase dramatically the isobutanol titer in *S. cerevisiae*.

Conclusion: Overexpression of related genes in isobutanol biosynthesis pathway and deletion of key genes that encode glycerol and ethanol biosynthesis is a promising strategy to increase isobutanol titer in *Saccharomyces cerevisiae*.

Keywords: *Saccharomyces cerevisiae*; Isobutanol; Ethanol; Glycerol; LDP1; GPD2; PDC6

Introduction

Environmental friendly biofuels gain great interests due to climate change and the need for renewable transportation fuels in recent years [1]. Ethanol has been received more attention as the most widely used biofuel [2]. However, compared to ethanol, higher alcohols have several advantages as next-generation transport fuels. And they are compatible with current infrastructure [3]. Isobutanol exhibits superior physicochemical properties such as higher energy density and lower hygroscopicity than ethanol [4,5]. Furthermore, it has higher octane number than the isomer n-butanol [6,7].

Saccharomyces cerevisiae has high tolerance to isobutanol up to 20 g/l and robustness during fermentation in harsh industrial conditions [8-10]. In addition, *S. cerevisiae* naturally produces small amount of isobutanol as byproduct from the catabolism of amino acids [11,12]. So *S. cerevisiae* has been considered as an attractive alternative host strain to produce isobutanol. Initially, glucose is converted to pyruvate via the process of glycolysis. Then pyruvate is converted to 2-ketoisovalerate (KIV) in the mitochondria by acetolactate synthase (Ilv2), acetohydroxyacid reductoisomerase (Ilv5) and dihydroxyacid dehydrates (Ilv3) [12].

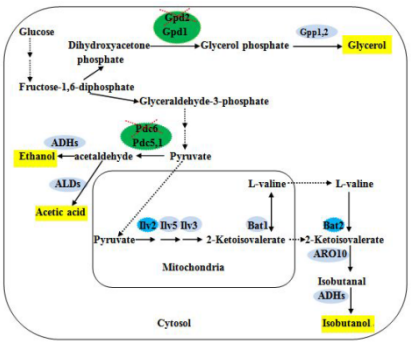

Figure 1: Isobutanol synthesis pathways in *S. cerevisiae*. Ilv2, the acetolactate synthase; Bat2, the branched-chain amino-acid aminotransferase; Gpd2, glycerol–3–phosphate dehydrogenase 2; Pdc6, pyruvate decarboxylase.

After that, KIV is further converted to L–valine by branched-chain amino-acid aminotransferase (Bat1) [10]. Furthermore, L–valine undergoes conversion to KIV by branched-chain amino-acid aminotransferase (Bat2). Finally, KIV converted to isobutanol (Figure 1).

Ilv2 catalyzes the first reaction in the synthesis of KIV in the mitochondria. KIV is the immediate precursor of valine [1]. BAT2 plays an important role in catalyzing the first reaction in the catabolism of valine in the cytosol [13]. PDC6 encodes pyruvate degradation in ethanol biosynthesis and GPD2 encodes glycerol phosphate formation. Carbon source flux mainly flows to ethanol and glycerol biosynthesis in isobutanol fermentation in *S. cerevisiae* [14]. To further improve isobutanol titer, we constructed engineered strains HZAL–13 (PGK1p–BAT2 gpd2Δ::RYUR) and HZAL–14 (PGK1p–BAT2 pdc6Δ::R gpd2Δ::RYUR). And the fermentation characters of these engineered strains were also investigated. HZAL–13 pILV2 exhibited a 445% increase (162.3 mg/l) in isobutanol production compared with control strain. And the maximum isobutanol titer of HZAL–14 pILV2 showed a 938% increase (309.3 mg/l) than that of control strain.

Materials and Methods

Yeast strains and media

The *S. cerevisiae* strains used in this study were all isogonics to W303–1A as described in table 1.

Strains	Complete Genotype	Reference or source
W303–1A	MATa leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100	Thomas and Rothstein [15]
HZAL–13	MATa leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 PGK1p-BAT2 gpd2Δ, RYUR	This study
HZAL–14	MATa leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 PGK1p-BAT2 pdc6Δ:: R gpd2Δ, RYUR	This study
W303–1A YEplac181	MATa leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100YEplac181–PGK1p–ILV2	This study
HZAL–13 pILV2	MATa leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 PGK1p-BAT2 gpd2Δ, RYUR YEplac181–PGK1p–ILV2	This study
HZAL–14 pILV2	MATa leu2-3, 112 ura3-1 trp1-92 his3-11, 15 ade2-1 can1-100 PGK1p-BAT2 pdc6Δ:: R gpd2Δ::RYUR YEplac181–PGK1p–ILV2	This study

Table 1: Strains used in this study.

The strains of *S. cerevisiae* cells were routinely grown in medium containing 2% peptone (w/v) and 1% yeast extract (w/v) supplemented with 2% glucose (w/v) as carbon source (YPD). Selective media SC [16] minus leucine or uracine were used for selection of transformants containing LEU2 or URA3 selective marker.

Growth conditions and experimental procedures

Incubation conditions were standardized at 30°C and 200 rev/min orbital shaking. Standard techniques were applied as described in Sambrook et al. [17]. For all gene–cloning experiments, PCR products were purified using phenol deproteinization and ethanol precipitation. Restriction and modification enzymes were used according to the manufacturers' instructions. Yeast transformation was performed by the lithium acetate method. *Escherichia coli* Top10' was used for subcloning. All yeast strains were maintained at 4°C on YPD plates and prepared monthly from a glycerol stock kept at –75°C.

Fermentation conditions

Microanaerobic cultivations were performed at 30°C in the unbaffled shake flasks kept at constant stirring speed of 100 rev/min with 100 ml medium (selective media SC minus leucine supplemented with 4% glucose (w/v) as carbon source). Initial biomass concentrations were set at OD_{600nm} 0.2 after inoculations. Fermentation experiments were performed in triplicate and one representative experiment was shown.

Plasmids and strains constructions

The plasmids and primers used in this study were described in Table 2 and Table 3, respectively.

Plasmids	Description	Reference or source
YEplac181	Ampr LEU2	Gietz and Sugino [18]
YIplac211	Ampr URA3	Gietz and Sugino [18]
pUC18–RYUR	Ampr	Zhang [19]
YEplac181–PGK1p–ILV2 (pILV2)	Ampr LEU2	This study
YIplac211–BAT2p–PGK1p–BAT2	Ampr URA3	This study
pUC18–PDC6P–RYUR–PDC6T	Ampr URA3	This study
pUC18–GPD2P–RYUR–GPD2T	Ampr URA3	This study

Table 2: Plasmids used in this study.

Primers	Oligonucleotide
ILV21to20-U	5'-GGATCCGTCGACATGATCAGACAATCTACGCT-3'
ILV2ORF-D	5'-GGGCCCCTGCAGCGTTTAGCTGGCTCCTGATG-3'
BAT2promoter-U	5'-GGGCCCGAGCTCCTCTTGTCTACAACACCAGC-3'
BAT2promoter-D	5'-GGGCCCGGATCCTCTAGGGGTGCCAAGGTCAT-3'
BAT2ORF-U	5'-GGGCCCGTCGACATGACCTTGGCACCCCTAGA-3'
BAT2ORF-D	5'-GGGCCCCTGCAGATACCGATAGGCCAGCACTA-3'
PGK1 promoter-U	5'-GGGCCCGGATCCAGGCATTTGCAAGAATTACTC-3'
PGK1 promoter-D	5'-GGGCCCGTCGACTGTTTTATATTTGTTGTAAA AAGTAG-3'
CPDC6P-U	5'-GGGCCCGAATTCATGCAGATCGGCTGTGGCAT-3'
CPDC6P-D	5'-GGGCCCGGATCCGCTCGCGAATCGCACCATAT-3'
CPDC6T-U	5'- CCCGGGCTGCAGAAGCCATTAGTAGTGTACTC-3'

CPDC6T-D	5'-CCCGGGAAGCTTGAGCCAAAGAGATGAGCCAA-3'
GPD2P-U	5'-CCCGGGAAGCTTCAGACGCAGCAGCAAGTAAC-3'
GPD2P-D	5'-CCCGGGCTGCAGGTAGAGAAGAGCTGCTGAAC-3'
GPD2T-U	5'-GGGCCCGGATCCAGGCAGTCTACCAGATAGTCT-3'
GPD2T-D	5'-GGGCCCGAATTCTGACTGGAGAGCCGTCAGTA-3'
Relevant restriction sites are underlined.	

Table 3: Primers used in this study.

Plasmid YA3in this study were all isogon-Iplac211–BAT2p–PGK1p–BAT2 construction

PGK1 promoter–U containing restriction enzyme site for *BamHI* in front of nucleotides 721 bp to 701 bp upstream of the start codon of PGK1, and PGK1 promoter–D containing restriction enzyme site for *SalI* in front of nucleotides 1 bp to 26 bp of the complementary strand upstream of the start codon of PGK1, were used to clone parts of the promoter gene of PGK1 by PCR with the Pyrobest DNA polymerase (TAKARA). The fragment was digested with *SalI* and *BamHI*, and ligated into the *SalI* and *BamHI* digestion sites of the integrative plasmid YIplac211, resulting in the plasmid YIplac211-PGK1p.

BAT2 promoter–U containing restriction enzyme site for SacI in front of nucleotides 942 bp to 923 bp upstream of the start codon of BAT2, and BAT2 promoter–D containing restriction enzyme site for *BamHI* in front of nucleotides 1 bp to 20 bp of the complementary strand upstream of the start codon of BAT2, were used to clone parts of the promoter gene of BAT2 promoter by PCR with the Pyrobest DNA polymerase (TAKARA). The fragment was digested with SacI and *BamHI*, and ligated into the SacI and *BamHI* digestion sites of the plasmid YIplac211–PGK1p, resulting in the plasmid YIplac211–BAT2p–PGK1p.

BAT2 ORF–U containing restriction enzyme site for *SalI* in front of nucleotides 1 bp to 20 bp upstream of the start codon of BAT2, and BAT2 ORF–D containing restriction enzyme site for *PstI* in front of nucleotides 1350 bp to 1331 bp of the complementary strand upstream of the start codon of BAT2, were used to clone parts of the promoter gene of BAT2 by PCR with the Pyrobest DNA polymerase (TAKARA). The fragment was digested with *SalI* and *PstI*, and ligated into the *SalI* and *PstI* digestion sites of the plasmid YIplac211–BAT2p–PGK1p, resulting in the plasmid YIplac211–BAT2p–PGK1p–BAT2. In this study, plasmid YIplac211–BAT2p–PGK1p is used to overexpress BAT2 gene.

The plasmid was linearized by digestion with *SpeI* before transformation of yeast to SC minus uracil medium plates using the lithium acetate method. Correct insertion of the plasmid into the BAT2 locus on chromosome was verified by PCR. For these purpose primers BAT2 promoter–U and BAT2 ORF-D were used. Loop-out of the URA3 marker gene by homologous recombination of the two direct BAT2 promoter sequences was obtained by cultivating the correct transformants on 5–FOA plates. Correct loop-out of the URA3 gene was verified by PCR, and the primers BAT2 promoter-U and BAT2 ORF–D were used. Correct engineered strains containing PGK1p-BAT2 in place of endogenous BAT2.

Plasmid YEplac181–PGK1p–ILV2 construction

PGK1p was amplified by PCR using primers PGK1 promoter–U (containing *BamHI* enzyme site) and PGK1 promoter–D (containing *SalI* enzyme site) and inserted into *SalI* and *BamHI* sites of shuttle plasmid YEplac181, resulting in plasmid YEplac181–PGK1p.

Primer Ilv21 to 20–U containing restriction enzyme site for *SalI* in front of nucleotides 1 bp to 20 bp upstream of the ATG start codon of ILV2, and primer ILV2ORF–D containing the restriction enzyme site for *PstI* in front of nucleotides of 2559 bp to 2540 bp downstream of the ATG start codon of ILV2, were used to clone a 2559 bp fragment of ILV2 by PCR with the Pyrobest DNA polymerase (TAKARA). The fragment was digested with *SalI* and *PstI*, and ligated into the *SalI* and *PstI* digestion sites of the plasmid YEplac181–PGK1p, resulting in plasmid YEplac181–PGK1p–ILV2. In this study, plasmid YEplac181–PGK1p–ILV2 is used to overexpress ILV2 gene. Plasmid YEplac181–PGK1p–ILV2 was also introduced into mutants using the lithium acetate method and the transformants were selected on SC minus leucine medium. By overexpressing of BAT2 and ILV2 we obtained engineered strain W303–1A YEplac181.

pUC18–GPD2P–RYUR–GPD2T plasmid construction

GPD2P–U containing restriction enzyme site for *HindIII* in front of nucleotides 954 bp to 935 bp upstream of the start codon of GPD2, and GPD2P–D containing restriction enzyme site for *PstI* in front of nucleotides 243 bp to 262 bp of the complementary strand upstream of the start codon of GPD2, were used to clone parts of the promoter gene of GPD2 promoter by PCR with the Pyrobest DNA polymerase (TAKARA). The fragment was digested with *HindIII* and *PstI*, and ligated into the *HindIII* and *PstI* digestion sites of the shuttle plasmid pUC18–RYUR, resulting in the plasmid pUC18–GPD2–RYUR.

GPD2T–U containing restriction enzyme site for *BamHI* in front of nucleotides 1238 bp to 1258 bp upstream of the start codon of GPD2, and GPD2T–D containing restriction enzyme site for *EcoRI* in front of nucleotides 1723 bp to 1704 bp of the complementary strand upstream of the start codon of GPD2, were used to clone parts of the promoter gene of GPD2 promoter by PCR with the Pyrobest DNA polymerase (TAKARA). The fragment was digested with *BamHI* and *EcoRI*, and ligated into the *BamHI* and *EcoRI* digestion sites of the plasmid pUC18–GPD2P–RYUR, resulting in the plasmid pUC18– GPD2P–RYUR–GPD2T.

Plasmid pUC18–GPD2P–RYUR–GPD2T was digested with *EcoRI* and *HindIII* and transformed into W303–1A YEplac181 using the lithium acetate method. Correct insertion of the plasmid into the GPD2 locus on chromosome was verified by PCR. For these purpose primers GPD2P–U and GPD2T–D were used. Finally, RYUR fragment including URA3 marker was pop out by homologous recombination of the two direct Repeat sequences was obtained by cultivating the correct transformations on 5-FOA plates. Correct loop-out of the URA3 gene was verified by PCR, and the primers GPD2P–U and GPD2T–D were used. Correct engineered strain was denoted as HZAL–13 pILV2.

Plasmid pUC18–PDC6T construction

CPDC6P-U containing restriction enzyme site for *EcoRI* in front of nucleotides 530 bp to 511 bp upstream of the start codon of PDC6, and CPDC6P-D containing restriction enzyme site for *BamHI* in front of nucleotides 31 bp to 50 bp of the complementary strand upstream of

the start codon of PDC6, were used to clone parts of the promoter gene of PDC6 promoter by PCR with the Pyrobest DNA polymerase (TARARA).The fragment was digested with *EcoRI* and *BamHI*, and ligated into the *EcoRI* and *BamHI* digestion sites of the shuttle plasmid pUC18-RYUR, resulting in the plasmid pUC18-PDC6P-RYUR.

CPDC6T-U containing restriction enzyme site for *PstI* in front of nucleotides 150 bp to 132 bp upstream of the start codon of PDC6, and CPDC6T-D containing restriction enzyme site for *HindIII* in front of nucleotides 2146 bp to 2127 bp of the complementary strand upstream of the start codon of PDC6, were used to clone parts of the promoter gene of PDC6 promoter by PCR with the Pyrobest DNA polymerase (TARARA). The fragment was digested with *PstI* and *HindIII*, and ligated into the *PstI* and *HindIII* digestion sites of the plasmid pUC18-PDC6P-RYUR, resulting in the plasmid pUC18-PDC6P-RYUR-PDC6T.

Plasmid pUC18-PDC6P-RYUR-PDC6T was linearized by digestion with *EcoRI* and *HindIII* before transformation of HZAL–13 pILV2 to SC minus uracil medium plates using the lithium acetate method. Correct insertion of the plasmid into the PDC6 locus on chromosome was verified by PCR. For these purpose primers CPDC6P-U and CPDC6T-D were used. Finally, RYUR fragment including URA3 marker was pop out by homologous recombination of the two direct Repeat sequences was obtained by cultivating the correct transformations on 5-FOA plates. Correct loop-out of the URA3 gene was verified by PCR, and the primers CPDC6P-U and CPDC6T-D were used. Correct engineered strain was denoted as HZAL–14 pILV2.

Analyses

Growth determination

Growth was followed by measuring the absorbance of the cultures at 600 nm in an INESA 721G spectrophotometer (INESA ANALYTICAL INSTRUMENT CO, LTD, China).

Measurement of glucose, isobutanol, ethanol, glycerol and acetic acid

The samples (0.002 l each) were centrifuged for 10 min at 15871 g and the resulting supernatants were frozen –20°C until analysis. Growth curves were measured using a UV–721G spectrophotometer. Concentration of glucose, ethanol, glycerol, and acetic acid concentrations were determined by a high–performance liquid chromatography (HPLC) system (Agilent Technologies 1260 Series) equipped with a Carbomix H–NP column and a refractive index (RI) detector. The column was eluted with 2.5 mM H_2SO_4 at a flow rate of 0.6 ml/min at 55°C, and RI detector was kept at 35°C. Isobutanol concentration was quantified by a gas chromatography (GC) system (Bruker 456-GC) equipped with a HP-INNOWAX column (length of 60 m, 0.32 mm of an inner diameter). The column temperature was controlled by 80°C for 10 min. The injector and detector temperatures were maintained at 200°C and 300°C.

Determination of dry weight

Samples (50 ml) were centrifuged at 15871 g for 10 min and washed twice with water, and subsequently the pellets were kept at 100°C for 24 h before temperature equilibration and weighing.

Results and Discussion

Growth characteristics of engineered strains

The fermentation properties of control strain (W303–1A YEplac181), HZAL–13 pILV2 and HZAL–14 pILV2 were studied under microaerobic condition. The growth rate of HZAL–13 pILV2 was similar to that of HZAl–14 pILV2 (Figure 2a). However, the growth rate of the two-engineered strains HZAL–13 pILV2 and HZAL–14 pILV2 were both slower than that of control strain. These results were further more supported by the fact that the consumption rates of glucose in HZAL–13 pILV2 and HZAL–14 pILV2 lagged a little compared to control strain (Figure 2b). Finally, the biomass concentrations in HZAL–13 pILV2 and HZAL–14 pILV2 at the end of the growth period were similar but both less than the control strain (Table 4).

Strains	Biomass concentration (g dl^{-1})
W303–1A YEplac181	0.121 ± 0.0012
HZAL–13 pILV2	0.1022 ± 0.0010
HZAL–14 pILV2	0.0924 ± 0.0009

Table 4: Biomass concentration of strains.

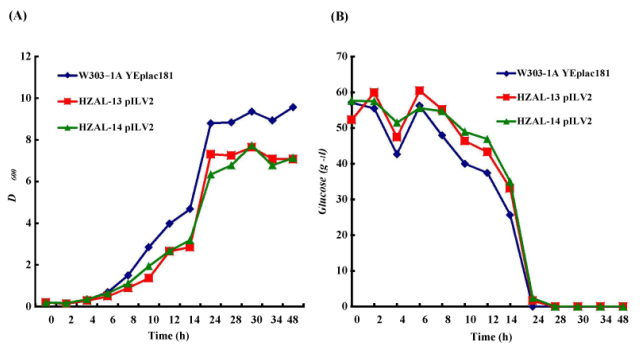

Figure 2: Growth curves and glucose consumption curves of strains carrying plasmids YEplac181 or YEplac181–PGK1p–ILV2. (A) Growth curves. (B) Glucose consumption curves. W303–1A YEplac181, ; HZAL–13 pILV2, ; HZAL–14 pILV2, . The means and standard deviations for triplet experiments are shown.

Isobutanol titer

Our results showed that isobutanol titers of HZAL–13 pILV2 and HZAL–14 pILV2 were both increased during microaerobic fermentations compared with control strain (Figure 3a). The maximum isobutanol titer of control strain was 29.8 mg/l. While HZAL–13 pILV2 exhibited a 445 % increase in isobutanol titer (162.3 mg/l) compared with control strain. Meanwhile the maximum isobutanol yield of HZAL–14 pILV2 was increased 938 % (309.3 mg/l) than that of control strain. One explanation is that GPD2 is the key gene to synthesize glycerol [20,21], deletion of GPD2 could decrease glycerol formation and help to further enhance the metabolic fluxes leading to isobutanol production. It was reported that overexpression of BAT2 would increase more carbon source flow to isobutanol biosynthesis direction

in *S. cerevisiae* [22-24]. It was also reported that deletion of PDC1 would increase isobutanol production in *S. cerevisiae* [25]. By deleting PDC6 and GPD2, parts of carbon source flux that would be used to form ethanol and glycerol has been used to synthesize isobutanol during microaerobic fermentations in HZAL–14 pILV2. Therefore, the integrative effect in isobutanol production was obtained with BAT2 and ILV2 overexpression and GPD2 and PDC6 deletion.

Production of ethanol

The maximum ethanol production of control strain (W303–1A YEplac181), HZAL–13 pILV2 and HZAL–14 pILV2 were 1481.4 mg/l, 1641.7 mg/l and 4040.9 mg/l, respectively (Figure 3b). Ethanol is the main product in *S. cerevisiae* [26]. This is the reason why ethanol production level was slightly increased after deleting GPD2 in HZAL–13 pILV2 than control strain. However, HZAL–14 pILV2 obtained a higher ethanol production compared with control strain. In addition to PDC6, PDC5 and PDC1 coding of pyruvate decarboxylase can also be used for catalyzing pyruvate into acetaldehyde. And Pdc5 and Pdc1 play major roles during ethanol fermentation [27,28]. The ethanol titer of HZAL–14 pILV2 was increased may probably due to enhancement of the functions of Pdc5 and Pdc1 after deleting GPD2 and PDC6 or any other reason. The precise molecular mechanisms need to be further investigated.

Formation of glycerol and acetic acid

We also investigated the effect of GPD2 and PDC6 deletion on the formation of other secondary metabolic products such as glycerol and acetic acid (Figures 3c-3d). The maximum glycerol formation of control strain, HZAL–13 pILV2 and HZAL–14 pILV2 were 257.7 mg/l, 275.5 mg/l and 448.8 mg/l, respectively. And the maximum acetic acid formation of control strain, HZAL–13 pILV2 and HZAL–14 pILV2 were 51.8 mg/l, 150.3 mg/l and 175.8 mg/l, respectively. The glycerol formation of HZAL–13 pILV2 was similar to that of control strain. However, the glycerol formation of HZAL–14 pILV2 increased 74% than that of control strain. On the other hand, acetic acid formation in HZAL–13 pILV2 and HZAL–14 pILV2 showed 190% increase and 239% increase, respectively, compared with the control strain.

Isobutanol exhibits superior physicochemical properties as an alternative biofuel and can be transported using current petroleum pipelines [3]. However, ethanol and glycerol are major unwanted by-products in isobutanol fermentation in *S. cerevisiae* [26]. To increase isobutanol titers in *S. cerevisiae*, two strains were obtained by deleting GPD2 and PDC6, and simultaneous overexpressing of BAT2 in *S. cerevisiae*. Microaerobic fermentations showed that the maximum isobutanol titers of HZAL-13 pILV2 and HZAL-14 pILV2 were 162.3 mg/l and 309.3 mg/l, respectively. Compared with control strain, isobutanol titers of HZAL-13 pILV2 and HZAL-14 pILV2 were increased 445 % and 938 %, respectively. In this study, only ILV2 (encoding acetolactate synthase which catalyze the first step in valine synthesis) was overexpressed. Isobutanol might further improved by simultaneously overexpressing ILV3 and IVL5.

Biomasses of HZAL–13 pILV2 and HZAL–14 pILV2 decreased markedly than that of control strain. Isobutanol titer of HZAL–14 pILV2 increased dramatically than that of control strain. While ethanol titer of HZAL–14 pILV2 increased markedly than that of control strain. These results are unexpected. One explanation perhaps is that GPD1, PDC5 and PDC1 still play important roles in HZAL–14 pILV2. Higher acetic acid titers in HZAL–13 pILV2 and HZAL–14 pILV2 perhaps to solve cofactors imbalance.

So far, there are many studies on improving isobutanol titers in *S. cerevisiae*. The accumulation of alcohols including isobutanol and ethanol is toxic to *S.cerevisiae*, which has limited the production capacity of many yeast strains in fermentation [26,29]. For this problem, investigation on improving isobutanol titer by adding potassium and hydroxide ions to the medium in which yeast grow has been reported recently [30]. There are also many other strategies. One strategy has been used for construction of higher isobutanol-producing titer strains of *S. cerevisiae* by re-locating the valine biosynthesis enzymes ILVs from the mitochondrial matrix into the cytosol [12]. Other strategies are overexpression or deletion of genes in isobutanol biosynthesis in *S. cerevisiae*. For example, overexpression of ILVs and BAT2 to increase flux in the isobutanol biosynthetic pathway [1]; deletion of BAT1 [10] or PDC1 [25] to eliminate competing pathways. But there have been no strategies about improving isobutanol titer by deleting GPD2 gene in *S. cerevisiae*. This is the first report about improving isobutanol titer by deletion of GPD2 and simultaneous deletion of PDC6 in *S. cerevisiae*. And this will provide guidance for future studies about improving isobutanol production.

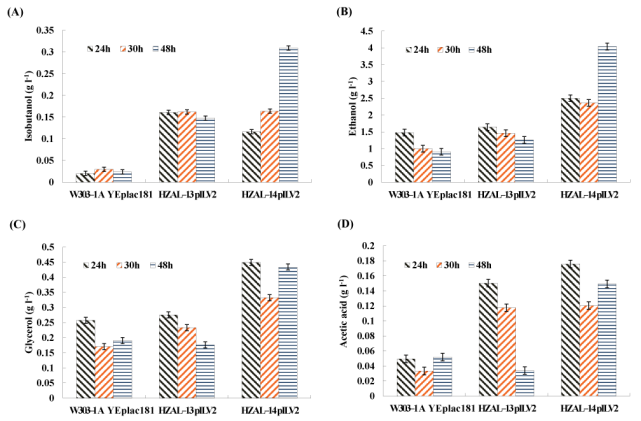

Figure 3: Changes in measured parameters of isobutanol titer, production of ethanol, formation of glycerol and acetic acid during batch fermentations of strains W303–1A YEplac181, HZAL–13 pILV2, HZAL–14 pILV2, with 40 g glucose l^{-1} as carbon source. (A) isobutanol titer, (B) production of ethanol, (C) formation of glycerol, (D) formation of acetic acid. The means and standard deviations for triplet experiments are shown.

In summary, the present study has demonstrated the proposed concept to increase the isobutanol titer by decreasing glycerol formation and ethanol biosynthesis in combination through deletion of PDC6 and GPD2 is feasible in *S. cerevisiae*.

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Conflict of Interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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