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Improving isobutanol production in metabolically engineered *Escherichia coli* by co-producing ethanol and modulation of pentose phosphate pathway

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Abstract Redox imbalance has been regarded as the key limitation for anaerobic isobutanol production in metabolically engineered *Escherichia coli* strains. In this work, the ethanol synthetic pathway was recruited to solve the NADH redundant problem while the pentose phosphate pathway was modulated to solve the NADPH deficient problem for anaerobic isobutanol production. Recruiting the ethanol synthetic pathway in strain AS108 decreased isobutanol yield from 0.66 to 0.29 mol/mol glucose. It was found that there was a negative correlation between aldehyde/alcohol dehydrogenase (AdhE) activity and isobutanol production. Decreasing AdhE activity increased isobutanol yield from 0.29 to 0.6 mol/mol. On the other hand, modulation of the glucose 6-phosphate dehydrogenase gene of the pentose phosphate pathway increased isobutanol yield from 0.29 to 0.41 mol/mol. Combination of these two strategies had a synergistic effect on improving isobutanol production. Isobutanol titer and yield of the best strain ZL021 were 53 mM and 0.74 mol/mol, which were 51 % and 12 % higher than the starting strain AS108, respectively. The total alcohol yield of strain ZL021 was 0.81 mol/mol, which was 23 % higher than strain AS108.

Keywords Isobutanol · Ethanol · Co-producing · *Escherichia coli* · NADPH

Introduction

Isobutanol is an important representative of higher chain alcohols which have received great attention for their potential usage as advanced biofuels [2, 3, 13]. Biosynthesis of these compounds from renewable and sustainable lignocellulosic biomass is considered to be a feasible, sustainable, and environment-friendly method to replace the petroleum-based transportation fuels [1, 2, 6, 16, 31].

By combining branched-chain amino acid synthetic pathway and Ehrlich pathway with 2-keto-isovalerate serving as a precursor, a synthetic pathway for isobutanol production from glucose has been constructed in *Escherichia coli* [2]. After inactivating native fermentation pathways competing for pyruvate and overexpressing key genes involved in converting pyruvate to isobutanol, the resulted *E. coli* strain produced 22 g/L isobutanol under microaerobic conditions [2]. However, this engineered *E. coli* strain could not grow and produce isobutanol under anaerobic conditions [2, 32]. The main reason was reported to be the redox imbalance problem when the pathway was designed to produce isobutanol as the sole fermentative product [31, 32].

NADH was produced as the reducing equivalent through glycolysis when *E. coli* was fermented under anaerobic conditions [5]. However, NADPH was the reducing equivalent required for the isobutanol production. Both keto acid reductoisomerase and some types of alcohol dehydrogenases were NADPH dependent. Thus, one or two moles of NADPH was required for producing one mole of isobutanol based on cofactor demand of the alcohol dehydrogenase [27]. When isobutanol was

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designed as the sole fermentation product, redox imbalance problem emerged and resulted in excess NADH while insufficient supply of NADPH. Several strategies had been developed to solve this problem [5, 20, 27, 31, 32]. Theoretical yield of isobutanol could be reached under anaerobic conditions when the cofactor specificities of keto acid reductoisomerase and alcohol dehydrogenase enzymes were changed from NADPH to NADH [5]. The membrane-bound transhydrogenase PntAB and NAD kinase were activated in combination to convert NADH to NADPH, leading to increase of anaerobic isobutanol yield from 0.66 to 0.92 mol/mol glucose [27]. Elementary mode (EM) analysis was also applied and the model predictions revealed that no EMs could co-produce cell biomass and isobutanol under anaerobic conditions in the engineered *E. coli* strain having all the native fermentative pathways deleted. As a result, it was infeasible to anaerobically produce isobutanol as the sole product. The isobutanol producing pathway must couple with other anaerobic pathways that could oxidize NADH to balance the reducing equivalent. When coupled with a pathway for ethanol production, the engineered strain could anaerobically produce 1.72 g/L isobutanol and the ratio between isobutanol and ethanol was 0.52 g/g. However, the insufficient supply of NADPH was still not solved in the engineered stain [31, 32].

In this work, to solve the cofactor imbalance problem for anaerobic isobutanol production, ethanol producing pathway was recruited in our previously engineered *E. coli* strain AS108 [27] to couple with isobutanol production for elimination of the excessive NADH. A new strategy was also used to increase the NADPH supply by modulation of the pentose phosphate pathway (PPP) for improving isobutanol production.

Materials and methods

Strains, media and growth conditions

Strains used in this study are listed in Table 1. During strain construction, cultures were grown aerobically at 30, 37, or 39 °C in Luria broth (per liter: 10 g Difco tryptone, 5 g Difco yeast extract and 5 g NaCl). Ampicillin (100 mg L^{-1}), kanamycin (50 mg L^{-1}), and chloramphenicol (34 mg L^{-1}) were used where appropriate.

Plasmids construction

To integrate *pflB* gene (encoding the pyruvate formate-lyase) at *ackA-pta* site, three plasmids were constructed. The *ackA-pta* gene operon and its neighboring 513 bp region were amplified from *E. coli* ATCC 8739 with primer set XZ-ackA-up/XZ-pta-down and cloned into the pEASY-Blunt vector to obtain plasmid pXZ023 [27]. A 4662 bp fragment was then obtained by the inside-out amplification using pXZ023 as template with primer set XZ-ackA-1/XZ-pta-2. This 4662 bp fragment was treated by the T4 polynucleotide kinase (New England Biolabs) and ligated with the *cat-sacB* cassette, which was amplified from pXZ-CS [29] using primer set cat-sacB-up/cat-sacB-down, to obtain plasmid pXZ024C. The kinase treated DNA fragment was also ligated with a DNA fragment containing *pflB* gene and its native regulation region (from −1 to −500 relative to translation start of *pflB*), which was amplified from *E. coli* ATCC 8739 with primer set pflB-up-PP/pflB-down-P, to obtain plasmid pXZ-pL001. PCR fragments amplified from pXZ024C and pXZ-pL001 with primer set XZ-ackA-up/XZ-pta-down were used for integration of *pflB* gene at *ackA-pta* site using the two-step homologous recombination method as described previously [17, 34]. Plasmids used to recover *adhE* gene (encoding the aldehyde/alcohol dehydrogenase) at its native site were constructed in the same manner. All plasmids are listed in Table 1 and primers in Table 2.

Gene integration and modulation

Gene integration and modulation were done seamlessly without leaving any foreign DNA using a two-step homologous recombination method [17, 34]. Red recombination technology (Gene Bridges GmbH, Dresden, Germany) was used to facilitate the homologous recombination [14].

Recovery of adhE and pflB genes in strain AS108

The *adhE* gene was recovered at its native site in strain AS108. The *cat-sacB* cassette was firstly amplified from pXZ021C with primer set XZ-adhE-up/XZ-adhE-down and inserted into the *adhE* site of the chromosome DNA. A DNA fragment containing the *adhE* gene was obtained by PCR amplification from *E. coli* ATCC 8739 with primer set XZ-adhE-up/XZ-adhE-down and used to replace the *cat-sacB* cassette by selection for resistance to sucrose. The resulted strain was designed as ZL002. The integration of *pflB* gene and its native promotor at *ackA-pta* site in strain ZL002 was done by the same method and the resulted strain was designed as ZL004.

Gene modulation with RBS library

The two-step homologous recombination method was also used for markerless modulation of gene expression in metabolically engineered *E. coli* strains. For *adhE* gene modulation with RBS library, the *cat-sacB* cassette was firstly amplified from pXZ-CS using a primer set of

Table 1 *Escherichia coli* strains and plasmids used in this work

	Relative characteristics	Sources or references
Strains		
ATCC 8739	Wild type	Lab collection
AS108	ATCC8739,Δ *ackA-pta*, Δ *adhE*, *frd*::*pck***-adhA*, *pflB*::M1-93-*kivD-ilvD*, *tdcDE*::M1-93-*alsS*, *ldhA*::M1-64-*alsS*, *mgsA*::M1-46-*ilvC*	[27]
M1-93	ATCC 8739, FRT-*Km*-FRT::M1-93::*lacZ*	[21]
ZL001	AS108, *adhE*::*cat-sacB*	This study
ZL002	ZL001, *adhE*	This study
ZL003	ZL002, *ackA-pta*::*cat-sacB*	This study
ZL004	ZL003, *ackA-pta*::*pflB*	This study
ZL005	ZL004, RBSL1-*zwf*	This study
ZL006	ZL004, RBSL2-*zwf*	This study
ZL007	ZL004, RBSL3-*zwf*	This study
ZL008	ZL004, RBSL4-*zwf*	This study
ZL009	ZL004, RBSL1- *adhE*	This study
ZL010	ZL004, RBSL2-*adhE*	This study
ZL015	ZL004, RBSL3-*adhE*	This study
ZL018	ZL004, RBSL4-*adhE*	This study
ZL019	ZL004, RBSL3-*adhE*, RBSL2-*zwf*	This study
ZL020	ZL004, RBSL3-*adhE*, RBSL3-*zwf*	This study
ZL021	ZL004, RBSL2-*adhE*, RBSL2-*zwf*	This study
ZL022	ZL004, RBSL2-*adhE*, RBSL3-*zwf*	This study
Plasmids		
pKD46	*bla γ β exo* (red recombinase), temperature-conditional replicon	[14]
pXZ-CS	*cat* gene of pACYC184 and *sacB* gene from *Bacillus subtilis* cloned into the pEASY-Blunt vector	[29]
pXZ020	*bla kan*; *adhE* (XZ-adhE-up/XZ-adhE-down) from *E. coli* ATCC8739 cloned into pEASY-Blunt vector	[27]
pXZ021C	*cat-sacB* cassette (cat-sacB-up/cat-sacB-down) from pXZ-CS cloned into *adhE* of pXZ020 (XZ-adhE-1/XZ-adhE-2)	This study
pXZ023	bla kan; *ackA-pta* (XZ-ackA-up/XZ-pta-down) from *E. coli* ATCC8739 cloned into pEASY-Blunt vector	[27]
pXZ024C	*cat-sacB* cassette (cat-sacB-up/cat-sacB-down) from pXZ-CS cloned into *ack-pta* of pXZ023 (XZ-ackA-1/XZ-pta-2)	This study
pXZ-pL001	*pflB* gene from *E. coli* ATCC8739 cloned into *ack-pta* of pXZ023 (XZ-ackA-1/XZ-pta-2)	This study

adhE-cat-sacB-up/adhE-cat-sacB-down and used to replace the native promoter of the *adhE* gene (from −1 to −110 relative to translational start codon of *adhE*). A RBS library was then obtained by PCR amplification from genomic DNA of *E. coli* M1-93 [21] using primer set of adhE-P-up/adhE-RBSL-down and used for the second recombination. The homologous arms used for recombination were directly generated by embedding the sequences in respective primers. The left homologous arm was the 50 nucleotides upstream of the *adhE* gene (from −111 to −161 relative to translational start codon of *adhE*), while the right arm was the 50 nucleotides downstream of translational start codon of *adhE* gene of *E. coli* ATCC 8739. The sequence of the RBS library was CAGGAGRNNNNNN. Primer set of adhE-YZ-up/adhE-YZ-down was designed based on the sequence outside of the homologous arms at chromosome and used to verify the resulted colonies. Four right colonies were selected for further fermentation. The *zwf* gene was modulated with the RBS library in the same manner.

Fermentation

For isobutanol production, fermentation were performed anaerobically as previously described [27]. Single clonies were picked from plates and inoculated into tubes containing 4 mL NBS (New Brunswick Scientific) mineral salts medium [33] with 2 % glucose, and grown at 37 °C and 250 rpm overnight. The resulted culture was re-transferred into a 250 mL flask containing 50 mL fresh NBS mineral salts medium with 2 % glucose, and grown at 37 °C and

Table 2 Primers used in this work

Primers	Sequence(5′–3′)
pflB gene integration at *ackA-pta* site	
XZ-ackA-up	CGGGACAACGTTCAAAACAT
XZ-pta-down	ATTGCCCATCTTCTTGTTGG
XZ-ackA-1	AACTACCGCAGTTCAGAACCA
XZ-pta-2	TCTGAACACCGGTAACACCA
cat-sacB-up	TGTGACGGAAGATCACTTCGCA
cat-sacB-down	TTATTTGTTAACTGTTAATTGTCCT
pflB-up-PP	ATATGACCGCAAATGGTCAATG
pflB-down-P	TTACATAGATTGAGTGAAGG
adhE gene integration at its local site	
XZ-adhE-up	CATGCTAATGTAGCCACCAAA
XZ-adhE-down	TTGCACCACCATCCAGATAA
XZ-adhE-1	TCCGGCTAAAGCTGAGAAAA
XZ-adhE-2	GTGCGTTAAGTTCAGCGACA
Modulation of *zwf* gene expression	
zwf-cat-sacB-up	ATCAGTTTTGCCGCACTTTGCGCGCTTTTCCCGTAATCGCACGGGTGGATAAGTGTGACGGAAGA TCACTTCGCA
zwf-cat-sacB-down	GCGCCGAAAATGACCAGGTCACAGGCCTGGGCTGTTTG CGTTACCGCCATTTATTTGTTAACTGTTAATTGTCCT
zwf-P-up	ATCAGTTTTGCCGCACTTTGCGCGCTTTTCCCGTAATCGCACGGGTGGATAAGTTATCTCTGGCGGTGTTGAC
zwf-RBSL-down	GCGCCGAAAATGACCAGGTCACAGGCCTGGGCTGTTTG CGTTACCGCCATNNNNNNYCTCCTGGTTTAAACGTACATG
zwf-YZ-up	GACTCACGGGTAATGACGAT
zwf-YZ-down	CATGGCAAAGTAGTTAATGG
Modulation of *adhE* expression	
adhE-cat-sacB-up	ATAACTCTAATGTTTAAACTCTTTTAGTAAATCACAGTGAGTGTGAGCGCTGTGACGGAAGATCACTTCGCA
adhE-cat-sacB-down	TTTACACGCTCTACGAGTGCGTTAAGTTCAGCGACATTAGTAACAGCCATTTATTTGTTAACTGTTAATTGTCCT
adhE-P-up	ATAACTCTAATGTTTAAACTCTTTTAGTAAATCACAGTGAGTGTGAGCGCTTATCTCTGGCGGTGTTGAC
adhE-RBSL-down	TTTACACGCTCTACGAGTGCGTTAAGTTCAGCGACATTAGTAACAGCCATNNNNNNYCTCCTGGTTTAAAC GTACATG
adhE-YZ-up	TTGCAGAATCGTGTCGCCGA
adhE-YZ-down	TGATCGATCCAGCCGATCAG

250 rpm to an OD_{550} of 3.0–5.0 to obtain the seed culture. The seed culture was inoculated into 50 ml NBS medium with 5 % glucose and 100 mM HEPES in 100 mL screw cap bottles with an initial OD_{550} of 0.1, and grown at 37 °C under static condition for 6 days. For each strain, fermentation was performed in triplicates independently.

Enzyme assay

Samples were taken at 48 h, which were at the late-exponential stage, for enzyme assay. Cells were collected (7000*g* for 8 min, 4 °C) and washed twice in 100 mM Tris–HCl buffer (pH 7.5). Activities were determined as described previously for Zwf [25] and AdhE [23]. Activity was reported in micromoles per milligram of protein per minute.

Analyses

Cell mass was estimated by measuring the optical density with a Shimadzu UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan) at 550 nm. Samples were taken at 144 h for measuring isobutanol, ethanol and glucose concentration in the fermentation broth. Samples were analyzed by Agilent 1200 high-performance liquid chromatography with an Aminex HPX-87C column (300 mm × 7.8 mm, 9.0 um, Bio-Rad, USA) using a refractive index detector (Agilent Technologies Inc., Santa Clara, CA, USA) [27]. A standard curve was prepared to determine the concentration of ethanol, isobutanol and glucose in the culture supernatants [27]. Samples were taken at 24 h, which were at the exponential stage, for measuring intracellular levels of NADPH as described previously [10]. Protein concentrations were

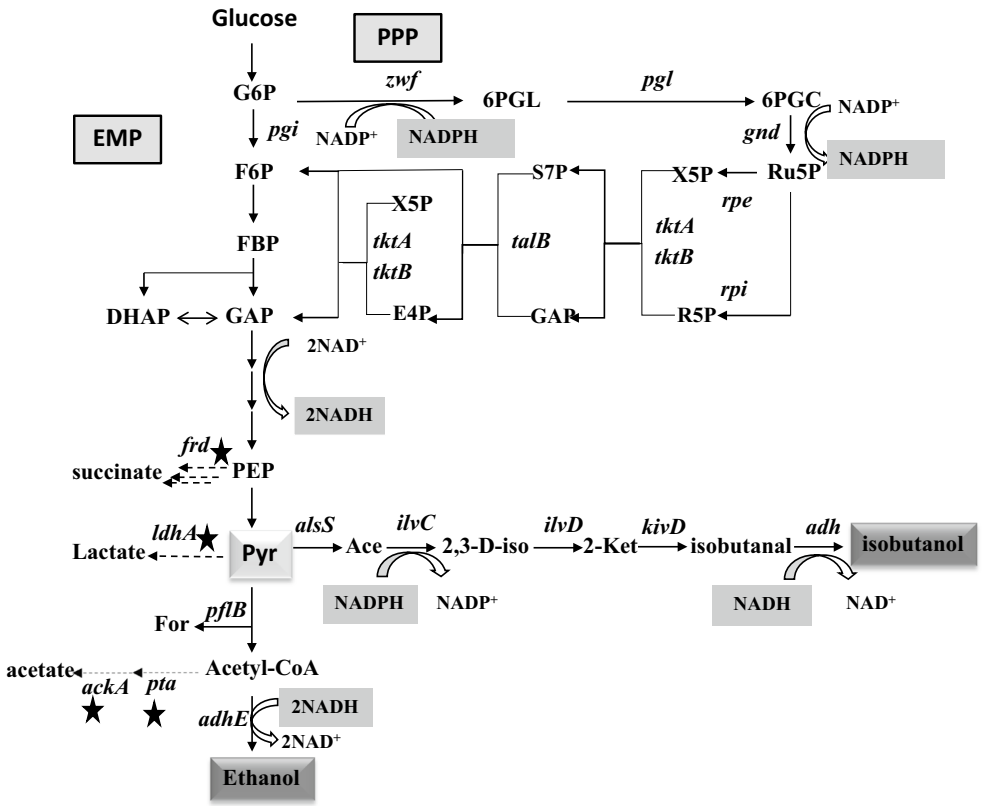

Fig. 1 Pathway designed for co-producing isobutanol and ethanol in engineered *E. coli*. Pentose phosphate pathway was modulated to increase the supply of NADPH. *Stars* indicated metabolic reactions that have been blocked by gene deletions. *G6P* glucose-6-phosphate, *F6P* fructose-6-phosphate, *FBP* fructose-1,6-bisphosphate, *GAP* glyceraldehyde-3-phosphate, *PEP* phosphoenolpyruvate, *For* formate, *6PGL* gluconolactone-6-phosphate, *6PGC* 6-phospho gluconate, *Ru5P* ribulose-5-phosphate, *X5P* xylurose-5-phosphate, *R5P* ribose-5-phosphate, *S7P* sedoheptulose-7-phosphate, *E4P* erythrose-4-phosphate, *Pyr* pyruvate, *Ace* acetolactate, *2,3-D-iso* 2,3-dihydroxy-isovalerate, *2-Ket* 2-ketoisovalerate

determined by the method of Bradford [8], with bovine serum albumin as a standard.

Results

Engineering *Escherichia coli* strain for co-producing isobutanol and ethanol

Escherichia coli strain AS108 was previously engineered for isobutanol production by recruiting essential genes responsible for converting pyruvate to isobutanol as well as inactivation of the native *E. coli* fermentative pathways [27]. Under anaerobic condition, this strain produced 35 mM isobutanol in 144 h and the isobutanol yield was 0.66 mol/mol glucose [27]. Since all the native fermentative pathways were inactivated and the isobutanol synthetic pathway required NADPH as the reducing equivalent, NADH produced by glycolysis could not be consumed. To consume the excessive NADH, an ethanol producing pathway was recruited by integration of the *adhE* gene at its original site and the *pflB* gene at the *ackA-pta* site in strain AS108 (Fig. 1). The resulting strain ZL004 produced 23 mM isobutanol and 36 mM ethanol in 144 h under anaerobic condition. The total alcohol yield increased to 0.74 mol/mol. However, the isobutanol yield of strain ZL004 decreased to 0.29 mol/mol, which was lower than that of AS108. In addition, the isobutanol yield was lower than the ethanol yield in strain ZL004. It was suggested that the carbon flux towards isobutanol production was less than that towards ethanol production, which was consistent with the previous reports [31, 32].

Improving isobutanol production by increasing NADPH supply

It was suggested that deficient supply of NADPH limited isobutanol production in strain ZL004. Pentose phosphate pathway was one of the three major ways for generating NADPH in *E. coli* [26]. The glucose 6-phosphate dehydrogenase gene (*Zwf*) was modulated to increase the NADPH supply for improving isobutanol production in strain ZL004.

Table 3 Sequences of the regulatory parts used in this work

Strains		Sequence (5′–3′)
Sequences of the regulatory parts for *zwf* gene		
ZL004	Wild type	TTTACAGTTTTCGCAAGCTCGTAAAAGCAGTACAGTGCACCGTAAGAAAATTACAAGTATACCCT GGCTTAAGTACCGGGTTAGTTAACTTAAGGAGAATGAC
ZL005	RBSL1-*zwf*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**AGACAGA**
ZL006	RBSL2-*zwf*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**AGAGCTA**
ZL007	RBSL3-*zwf*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**AAGTAAA**
ZL008	RBSL4-*zwf*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**ATGTATC**
Sequences of the regulatory parts for *adhE* gene		
ZL004	Wild type	GAGTAAGCTTTTGATTTTCATAGGTTAAGCAAATCATCACCGCACTGACTATACTCTCGTATTCGA GCAGATGATTTACTAAAAAAGTTTAACATTATCAGGAGAGCATT
ZL009	RBSL1-*adhE*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**ATTGCGA**
ZL010	RBSL2-*adhE*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**AGCATGA**
ZL015	RBSL3-*adhE*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**CCTAAGG**
ZL018	RBSL4-*adhE*	TTATCTCTGGCGGTGTTGACAAGAGATAACAACGTTGATATAATTGAGCCCGTATTGTTAGCAT GTACGTTTAAACCAGGAG**ATACGGA**

Different sequences produced by RBS library are in bold

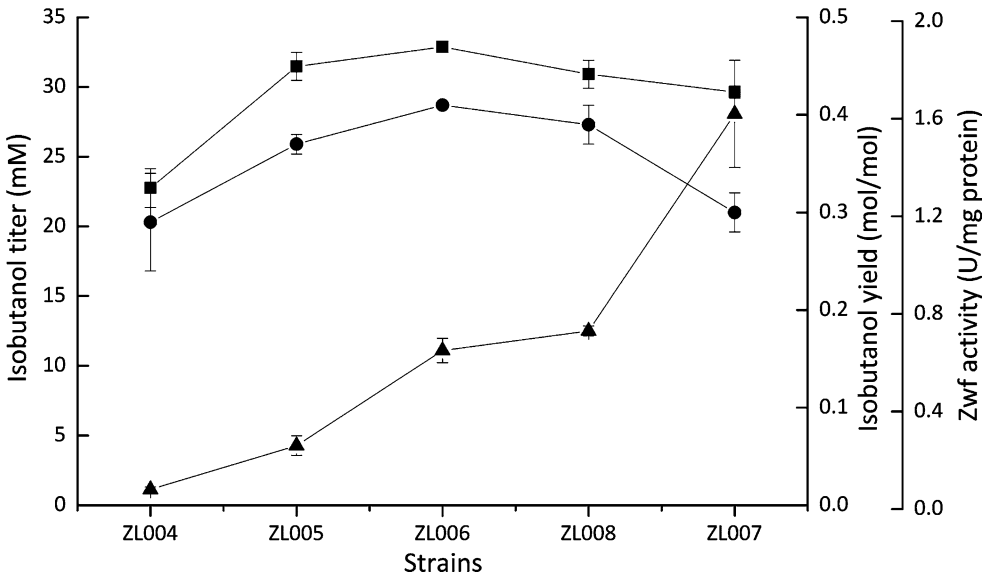

Fig. 2 The relationship between isobutanol production and glucose 6-phosphate-1-dehydrogenase (Zwf) activities. Samples were taken at 48 h for measuring Zwf activities. Samples were taken at 144 h for measuring isobutanol titer and yield. *Squares* represented the isobutanol titer. *Circles* represented the isobutanol yield. *Triangle* represented the Zwf activities

A RBS library [11] was constructed for modulation of *zwf* gene in strain ZL004. Four representative strains (ZL005, ZL006, ZL007 and ZL008) having different RBS sequences (Table 3) and Zwf activities (Fig. 2) were selected for investigating isobutanol production. There was a positive correlation between Zwf activity and isobutanol production when Zwf activity was equal to or less than 0.65 U/mg protein (Fig. 2). However, excessive Zwf activity (higher than 0.65 U/mg) decreased isobutanol production (Fig. 2). The best strain ZL006 produced 33 mM isobutanol with a yield of 0.41 mol/mol, which were 43 % and 41 % higher than the parent strain ZL004, respectively. Strain ZL007 having the highest Zwf activity (1.62 U/mg protein) produced 30 mM isobutanol with a yield of 0.3 mol/mol, which were 9 % and 27 % lower than strain ZL006, respectively.

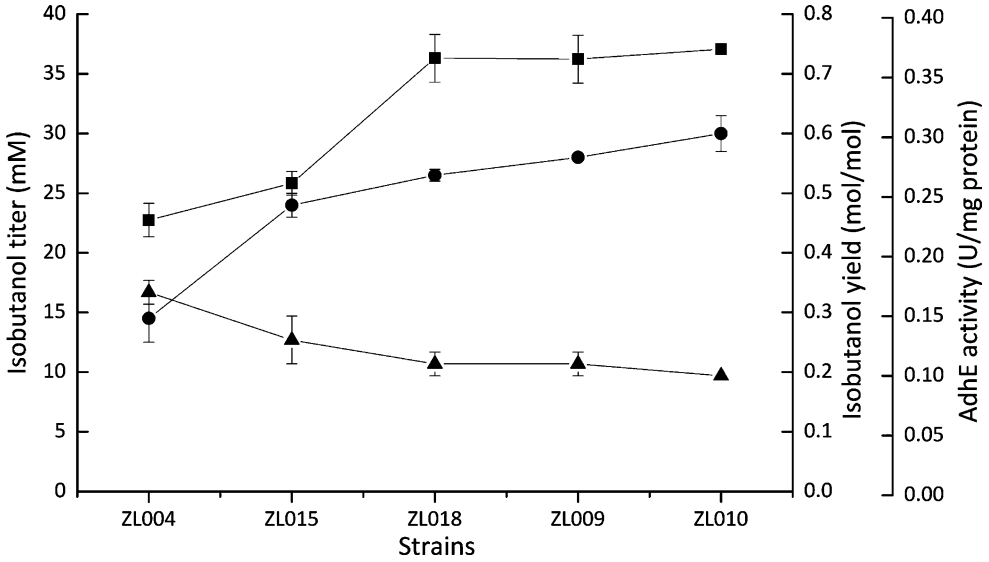

Fig. 3 The relationship between isobutanol production and aldehyde/alcohol dehydrogenase (AdhE) activities. Samples were taken at 48 h for measuring AdhE activities. Samples were taken at 144 h for measuring isobutanol titer and yield. *Squares* represented the isobutanol titer. *Circles* represented the isobutanol yield. *Triangle* represented the AdhE activities

Improving isobutanol production by decreasing AdhE activity

Alcohol dehydrogenase AdhE has been reported to play an important role in the co-production of isobutanol and ethanol under anaerobic conditions [32]. It was suggested that an appropriate AdhE activity was important for improving isobutanol production. A RBS library [11] was constructed for modulation of *adhE* gene in strain ZL004. Four strains (ZL009, ZL010, ZL015 and ZL018) having different RBS sequences (Table 3) and AdhE activities (Fig. 3) were selected for investigating isobutanol production. There was a negative correlation between AdhE activity and isobutanol production (Fig. 3). The best strain ZL010, which had the lowest AdhE activity (0.1 U/mg protein), produced 37 mM isobutanol with a yield of 0.6 mol/mol, which were 61 % and 107 % higher than the parent strain ZL004, respectively.

Improving isobutanol production by modulating *zwf* and *adhE* genes in combination

In order to investigate whether increasing Zwf activity and decreasing AdhE activity had a synergistic effect on improving isobutanol production, the *zwf* and *adhE* genes were modulated in combination. Regulatory parts leading to high, medium and low activities of Zwf and AdhE were selected, and 9 combinations were obtained (Fig. 4).

The highest ethanol titer and yield were obtained in strain ZL004 which had the highest AdhE activity and lowest Zwf activity. The highest isobutanol titer and yield were obtained in strain ZL021 which had the lowest AdhE activity and a medium Zwf activity. Strain ZL021 produced 53 mM isobutanol, which were 61 % higher than strain ZL006 (the best strain when increasing Zwf activity alone) and 43 % higher than strain ZL010 (the best strain when decreasing AdhE activity alone). In addition, isobutanol yield of strain ZL021 was 0.74 mol/mol, which were 80 % and 23 % higher than strain ZL006 and ZL010, respectively. It was thus suggested that increase of NADPH supply and reduction of carbon flux towards ethanol had a synergistic effect on improving isobutanol production. The isobutanol titer and yield of strain ZL021 were 51 % and 12 % higher than strain AS108, respectively. In addition, the total alcohol yield of strain ZL021 was 0.81 mol/mol, which was 23 % higher than strain AS108.

Discussion

Due to its great potential as a next-generation biofuel, isobutanol has attracted special interests in recent years [22]. Production of isobutanol from renewable resources had been realized in several microorganisms, such as *E. coli* and *Saccharomyces cerevisiae* [2, 4, 9]. Comparing to the high isobutanol titer produced by metabolically engineered *E. coli* strains [2, 5, 27], the isobutanol titer obtained by engineered yeast was still low [4, 9, 28]. The main reason was suggested to be associated with the complicated subcellular structure of yeast. The upstream part (from pyruvate to 2-ketoisovalerate) of the biosynthetic pathway was confined to mitochondria [4, 18], while the downstream

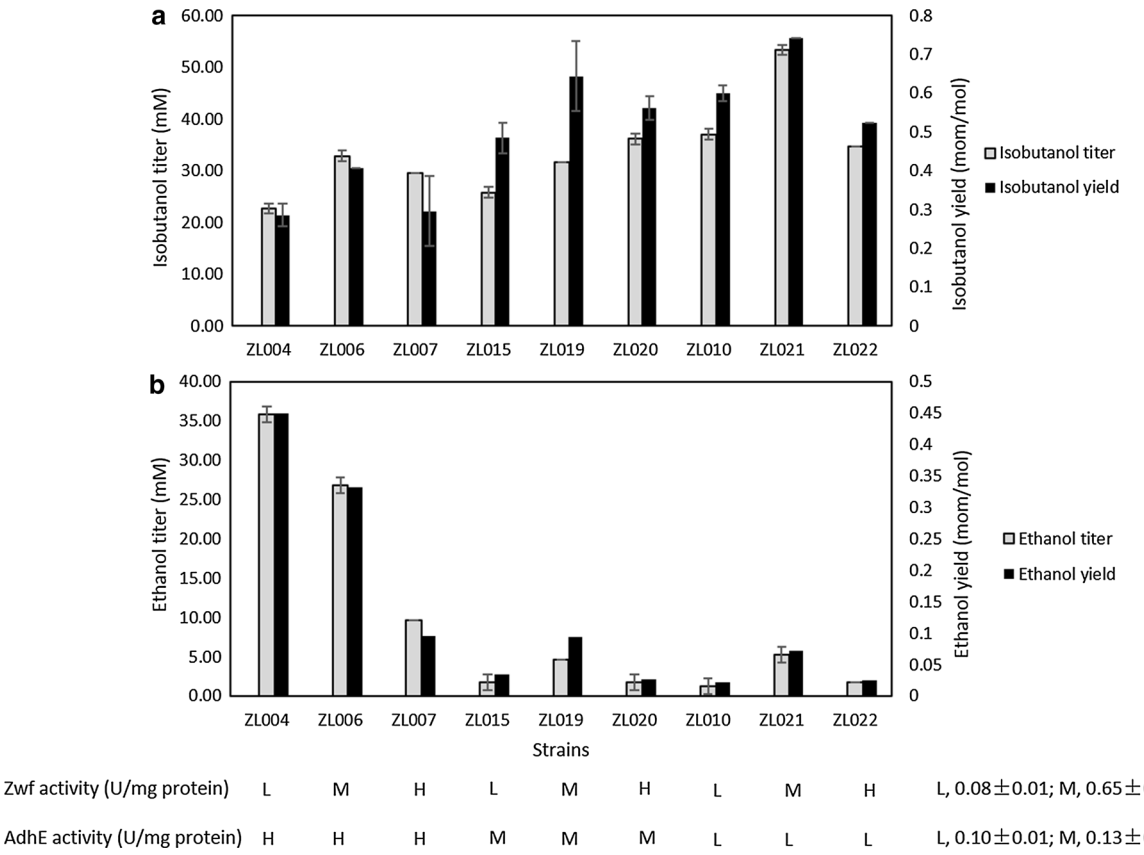

Fig. 4 Improvement of isobutanol production by co-producing ethanol and modulating the *zwf* gene in combination. Regulatory parts leading to high (*H*), medium (*M*), and low (*L*) activities of Zwf and AdhE were selected and nine combinations were obtained. **a** Isobutanol titers and yields of the nine strains. **b** Ethanol titers and yields of the nine strains. *Zwf* glucose 6-phosphate-1-dehydrogenase, *AdhE* aldehyde/alcohol dehydrogenase

part (from 2-ketoisovalerate to isobutanol) was confined to cytoplasm [4, 15]. Isobutanol productivity was greatly reduced by the limitation of intermediates transport between subcellular fractions. In contrast, production of isobutanol was easier in *E. coli* due to its simpler cellular structure and great progress had been achieved for microaerobic isobutanol production [2, 5, 27]. On the other hand, the isobutanol titer obtained by engineered *E. coli* was still limited due to the toxicity of the solvent. *S. cerevisiae* was more tolerant to isobutanol than *E. coli*, and might be more suitable for isobutanol production with high titer.

Although microaerobic isobutanol production in *E. coli* was very efficient [2, 5, 27], anaerobic isobutanol production was much more difficult and redox imbalance problem was the main limitation. NADH was the common reducing equivalent, which was produced through glycolysis, under anaerobic glucose fermentation condition. However, NADPH was the reducing equivalent required for isobutanol production due to the cofactor specificities of keto acid reductoisomerase and alcohol dehydrogenase. Thus, NADH was redundant while NADPH was deficient.

Accumulation of NADH would lead to growth retardation. To solve this problem, an engineering strategy was developed based on computer simulation models [31, 32]. Isobutanol production was forced to combine with other anaerobic pathways for oxidizing the excess NADH, such as pathway for ethanol production. However, the isobutanol yield and molar ratio of isobutanol and ethanol in the resulting strains were lower than the predicted values exhibited by the model analysis. Fermentation results indicated that ethanol was the major product and less carbon flux was directed towards isobutanol synthesis. It was suggested that the NADH-dependent aldehyde/alcohol dehydrogenase AdhE played a key role for isobutanol production. The same problem was found in our work. Recruiting AdhE led to decreased isobutanol production in strain ZL004. Decreasing AdhE activity from 0.17 to 0.1 U/mg protein led to increase of isobutanol yield from 0.26 to 0.6 mol/mol glucose. It was demonstrated that modulation of the AdhE activity was a good strategy to balance the distribution of carbon flux between isobutanol and ethanol pathways.

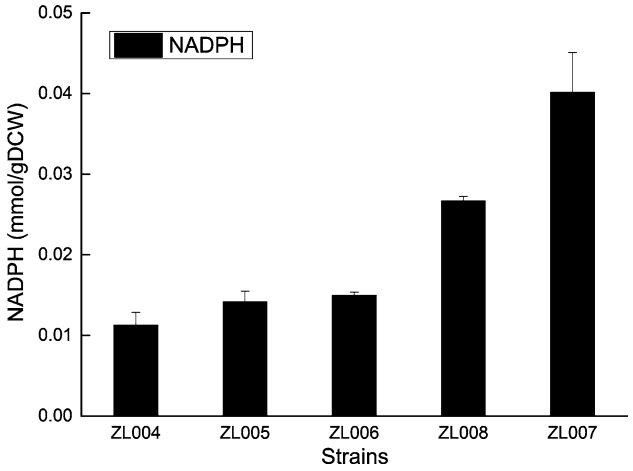

Fig. 5 The intracellular levels of NADPH in strains having the *zwf* gene modulated. Samples were taken at 24 h for measuring the NADPH concentration

Sufficient NADPH supply was another key factor for efficient isobutanol production. Even the NADH-dependent alcohol dehydrogenase from *Lactococcus lactis* was used, one molar NADPH was still needed by the keto acid reductoisomerase to produce one molar isobutanol. NADPH was produced by three major ways including the pentose phosphate pathway, tricarboxylic acid cycle and membrane-bound transhydrogenase PntAB [26]. Several previous studies have been focused on the activation of transhydrogenase PntAB alone or in combination with NAD kinase to convert NADH to NADPH in vivo [5, 7, 12, 27]. Pentose phosphate pathway contributed 35–45 % of NADPH for biosynthesis [5, 26, 27]. It has been reported that the intracellular level of NADPH can be increased by over-expression of the *zwf* gene [19]. In this study, the *zwf* gene was modulated by a RBS library and strains with different Zwf activities were obtained. There was a positive relationship between Zwf activities and intracellular levels of NADPH (Fig. 5). Isobutanol yield increased 43 % in the best strain having *zwf* gene modulated, demonstrating that increasing Zwf activity did increase the carbon flux towards pentose phosphate pathway and increase NADPH supply. It should be noted that excessive Zwf activities had negative effects on isobutanol production (Fig. 2). 6-Phosphoglucono-δ-lactone was the catalytic product of Zwf enzyme and was a toxic intermediate which could react with endogenous nucleophiles [24, 30]. It was suggested that excessive Zwf activity would lead to accumulation of the toxic 6-phosphoglucono-δ-lactone if activities of the downstream enzymes of the pentose phosphate pathway were not increased, thus leading to decreased isobutanol production. It should also be noted that one carbon is lost to carbon dioxide when one glucose is metabolized through the pentose phosphate pathway. The theoretical maximum isobutanol yield would decrease from 1 to 0.83 mol/mol glucose if all the carbon fluxes go through the pentose phosphate pathway.

In summary, a combination strategy was developed in this work to solve both the NADH redundant and NADPH deficient problems during anaerobic isobutanol production in engineered *E. coli* strains. Recruiting ethanol synthetic pathway and modulating AdhE activity was used to solve the NADH redundant problem, while modulating Zwf of the pentose phosphate pathway was used to solve the NADPH deficient problem. Combination of these two strategies had a synergistic effect on improving isobutanol production, leading to 51 % and 12 % increase of the isobutanol titer and yield comparing to the starting strain AS108, respectively.

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