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Activating transhydrogenase and NAD kinase in combination for improving isobutanol production

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ABSTRACT

Isobutanol is an excellent alternative biofuel. Fermentative production of isobutanol had been realized in several microorganisms by combining branched-chain amino acids synthetic pathway and Ehrlich pathway. In contrast to using plasmid overexpression and inducible promoters, genetically stable *Escherichia coli* strains for isobutanol production were constructed in this work by integrating essential genes into chromosome. A chromosome-based markerless gene modulation method was then developed for fine-tuning gene expression with multiple regulatory parts to improve isobutanol production. There was also a cofactor imbalance problem for anaerobic isobutanol synthesis. NADPH is the reducing equivalent required for isobutanol production, while the common reducing equivalent under anaerobic condition is NADH. Two strategies were used to modulate expression of transhydrogenase (*pntAB*) and NAD kinase (*yfjB*) genes to increase NADPH supply for improving isobutanol production. Plasmid overexpression of *pntAB* and *yfjB* genes either individually or in combination had little effect on isobutanol production. In contrast, modulating *pntAB* and *yfjB* gene expression in chromosome with multiple regulatory parts identified optimal modulators under aerobic and anaerobic conditions, respectively, and improved isobutanol production. Modulating *pntAB* gene alone led to 20% and 8% increase of anaerobic isobutanol titer and yield. Although modulating *yfjB* gene alone had nearly no effect, modulating *pntAB* and *yfjB* genes in combination led to 50% and 30% increase of isobutanol titer and yield in comparison with modulating *pntAB* gene alone. It was also found that increasing *pntAB* gene expression alone had a threshold for improving anaerobic isobutanol production, while activating NAD kinase could break through this threshold, leading to a yield of 0.92 mol/mol. Our results suggested that transhydrogenase and NAD kinase had a synergistic effect on increasing NADPH supply and improving anaerobic isobutanol production. This strategy will be useful for improving production of target compounds using NADPH as reducing equivalent within their synthetic pathways. In addition, combined activation of PntAB and YfjB led to 28% and 22% increase of aerobic isobutanol titer and yield, resulting in production of 10.8 g/L isobutanol in 24 h with a yield of 0.62 mol/mol.

1. Introduction

Although ethanol has been the most widely used biofuel, this compound has several severe problems as an alternative to gasoline, such as low energy density and high hygroscopicity leading to storage and transportation problems (Atsumi et al., 2008b). In contrast, higher-chain alcohols are better than ethanol because they (1) have higher energy densities similar to gasoline, (2) can be transported using current petroleum pipelines and (3) have a low hygroscopicity and vapor pressure so that they are compatible with existing engines and can be mixed with gasoline in high proportion (Atsumi et al., 2008a, 2008b; Carter et al., 2012; Connor and Liao, 2009; Lan and Liao, 2011; Nicolaou et al., 2010; Reyes et al., 2012; Shen and Liao, 2008). Isobutanol is a representative higher-chain alcohol. Except for being used as biofuel, isobutanol also has many industrial applications, such as solvent, paint additives and ink ingredient. It can also be dehydrated to produce butenes, which is a bulk chemical used for production of synthetic rubber (Wang et al., 2012).

A synthetic pathway for isobutanol production from glucose had been created by combining branched-chain amino acids synthetic pathway and Ehrlich pathway with 2-keto-isovalerate serving as a precursor (Atsumi et al., 2008b; Smith and Liao, 2011; Fig. 1). Pyruvate was converted to 2-keto-isovalerate by acetolactate synthase, keto-acid reductoisomerase (IlvC) and dihydroxy-acid dehydratase (IlvD). 2-keto-isovalerate was then

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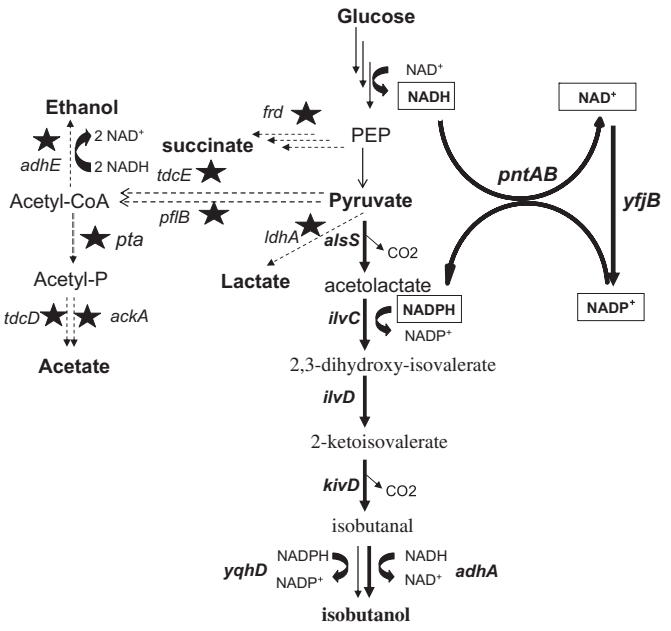

Fig. 1. Isobutanol synthetic pathway constructed in *Escherichia coli* (Atsumi et al., 2008b). NADH and $NADP^+$ were converted to NADPH and NAD^+ by transhydrogenase PntAB and $NADP^+$ was regenerated by NAD kinase YfjB. Genes and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate-lyase; *frd*, fumarate reductase; *ackA*, acetate kinase; *pta*, phosphate acetyltransferase; *adhE*, alcohol dehydrogenase; *tdcE*, 2-ketobutyrate formate-lyase/pyruvate formate-lyase; *tdcD*, propionate kinase; *alsS*, acetolactate synthase of *Bacillus subtilis*; *ilvC*, ketol-acid reductoisomerase; *ilvD*, dihydroxy-acid dehydratase; *kivd*, 2-keto acid decarboxylase; *adhA*, alcohol dehydrogenase of *Lactococcus lactis*; *yqhD*, alcohol dehydrogenase of *E. coli*; *pntAB*, membrane-bound transhydrogenase; *yfjB*, NAD kinase.

converted to isobutanol by 2-keto acid decarboxylase (KivD) and alcohol dehydrogenase (Fig. 1). By inactivating native fermentation pathways competing for pyruvate, the engineered *Escherichia coli* strain produced about 22 g/L isobutanol within 112 h under a micro-aerobic condition (Atsumi et al., 2008b). Isobutanol production was also reached in other organisms, such as *Corynebacterium glutamicum* (Smith et al., 2010; Blombach et al., 2011), *Synechococcus elongatus* (Atsumi et al., 2009) and *Bacillus subtilis* (Li et al., 2011).

Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is the reducing equivalent required for production of isobutanol (Fig. 1). Both keto-acid reductoisomerase and alcohol dehydrogenase are NADPH-dependent, and two equivalents of NADPH are required for conversion of pyruvate to isobutanol. Although NADH-dependent alcohol dehydrogenase, such as AdhA of *Lactococcus lactis*, can be recruited to reduce NADPH dependence (Atsumi et al., 2010), one equivalent of NADPH is still required for isobutanol synthesis.

There are three major ways for generation of NADPH: pentose phosphate pathway (PPP), tricarboxylic acid cycle (TCA) and membrane-bound transhydrogenase (PntAB) (Sauer et al., 2004). During aerobic conditions, 35–45% of NADPH that is required for biosynthesis was produced via PntAB, whereas PPP and TCA cycle contributed 35–45% and 20–25%, respectively (Sauer et al., 2004). Most of previous work used aerobic conditions for isobutanol production (Atsumi et al., 2008b; Baez et al., 2011; Smith et al., 2010; Blombach et al., 2011). PPP and TCA cycle are usually not functional under anaerobic condition (Bastian et al., 2011), and the only source for NADPH is transhydrogenase. In contrast, the common reducing equivalent under anaerobic condition is NADH, which is produced through glycolysis (Bastian et al., 2011). This cofactor imbalance problem needs to be solved for efficient isobutanol production. Over-expressing *pntAB* gene had been used to increase NADPH supply for improving isobutanol production either in *E. coli* (Bastian et al., 2011) or in *C. glutamicum* (Blombach et al., 2011).

PntAB is a membrane-bound proton translocating pyridine nucleotide transhydrogenase which transfers a hydride from NADH to $NADP^+$ with the concurrent production of NADPH and NAD^+, powered by the proton motive force (Clarke and Bragg, 1985; Sauer et al., 2004). In order to recycle NAD^+ for continuous transhydrogenation, NAD^+ needs to be converted to $NADP^+$ (Fig. 1). In this work, NAD kinase, which catalyzes phosphorylation of NAD^+ to $NADP^+$, was activated together with transhydrogenase, leading to a synergestic effect on increasing NADPH supply and improving anaerobic isobutanol production.

2. Materials and methods

2.1. Strains, media, and growth conditions

Strains used in this study were listed in Table 1. During strain construction, cultures were grown aerobically at 30 °C, 37 °C, or 39 °C in Luria broth (per liter: 10 g Difco tryptone, 5 g Difco yeast extract. and 10 g NaCl) containing 20 g glucose/L. Ampicillin (100 mg L^{-1}), kanamycin (25 mg L^{-1}), and chloramphenicol (17 mg L^{-1}) were used where appropriate.

2.2. Plasmids construction

2.2.1. Plasmids for gene deletion

The *ldhA* gene and neighboring 800 bp were amplified from *E. coli* ATCC8739 with primer set XZ-ldhA-up/XZ-ldhA-down and cloned into the pEASY-Blunt vector to obtain plasmid pXZ001 (Table S1). This plasmid DNA was served as a template for inside-out amplification with primer set XZ-ldhA-1/XZ-ldhA-2. The resulting 4800 bp DNA fragment was ligated with *cat-sacB* cassette, which was amplified from pLOI4162 (Jantama et al., 2008) using primer set cat-sacB-up/down, to obtain plasmid pXZ002 (Table S1). This 4800 bp DNA fragment was also treated by T4 polynucleotide kinase (New England Biolabs) and self-ligated to obtain plasmid pXZ003 (Table S1). PCR fragments amplified from pXZ002 and pXZ003 with primer set XZ-ldhA-up/down were used to delete *ldhA* gene by the two-step recombination method described previously (Jantama et al., 2008; Zhang et al., 2007). Plasmids for deletion of pyruvate formate-lyase (*pflB*), fumarate reductase (*frd*), acetate kinase (*ackA*), phosphate acetyltransferase (*pta*), alcohol dehydrogenase (*adhE*), 2-ketobutyrate formate-lyase/pyruvate formate-lyase (*tdcE*), propionate kinase (encoded by *tdcD*) and methylglyoxal synthase (*mgsA*) genes were constructed in a same manner. All primers were listed in Table S2, and plasmids constructed were listed in Table S1.

2.2.2. Plasmids for gene integration

Plasmid pXZ618 was constructed for integration of *kivD-ilvD* operon at *pflB* site. The *kivD* gene was amplified from *L. lactis* IL1403 with primer set kivD-F-KpnI/R-XbaI and digested by KpnI and XbaI. The *ilvD* gene was amplified from *E. coli* MG1655 with primer set ilvD-F-XbaI/R-SalI and digested by XbaI. A mutant of *E. coli pck* promoter (*pck**, containing a G to A transition at position −64 relative to the ATG start codon) was constructed as described previously (Zhang et al., 2009a) and digested by KpnI. These three DNA fragments were ligated together by Quick Ligase (New England Biolabs), and ligated with a DNA fragment amplified from plasmid pXZ015 with primer set XZ-pflB-1/XZ-pflB-2, resulting in plasmid pXZ618.

Plasmid pXZ619 was constructed for integration of *adhA* gene at *frd* site. The *adhA* gene was amplified from *L. lactis* IL1403 with

Table 1
Strains used in this work.

Strains	Relevant characteristic[a]	Sources
E. coli ATCC 8739	Wild type	Zhang et al. (2009b)
E. coli MG1655	Wild type	Lab collection
Lactococus lactis IL1403	Wild type	Lab collection
Bacillus subtilis 168	Wild type	Lab collection
XZ-T010	△*ldhA*, △*pflB*, △*frd*, △*ackA-pta*	This work
AS6	XZ-T010, *pflB::pck*-kivD-ilvD*	This work
AS18	AS6, *frd::pck*-adhA*	This work
AS29	AS18, *pflB::M1-93-kivD-ilvD*	This work
AS74	AS29, △*adhE*, △*tdcDE*	This work
AS75	AS74, *tdcDE::alsS*	This work
AS77	AS74, *tdcDE::M1-93-alsS*	This work
AS78	AS74, *tdcDE::M1-37-alsS*	This work
AS79	AS74, *tdcDE::M1-46-alsS*	This work
AS80	AS74, *tdcDE::M1-30-alsS*	This work
AS81	AS74, *tdcDE::M1-64-alsS*	This work
AS82	AS74, *tdcDE::M1-12-alsS*	This work
AS83	AS74, *ldhA::alsS*	This work
AS84	AS74, *ldhA::M1-93-alsS*	This work
AS85	AS74, *ldhA::M1-37-alsS*	This work
AS86	AS74, *ldhA::M1-46-alsS*	This work
AS87	AS74, *ldhA::M1-30-alsS*	This work
AS88	AS74, *ldhA::M1-64-alsS*	This work
AS89	AS74, *ldhA::M1-12-alsS*	This work
AS105	AS77, *ldhA::M1-64-alsS*	This work
AS106	AS105, *mgsA::M1-93-ilvC*	This work
AS107	AS105, *mgsA::M1-37-ilvC*	This work
AS108	AS105, *mgsA::M1-46-ilvC*	This work
AS109	AS105, *mgsA::M1-30-ilvC*	This work
AS142	AS108, *M1-93-pntAB*	This work
AS143	AS108, *M1-37-pntAB*	This work
AS144	AS108, *M1-46-pntAB*	This work
AS145	AS108, *M1-30-pntAB*	This work
AS147	AS108, *M1-37-yfjB*	This work
AS148	AS108, *M1-46-yfjB*	This work
AS149	AS108, *M1-30-yfjB*	This work
AS165	AS108, *M1-93-pntAB, M1-37-yfjB*	This work
AS166	AS108, *M1-93-pntAB, M1-46-yfjB*	This work
AS167	AS108, *M1-93-pntAB, M1-30-yfjB*	This work
AS172	AS108, *M1-30-pntAB, M1-37-yfjB*	This work
AS173	AS108, *M1-30-pntAB, M1-46-yfjB*	This work
AS174	AS108, *M1-30-pntAB, M1-30-yfjB*	This work
AS225	AS108, *M1-46-pntAB, M1-37-yfjB*	This work
AS226	AS108, *M1-46-pntAB, M1-30-yfjB*	This work

[a] The abbreviation *pck** represents a mutant of *E. coli pck* promoter (G to A at −64 relative to the ATG start) (Zhang et al., 2009a). M1-93, M1-37, M1-46, M1-30, M1-64 and M1-12 were artificial regulatory parts constructed previously, which strengths were 5, 2.5, 1.7, 0.8, 0.4 and 0.1 times of induced *E. coli lacZ* promoter (Lu et al., 2012).

primer set adhA-F-XbaI/R-SalI and digested by XbaI. A mutant of *E. coli pck* promoter (*pck**) was constructed as described previously (Zhang et al., 2009a) and digested by XbaI. These two DNA fragments were ligated together by Quick Ligase (New England Biolabs), and ligated with a DNA fragment amplified from plasmid pXZ005 with primer set XZ-frdC-1/XZ-frdB-2, resulting in plasmid pXZ619.

For integration of *alsS* gene at *ldhA* site, the *alsS* gene was amplified from *B. subtilis* using primer set alsS-F-SacI/R-BamHI and ligated with a DNA fragment amplified from plasmid pXZ001 with primer set XZ-ldhA-1/XZ-ldhA-2, resulting in plasmid pXZ664. Plasmids for integration of *alsS* gene at *tdcDE* site and integration of *ilvC* gene at *mgsA* site were constructed in a same manner.

2.2.3. Plasmids for gene overexpression

Plasmid pACYC184-M was constructed for gene expression. DNA fragment 1 was amplified from pTrc99A using primer set 99A-F1-PacI-SpeI-NdeI/99A-R1-PacI. This fragment was further digested by DpnI and phosphorylated by T4 polynucleotide kinase (NEB) to provide a phosphorylated end for ligation. DNA fragment 2 was amplified from pACYC184 using primer set 184-F2/184-R2, digested by DpnI and ligated with phosphorylated DNA fragment 1, resulting in pACYC184-M.

The *pntAB* gene was amplified from *E. coli* MG1655 with primer set *pntAB*-F-BamHI/R-PstI, digested by *Bam*HI and *Pst*I, and cloned into pACYC184M at *Bam*HI and *Pst*I sites, resulting in pXZ604. The *yfjB* gene was amplified from *E. coli* MG1655 with primer set yfjB-F-SacI/R-BamHI, digested by *Sac*I and *Bam*HI, and cloned into pACYC184M at *Sac*I and *Bam*HI sites, resulting in pXZ606. Two DNA fragments containing digested *pntAB* and *yfjB* genes were cloned together into pACYC184M at *Pst*I and *Sac*I sites, resulting in pXZ607.

The *alsS* gene was amplified from *B. subtilis* 168 with primer set alsS-F-SacI/R-BamHI, and digested by *Sac*I and *Bam*HI. The *ilvC* gene was amplified from *E. coli* MG1655 with primer set ilvC-F-BamHI/R-PstI, and digested by *Bam*HI and *Pst*I. These two DNA fragments were then cloned into pACYC184M at *Sac*I and *Pst*I sites, resulting in plasmid pXZ116.

The *kivD* gene was amplified from *L. lactis IL1403* with primer set kivD-F-KpnI/R-XbaI, and digested by *Kpn*I and *Xba*I. The *ilvD* gene was amplified from *E. coli* MG1655 with primer set ilvD-F-XbaI/R-SalI, and digested by *Xba*I and *Sal*I. These two DNA fragments were then cloned into pTrc99A at *Kpn*I and *Sal*I sites, resulting in pXZ112. This plasmid was digested by *Sal*I and treated with T4 DNA polymerase to produce a blunt end, which was then ligated with a DNA fragment containing *adhA* gene amplified from *L. lactis IL1403* with primer set adhA-F-XbaI/R-SalI, resulting in plasmid pXZ605.

2.3. Gene deletion, integration and modulation

Gene deletion and gene integration were done seamlessly without leaving any foreign DNA as described previously (Zhang et al., 2007; Jantama et al., 2008).

Artificial regulatory parts were used for modulation of gene expression directly in chromosome. These regulatory parts were selected from a previously constructed mRNA stabilizing region (mRS) library (Lu et al., 2012), which had the same promoter (P2-15) and RBS (*E. coli lacZ*) sequence and differed in the sequence between promoter and RBS region (Fig. S1). Strengths of regulatory parts M1-93, M1-37, M1-46, M1-30, M1-64 and M1-12 were 5, 2.5, 1.7, 0.8, 0.4 and 0.1 times of induced *E. coli lacZ* promoter when cultivated in LB medium.

A two-step recombination method was developed for markerless modulation of *kivD-ilvD* operon (Fig. 4). In the first recombination, *cat-sacB* cassette was amplified with primer set XZ-pflB-up/kivD-sacB-down and used to replace *pck** promoter of *kivD-ilvD* operon in strain AS18. In the second recombination, artificial regulatory parts M1-93 was amplified with a primer set pflB-up-P/kivD-RBS-down and used to replace *cat-sacB* cassette by selection for resistance to sucrose. Cells containing *sacB* gene accumulate levan during incubation with sucrose and are killed. Surviving recombinants are highly enriched for loss of *cat-sacB* cassette (Jantama et al., 2008; Zhang et al., 2007). The *alsS* and *ilvC* genes were modulated in a same manner.

For *pntAB* and *yfjB* genes, their expressions were modulated with different regulatory parts by a one-step recombination method as described previously (Lu et al., 2012).

Red recombinase technology (Gene Bridges GmbH, Dresden, Germany) was used to facilitate chromosomal gene deletion, integration and modulation (Datsenko and Wanner, 2000).

2.4. Real-time PCR analysis of pntAB gene expression

Total RNA was prepared from cells harvested during mid-log phase of growth using RNeasy Plant Mini Kit (QIAGEN) and digestion with DNaseI (QIAGEN). cDNA was prepared with RevertAid Premium First Strand cDNA Synthesis Kit (Thermo Scientific, USA) using 500 ng total RNA as template. Samples were analyzed using Bio-Rad CFX Connect Real Time System with iQ SYBR Green Supermix RT-PCR Kit (Bio-Rad Laboratories, Hercules, CA). Primer sets (Table S2) used for real-time PCR were designed by Beacon Design 7.0 software (PREMIER Biosoft International). 16S rRNA gene fragments were amplified as an internal control and three biological replicates were performed. Relative gene expression was calculated using the comparative critical threshold cycle (ΔΔCT) method with PCR efficiency (Livak and Schmittgen, 2001).

2.5. Fermentation

Single colonies were picked from plates and inoculated into 250 ml flasks containing 50 ml NBS (New Brunswick Scientific) mineral salts medium (Zhang et al., 2009b) with 5% glucose, and grown at 37 °C and 200 rpm for 12 h. For aerobic fermentation, seed culture was inoculated into 250 mL flasks containing 50 mL NBS medium with 5% glucose and 100 mM MOPS, and grown at 37 °C and 250 rpm for 24 h. For anaerobic fermentation, seed culture was inoculated into 100 mL screw cap bottles containing 50 mL NBS medium with 5% glucose and 100 mM MOPS, and grown at 37 °C without shaking for 6 days. For cultivating in rich medium, 10 g/L yeast extract was added into the NBS medium. For a two-stage fermentation process, strains were first cultivated aerobically in NBS medium to an OD550 of 3.0–5.0, and then shifting to anaerobic conditions by flushing with oxygen-free atmosphere in a 100 mL screw cap bottles. The cultures were then incubated without shaking at 37 °C, and were swirled twice a day. Samples were taken at the time of the anaerobic shift and 24 h after induction. For each strain, three independent fermentations were performed.

2.6. Analysis

Cell mass was estimated by measuring the optical density at 550 and 600 nm. Isobutanol was analyzed by Agilent 1200 high performance liquid chromatography with Aminex HPX-87H column using a refractive index detector (Agilent Technologies Inc., Santa Clara, CA, USA). Internal standard was added for verification of isobutanol concentration. The concentration of glucose and other fermentation products were also measured by HPLC with Aminex HPX-87H column.

3. Result

3.1. Construction of strain AS108 for isobutanol production

The native fermentation pathways, including lactate dehydrogenase, pyruvate formate-lyase, fumarate reductase, acetate kinase and phosphate acetyltransferase, which compete for pyruvate with isobutanol synthetic pathway, were first inactivated in *E. coli* ATCC 8739, resulting in strain XZ-T010. The *kivD-ilvD* operon, together with a mutant *E. coli pck* promoter (*pck**), were integrated into chromosome of strain XZ-T010 at *pflB* site, resulting in strain AS6. The *adhA* gene of *L. lactis*, together with *pck** promoter, were further integrated into AS6 at *frd* site, resulting in strain AS18 (Table 1, Fig. 2). This strain produced 3.7 mM isobutanol with a yield of 0.04 mol/mol under aerobic condition,

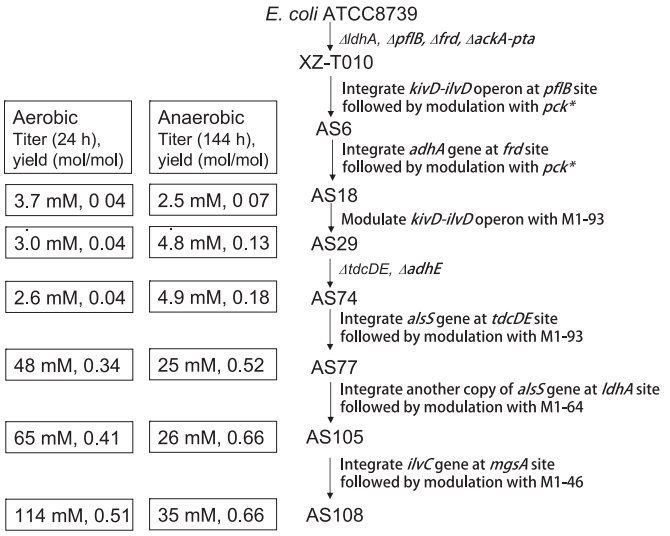

Fig. 2. Diagram summarizing steps in the metabolic engineering of *E. coli* for isobutanol production.

and produced 2.5 mM isobutanol with a yield of 0.07 mol/mol under anaerobic condition (Fig. 2).

In order to improve isobutanol production, the expression of *kivD-ilvD* operon was modulated with a previously constructed regulatory part M1-93. The resulting strain AS29 produced 3.0 mM isobutanol with a yield of 0.04 mol/mol under aerobic condition, and produced 4.8 mM isobutanol with a yield of 0.13 mol/mol under anaerobic condition (Fig. 2).

Although pyruvate formate-lyase, acetate kinase and phosphate acetyltransferase were inactivated, acetate and ethanol still accumulated in strain AS29. It was assumed that 2-ketobutyrate formate-lyase/pyruvate formate-lyase (encoded by *tdcE*) was activated, which converted pyruvate to acetyl-CoA. Acetyl-CoA could be converted either to ethanol by alcohol dehydrogenase (encoded by *adhE*), or to acetate by an alternative phosphate acetyltransferase (encoded by *eutD*) and propionate kinase (encoded by *tdcD*). In order to eliminate acetate and ethanol production, *tdcDE* and *adhE* genes were deleted in AS29. The resulting strain AS74 produced 2.6 mM isobutanol with a yield of 0.04 mol/mol under aerobic condition, and produced 4.9 mM isobutanol with a yield of 0.18 mol/mol under anaerobic condition (Fig. 2).

Acetolactate synthase (AHAS) was reported as the rate-limiting step during valine synthesis in *E. coli* (Park et al., 2007, 2010). In order to increase AHAS activity, *B. subtilis alsS* gene was integrated into AS74 at *tdcDE* site, followed by modulation of its expression with 6 regulatory parts (M1-93, M1-37, M1-46, M1-30, M1-64 and M1-12), resulting in strains AS77 to AS82 (Table 1). AS77, having *alsS* gene modulated by M1-93, had the highest isobutanol titer among all modulated strains. This strain produced 48 mM isobutanol with a yield of 0.34 mol/mol under aerobic condition, and produced 25 mM isobutanol with a yield of 0.52 mol/mol under anaerobic condition (Fig. 2). Both titer and yield increased significantly after utilizing AlsS, suggesting that conversion of pyruvate to acetolactate was the rate-limiting step for isobutanol synthesis. In order to further increase AHAS activity, a second copy of *alsS* gene together with regulatory part M1-64 were integrated into AS77 at *ldhA* site, resulting in strain AS105 (Table 1). This strain produced 65 mM isobutanol with a yield of 0.41 mol/mol under aerobic condition, and produced 26 mM isobutanol with a yield of 0.66 mol/mol under anaerobic condition (Fig. 2).

Keto-acid reductoisomerase (IlvC) catalyzes the first reducing reaction in the isobutanol synthetic pathway. In order to investigate whether increasing IlvC activity could improve isobutanol production, the *ilvC* gene was integrated into AS105 at *mgsA* site followed by modulation of its expression with 4 regulatory parts (M1-93, M1-37, M1-46 and M1-30), resulting in strains AS106 to AS109 (Table 1). AS108, having *ilvC* gene modulated with M1-46, had the highest isobutanol titer among all modulated strains. This strain produced 114 mM isobutanol with a yield of 0.51 mol/mol under aerobic condition, and produced 35 mM isobutanol with a yield of 0.66 mol/mol under anaerobic condition (Fig. 2).

3.2. Activating transhydrogenase and NAD kinase through plasmid overexpression for improving isobutanol production

Plasmid overexpression was first used to increase transhydrogenase and NAD kinase activities for improving isobutanol production. Under anaerobic condition, overexpression of *pntAB* and *yfjB* genes individually decreased isobutanol titer by 15% and 4%, while isobutanol yield increased 5% and 12%, respectively (Table 2). Overexpression of *pntAB* and *yfjB* genes in combination decreased isobutanol titer and yield by 15% and 9%, respectively (Table 2). Under aerobic condition, overexpression of *pntAB* and

Table 2
Production of isobutanol by *E. coli* strains having *pntAB* and *yfjB* genes modulated under anaerobic condition.

Strain[a]	Genetic modification	OD_{550}	Glucose used (mM)	Titer (mM)	Improvement [b]	Yield (mol/mol)	Improvement [b]
Plasmid expression							
AS108	pACYC184M	0.36	41 ± 4	26 ± 1	1	0.63 ± 0.01	1
AS108	pACYC184M-*pntAB*	0.3	33 ± 1	22 ± 1	−0.15	0.66 ± 0.02	0.05
AS108	pACYC184M-*yfjB*	0.36	36 ± 3	25 ± 0	−0.04	0.70 ± 0.02	0.12
AS108	pACYC184-*yfjB-pntAB*	0.27	37 ± 3	22 ± 1	−0.15	0.60 ± 0.03	−0.09
Chromosome modulation							
AS108		0.5	53 ± 3	35 ± 2	1	0.66 ± 0.01	1
Modulation of *pntAB*							
AS142	*M1-93-pntAB*	0.63	58 ± 0	40 ± 1	0.14	0.69 ± 0.00	0.05
AS143	*M1-37-pntAB*	0.55	59 ± 2	41 ± 1	0.17	0.69 ± 0.01	0.05
AS144	*M1-46-pntAB*	0.4	37 ± 3	23 ± 2	−0.34	0.62 ± 0.01	−0.06
AS145	*M1-30-pntAB*	0.51	59 ± 1	42 ± 1	0.2	0.71 ± 0.00	0.08
Modulation of *yfjB*							
AS147	*M1-37-yfjB*	0.53	49 ± 1	34 ± 1	−0.03	0.70 ± 0.01	0.06
AS148	*M1-46-yfjB*	0.54	51 ± 1	36 ± 1	0.03	0.70 ± 0.01	0.06
AS149	*M1-30-yfjB*	0.52	45 ± 3	34 ± 1	−0.03	0.76 ± 0.01	0.15
Combinatorial modulation of *pntAB* and *yfjB* genes							
AS165	*M1-93-pntAB, M1-37-yfjB*	0.75	76 ± 3	62 ± 1	0.77	0.82 ± 0.00	0.24
AS166	*M1-93-pntAB, M1-46-yfjB*	0.72	68 ± 1	63 ± 1	0.8	0.92 ± 0.00	0.39
AS167	*M1-93-pntAB, M1-30-yfjB*	0.7	80 ± 2	66 ± 1	0.89	0.83 ± 0.00	0.26
AS172	*M1-30-pntAB, M1-37-yfjB*	0.69	71 ± 2	44 ± 0	0.26	0.62 ± 0.01	−0.06
AS173	*M1-30-pntAB, M1-46-yfjB*	0.68	75 ± 1	50 ± 1	0.43	0.67 ± 0.01	0.02
AS174	*M1-30-pntAB, M1-30-yfjB*	0.69	69 ± 4	44 ± 1	0.26	0.64 ± 0.01	−0.03

[a] Strains were cultivated in 100 mL screw cap bottles containing 50 mL NBS medium with 5% glucose and 100 mM MOPS, and grown at 37 °C without shaking for 6 days.
[b] Improvement of titer and yield was calculated by comparing with parent strain AS108.

Table 3
Production of isobutanol by *E. coli* strains having *pntAB* and *yfjB* genes modulated under aerobic condition.

Strain[a]	Genetic modification	OD_{550}	Glucose used (mM)	Titer (mM)	Improvement [b]	Yield (mol/mol)	Improvement [b]
Plasmid expression							
AS108	pACYC184M	4.1	172 ± 1	81 ± 0	1	0.47 ± 0.00	1
AS108	pACYC184M-*pntAB*	4.01	165 ± 1	78 ± 1	−0.04	0.47 ± 0.01	0
AS108	pACYC184M-*yfjB*	4.19	163 ± 2	80 ± 1	−0.01	0.49 ± 0.01	0.04
AS108	pACYC184-*yfjB-pntAB*	3.98	170 ± 0	80 ± 0	−0.01	0.47 ± 0.00	0
Chromosome integrated							
AS108		8.75	224 ± 2	114 ± 1	1	0.51 ± 0.01	1
Modulation of *pntAB*							
AS142	*M1-93-pntAB*	8.9	238 ± 1	112 ± 1	−0.02	0.47 ± 0.00	−0.08
AS143	*M1-37-pntAB*	8.5	236 ± 0	111 ± 2	−0.03	0.47 ± 0.01	−0.08
AS144	*M1-46-pntAB*	8.62	238 ± 3	138 ± 2	0.21	0.58 ± 0.01	0.14
AS145	*M1-30-pntAB*	8	240 ± 2	115 ± 2	0.01	0.48 ± 0.00	−0.06
Modulation of *yfjB*							
AS147	*M1-37-yfjB*	8.51	240 ± 1	120 ± 2	0.05	0.5 ± 0.00	−0.02
AS148	*M1-46-yfjB*	8.56	238 ± 2	112 ± 4	−0.02	0.47 ± 0.01	−0.08
AS149	*M1-30-yfjB*	8.77	240 ± 0	120 ± 1	0.05	0.5 ± 0.00	−0.02
Combinatorial modulation of *pntAB* and *yfjB* genes							
AS225	*M1-46-pntAB, M1-37-yfjB*	8.52	235 ± 1	146 ± 2	0.28	0.62 ± 0.00	0.22
AS226	*M1-46-pntAB, M1-30-yfjB*	8.5	235 ± 3	146 ± 2	0.28	0.62 ± 0.00	0.22

[a] Strains were cultivated in 250 mL flasks containing 50 mL NBS medium with 5% glucose and 100 mM MOPS, and grown at 37 °C and 250 rpm for 24 h.
[b] Improvement of titer and yield was calculated by comparing with parent strain AS108.

yfjB genes either individually or in combination had nearly no effects on isobutanol titer and yield (Table 3).

3.3. Modulating pntAB gene in chromosome for improving isobutanol production

The *pntAB* gene of strain AS108 was modulated with 4 regulatory parts (M1-93, M1-37, M1-46 and M1-30), resulting in strains AS142 to AS145 (Table 1). Under anaerobic condition, similar improvement of isobutanol titer (14% to 20%) and yield (5% and 8%) were obtained in all modulated strains except AS144 which was modulated with M1-46 (Table 2). Titer and yield decreased 34% and 6% in strain AS144. The *pntAB* gene expression levels of the modulated strains were compared with parent strain AS108 through RT-PCR analysis. M1-93 led to the biggest increase of expression level (40-fold). M1-37 and M1-30 led to 25-fold increase, while M1-46 led to the smallest increase of expression level (20-fold) (Fig. 5A).

Under aerobic condition, isobutanol titer and yield decreased in all modulated strains except AS144 (Table 3). Titer and yield increased 21% and 14% in strain AS144. M1-46 led to the biggest increase of expression level (450-fold), while other regulatory parts led to 70 to 150-fold increase (Fig. 5B).

3.4. Modulating yfjB gene in chromosome for improving isobutanol production

The *yfjB* gene of strain AS108 was modulated with 3 regulatory parts (M1-37, M1-46 and M1-30), resulting in strains AS147 to AS149 (Table 1). Under anaerobic condition, strains with *yfjB* gene modulated had nearly the same isobutanol titer with the parent strain (from −3% to 3%), while yield increased in all modulated strains from 6% to 15% (Table 2). Under aerobic conditions, isobutanol titer increased 5% when modulated with M1-37 and M1-30, while yield decreased 2% to 8% in all modulated strains (Table 3).

3.5. Modulating pntAB and yfjB genes in combination for improving isobutanol production

In order to investigate whether transhydrogenase and NAD kinase had a synergistic effect on improving isobutanol production, *yfjB* gene was further modulated in strains having activated transhydrogenase. The *yfjB* gene of strain AS142 was modulated with 3 regulatory parts (M1-37, M1-46 and M1-30), resulting in strains AS165 to AS167 (Table 1). Under anaerobic condition, isobutanol titer and yield increased in all 3 modulated strains compared to parent strain AS142 (Table 2). The best strain AS166 produced 63 mM isobutanol with a yield of 0.92 mol/mol. Titer was 50% higher than parent strain AS142, and 80% higher than un-modulated strain AS108 (Table 2; Fig. 3). Yield was 30% higher than parent strain AS142, and 39% higher than un-modulated strain AS108 (Table 2; Fig. 3). The *yfjB* gene of strain AS145 was also modulated with these 3 regulatory parts, resulting in strains AS172 to AS174 (Table 1). Isobutanol titer and yield were all lower than AS142 derived strains (Table 2).

Under aerobic condition, only modulation of *pntAB* with M1-46 (AS144) resulted in improved isobutanol production. Thus, *yfjB* gene of strain AS144 was modulated with two regulatory parts (M1-37 and M1-30), resulting in strains AS225 and AS226 (Table 1). Isobutanol titer and yield increased in both modulated strains compared to parent strains AS144 (Table 3). The best strain AS226 produced 146 mM isobutanol with a yield of 0.62 mol/mol (Table 3). Titer was 6% higher than parent strain AS144, and 28% higher than un-modulated strain AS108 (Table 3; Fig. 3). Yield was 7% higher than parent strain AS144, and 22% higher than un-modulated strain AS108 (Table 3; Fig. 3).

3.6. Comparison of isobutanol production with different fermentation conditions

Strains AS108, AS226 and AS166 were cultivated in rich medium to investigate the effect of different culture medium on isobutanol production. Under aerobic condition, AS108 produced 43 mM isobutanol with a yield of 0.22 mol/mol in rich medium.

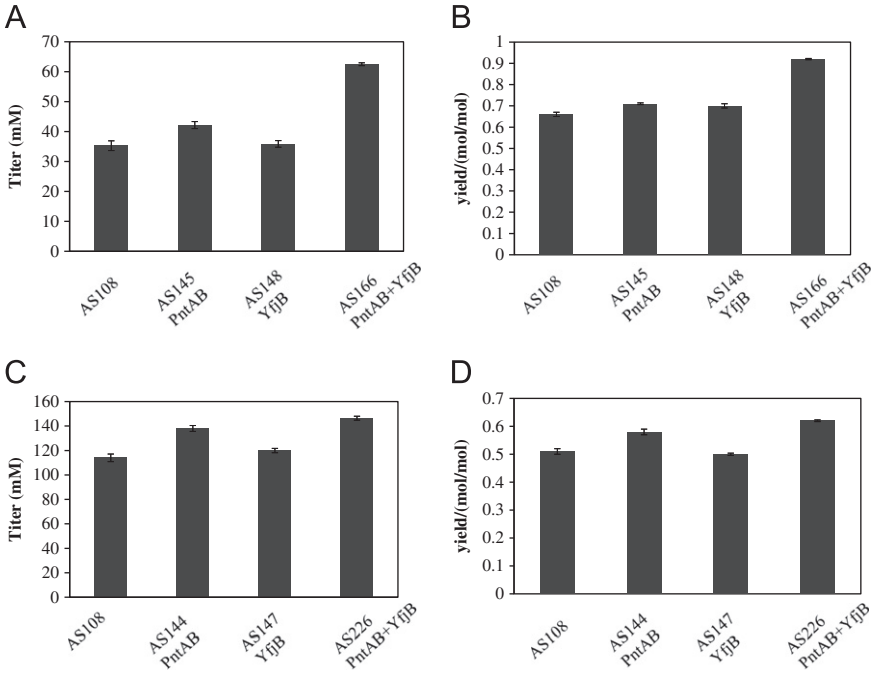

Fig. 3. Improvement of isobutanol titer and yield by activating transhydrogenase and NAD kinase. (A) and (B) Anaerobic condition; (C) and (D) Aerobic condition.

Titer and yield decreased 62% and 57% compared to mineral salts medium (Table S3). AS226 produced 45 mM isobutanol with a yield of 0.23 mol/mol in rich medium. Titer and yield decreased 69% and 63% compared to mineral salts medium (Table S3).

Under anaerobic condition, AS108 produced 12 mM isobutanol with a yield of 0.18 mol/mol in rich medium. Titer and yield decreased 66% and 73% compared to mineral salts medium (Table S3). AS166 produced 17 mM isobutanol with a yield of 0.2 mol/mol in rich medium. Titer and yield decreased 73% and 78% compared to mineral salts medium (Table S3).

In order to investigate the effect of different fermentation processes on isobutanol production, a two-stage process as described previously (Bastian et al., 2011) was used to cultivate AS166 for isobutanol production. This strain produced 75 mM isobutanol in 24 h. Productivity and yield were 0.07 g/L h OD_{600} and 1.09 mol/mol, which were 46% and 18% higher than using one-step anaerobic process, respectively (Table S5).

4. Discussion

4.1. Increasing NADPH supply to solve cofactor imbalance problem for anaerobic isobutanol production

Under anaerobic condition, isobutanol production was limited due to cofactor imbalance of the synthetic pathway (Bastian et al., 2011). Recently, this cofactor imbalance problem was solved by engineering keto-acid reductoisomerase and alcohol dehydrogenase (Bastian et al., 2011). The cofactor specificity of these two enzymes was changed from NADPH to NADH, and theoretical yield was obtained under anaerobic condition. Over-expressing *pntAB* gene together with engineering keto-acid reductoisomerase also led to near theoretical yield.

In this work, this cofactor imbalance problem for anaerobic isobutanol production was solved by increasing NADPH supply through activating membrane-bound transhydrogenase and NAD kinase in combination. Although over-expressing either *pntAB* or *yfjB* gene individually had been commonly used to increase NADPH supply (Bastian et al., 2011; Blombach et al., 2011; Lee et al., 2010; Li et al., 2009), to our knowledge, these two genes had not been utilized in combination. NAD kinase (YfjB) is a critical enzyme for controlling NAD(H) and NADP(H) balance (Shi et al., 2009). $NADP^+$ is mainly produced by phosphorylation of NAD^+, suggesting that NAD kinase may play an important role in regulation of $NADP^+$ turnover and size of the $NADP^+$ pool (Kawai et al., 2001). NAD^+ produced by PntAB can be phosphorylated to $NADP^+$ by YfjB, pushing the reaction direction towards NADPH synthesis (Fig. 1). Although activating NAD kinase alone had little effect on anaerobic isobutanol production, activating transhydrogenase and NAD kinase in combination led to 50% and 30% increase of isobutanol titer and yield, respectively, comparing to activating transhydrogenase alone. Our results suggested that transhydrogenase and NAD kinase had a synergistic effect on increasing NADPH supply and improving anaerobic isobutanol production. This strategy will be useful for improving production of target compounds using NADPH as reducing equivalent within their synthetic pathways.

4.2. Strategies for modulating gene expression

Although plasmid overexpression was commonly used to control expression of essential genes for improving production of target compounds, this strategy rarely reached optimal transcript level, and appeared to be unsuccessful in most cases (Keasling, 2008). Plasmid overexpression has several disadvantages for engineering of genetically stable strains (Keasling, 2008). Plasmid maintenance is a metabolic burden on the host cell, especially for high-copy number plasmids (De la Cueva-Mendez and Pimentel, 2007), and only few natural unit-copy plasmids have the desirable genetic stability (Keasling, 2008). It was found that plasmid overexpression of *pntAB* and *yfjB* genes either individually or in combination had little effect on isobutanol production (Tables 2 and 3).

On the other hand, gene expression can be modulated directly in chromosome. It has been demonstrated that modulating gene expression by multiple regulatory parts with varied strength would have more opportunities to obtain better production. The 1-deoxy-D-xylulose-5-phosphate synthase gene (*dxs*) was modulated by several artificial promoters to obtain optimal strength for maximum lycopene production (Alper et al., 2005). The glyceraldehyde-3-phosphate dehydrogenase gene (*gapA*) was modulated by three artificial promoters to obtain optimal strength for maximum glycerol production (Meynial-Salles et al., 2005). Multiple regulatory parts with varied strength were thus used to modulate *pntAB* and *yfjB* gene expression to obtain maximum isobutanol production. It was found that modulating *pntAB* gene with M1-46 resulted in the lowest isobutanol production among all modulated strains under anaerobic condition, while it led to the highest isobutanol production under aerobic condition (Tables 2 and 3). RT-PCR analysis found that M1-46 resulted in the lowest expression level of *pntAB* gene under anaerobic condition, while it led to the highest expression level under aerobic condition (Fig. 5). Our results suggested that different cultivation conditions (aerobic and anaerobic) had big influences on the expression level of a same regulatory part.

Strengths of regulatory parts M1-93, M1-37, M1-46 and M1-30 were 5, 2.5, 1.7 and 0.8 times of induced *E. coli lacZ* promoter when cultivated aerobically in LB medium. However, relative strengths of these regulatory parts changed significantly when used to modulate *pntAB* gene. It was suggested that expression level of a specific gene depended on both the sequence of regulatory part and sequence of gene itself. Different gene sequences might have an influence on the 5′ secondary structures between the transcription and translation start sites, thus causing varied transcript stability (Carrier and Keasling, 1999).

On the other hand, although modulating *pntAB* gene with M1-93 led to 60% higher expression level than M1-30 under anaerobic condition, similar isobutanol production was obtained (Table 2). For combined modulation of *pntAB* and *yfjB* genes, strains having *pntAB* gene modulated with M1-30 (AS172, AS173 and AS174) had yield indistinguishable from the parent strain, while isobutanol yields increased 24% to 39% in strains having *pntAB* gene modulated with M1-93 (AS165, AS166 and AS167). It was suggested that increasing transhydrogenase activity alone had a threshold for improving anaerobic isobutanol production, while activating NAD kinase in combination would break through this threshold.

4.3. Markerless gene modulation method

All the previous chromosome-based gene modulation method utilized Red recombination technology (Datsenko and Wanner, 2000) with the aid of FRT-antibiotic-FRT cassette (Alper et al., 2005; Meynial-Salles et al., 2005). Although the antibiotic marker can be removed by flippase treatment, a FRT scar still remained in chromosome. The recombination efficiency and opportunity to obtain correct recombinants decreased significantly when FRT scar sequences continuously accumulated, since FRT sequences have the possibility of self-recombination (Jantama et al., 2008). When gene deletion was performed in an engineered *E. coli* strain with three FRT scar sequences remained in chromosome, only

1 correct recombinant was obtained by screening 1000 colonies (data not shown). A two-step recombination method for gene deletion and gene integration had been developed by Ingram and his colleagues (Jantama et al., 2008; Zhang et al., 2007). The sucrose counter-selection strategy by levansucrase (SacB) was also utilized here for developing the markerless gene modulation method. Based on the regulatory parts libraries constructed previously (Lu et al., 2012), only two pairs of primers were needed to amplify DNA fragments for recombination, which can avoid laborious plasmid construction (Fig. 4).

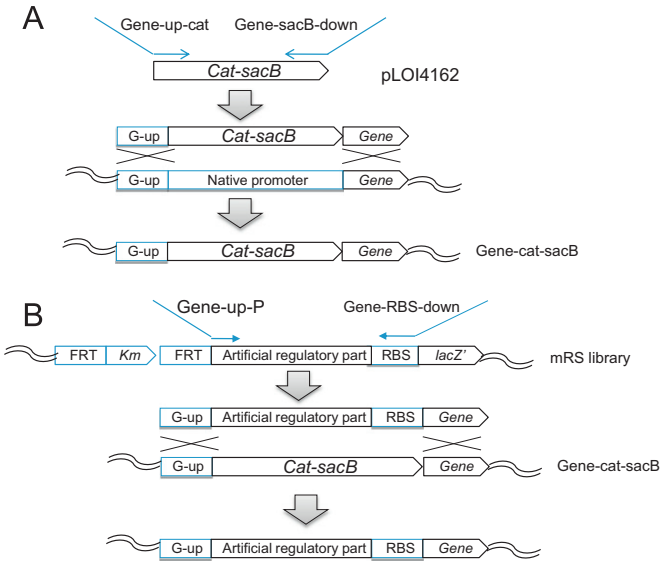

Fig. 4. Two-step recombination method for modulating gene expression in *E. coli* chromosome by artificial regulatory parts. (A) The first recombination step, *cat-sacB* cassette was amplified and used to replace the native promoter of target gene. (B) The second recombination step, different artificial regulatory parts were amplified and used to replace *cat-sacB* cassette.

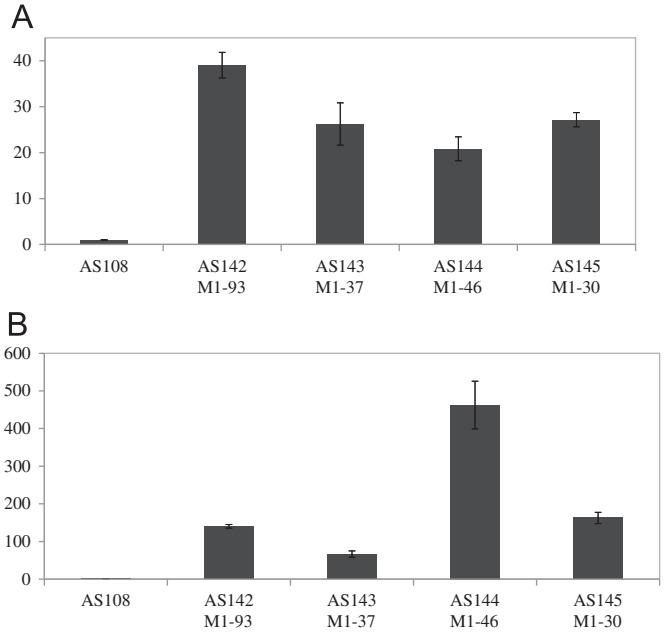

Fig. 5. Relative transcriptional level of *pntAB* gene after modulating with different regulatory parts. (A) Anaerobic condition; (B) Aerobic condition.

4.4. Effects of different fermentation conditions on isobutanol production

Although theoretical yield for anaerobic isobutanol production was obtained by Arnold and her colleagues (Bastian et al., 2011), there are still two aspects that can be improved. Rich medium (10 g/L yeast extract) was used in their experiments, which would increase costs for both production and down-stream purification process. On the other hand, a two-stage process (aerobic cell growth followed by anaerobic production) was used for isobutanol production. The total yield would be lower than 100% considering carbon loss during aerobic cell growth.

Strains constructed in this work were cultivated in rich medium for comparison with mineral salts medium. However, isobutanol production decreased significantly under both aerobic and anaerobic conditions (Table S3). Conversion of pyruvate to acetolactate was the rate-limiting step for isobutanol synthesis. There were only two copies of *alsS* gene in our constructed isobutanol producing strains. Activity of acetolactate synthase of *B. subtilis* decreased 45% when 10 mM valine was present (Table S4). It was suggested that branched-chain amino acids in rich medium would inhibit acetolactate synthase activity, thus leading to decreased isobutanol production. High isobutanol titer and yield were obtained when using rich medium by Arnold and her colleagues (Bastian et al., 2011). This might be due to high acetolactate synthase activity in their isobutanol producing strains, which had multiple copies of *alsS* gene in plasmid.

Strains were also cultivated with a two-stage process for comparison with one-step anaerobic process. Cultivating strain AS166 with a two-stage process resulted in 46% increase of isobutanol productivity comparing with one-step anaerobic process (Table S5). Surprisingly, isobutanol yield was higher than theoretical maximum when using two-stage process (Table S5). Glucose, isobutanol and other intermediates of the glycolysis pathway and isobutanol synthetic pathway accumulated in the cytoplasm at the time of the anaerobic shift (Table S8). These intracellular metabolites biased the calculated isobutanol yield of the anaerobic process.

4.5. Stability of isobutanol producing strains

Genetically stable isobutanol producing strains were obtained in this work by integrating essential genes into *E. coli* chromosome followed by modulating their expression with multiple regulatory parts. In order to demonstrate stability of our constructed strains, AS226 was serially transferred for 20 times under aerobic condition, leading to 139 generations. The resulting strain AS226-20 produced 141 mM isobutanol with a yield of 0.65 mol/mol under aerobic condition, which was almost the same as the parent strain (Table S6). On the other hand, AS166 was serially transferred for 10 times under anaerobic condition, leading to 43 generations. The resulting strain AS166-10 produced 65 mM isobutanol with a yield of 0.92 mol/mol under anaerobic condition, which was also almost the same as the parent strain (Table S6).

Genetic stability could come at a cost in gene copy number and thus enzyme activities. In order to investigate whether increasing gene copy numbers could improve isobutanol production, strain AS166 was transformed with two plasmids (pXZ116 and pXZ605) containing all five genes responsible for converting pyruvate to isobutanol. Two empty plasmids (pTrc99A and pACYC184M) were also transformed into AS166 as a control. Two-stage fermentation process was used for comparison of isobutanol production by these two strains. Isobutanol productivity increased 36% (from 0.059 to 0.08 g/L h OD_{600}) with plasmid over-expressing isobutanol synthetic genes, while yield increased 21% (from 0.95 to 1.15 mol/mol) (Table S7). These results demonstrated that

reduced gene copy number did limit isobutanol production. Gene copy numbers need to be increased in chromosome to further improve isobutanol production.

4.6. Increasing NADPH supply for aerobic isobutanol production

Activating transhydrogenase alone or together with NAD kinase were also favorable for aerobic isobutanol production (Table 3), suggesting that NADPH provided by pentose phosphate pathway and tricarboxylic acid cycle under aerobic condition was not enough for isobutanol production. The theoretical isobutanol yield per glucose under aerobic condition is 0.86 mol/mol (Bastian et al., 2011), which requires large amount of carbon fluxes going through pentose phosphate pathway. Although activating transhydrogenase and NAD kinase in combination increased isobutanol yield from 0.51 to 0.63 mol/mol, it was still far away from the theoretical maximum. Increasing carbon fluxes through pentose phosphate pathway needs to be done to further improve isobutanol yield under aerobic condition.

5. Conclusions

Genetically stable *E. coli* strains for isobutanol production were obtained by integrating essential genes into chromosome, followed by modulating their expression with multiple regulatory parts in chromosome to improve isobutanol production. Cofactor imbalance problem for anaerobic isobutanol production was then solved through activating transhydrogenase and NAD kinase to increase NADPH supply. Our work demonstrated that increasing PntAB activity alone had a threshold for improving anaerobic isobutanol production, while combination of PntAB and YfjB had a synergistic effect on improving anaerobic isobutanol production, leading to a yield of 0.92 mol/mol. Activating PntAB and YfjB also increased aerobic isobutanol titer and yield by 28% and 22%, leading to production of 10.8 g/L isobutanol in 24 h with a yield of 0.62 mol/mol.

Competing interests

This work has been included in a patent application by Tianjin Institute of Industrial Biotechnology.

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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.ymben.2012.11.008.

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