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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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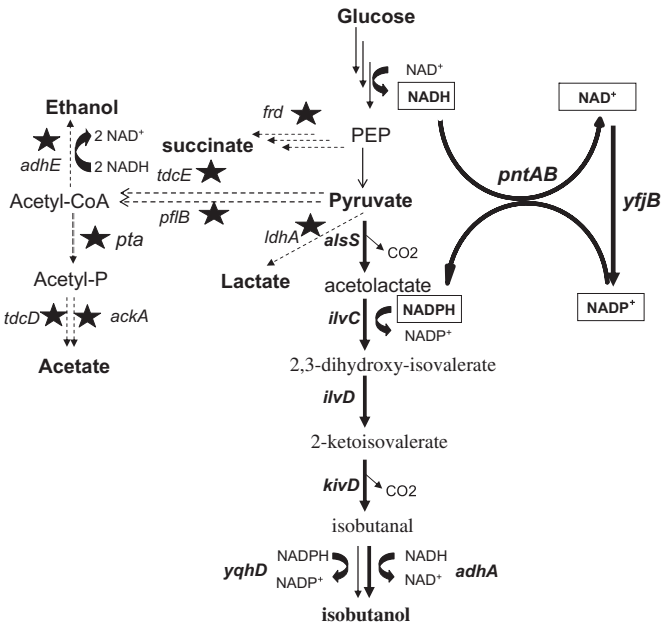

Fig. 1. Isobutanol synthetic pathway constructed in *Escherichia coli* (Atsumi et al., 2008b). NADH and $NADP^+$ were converted to NADPH and NAD^+ by transhydrogenase PntAB and $NADP^+$ was regenerated by NAD kinase YfjB. Genes and enzymes: *ldhA*, lactate dehydrogenase; *pflB*, pyruvate formate-lyase; *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