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Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in *Escherichia coli*

Sabine Bastian [a], Xiang Liu [a,1], Joseph T. Meyerowitz [a], Christopher D. Snow [a], Mike M.Y. Chen [a], Frances H. Arnold [a,*]

[a] Division of Chemistry and Chemical Engineering, California Institute of Technology, Mail code 210-41, Pasadena, CA 91125, USA

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ABSTRACT

2-methylpropan-1-ol (isobutanol) is a leading candidate biofuel for the replacement or supplementation of current fossil fuels. Recent work has demonstrated glucose to isobutanol conversion through a modified amino acid pathway in a recombinant organism. Although anaerobic conditions are required for an economically competitive process, only aerobic isobutanol production has been feasible due to an imbalance in cofactor utilization. Two of the pathway enzymes, ketol-acid reductoisomerase and alcohol dehydrogenase, require nicotinamide dinucleotide phosphate (NADPH); glycolysis, however, produces only nicotinamide dinucleotide (NADH). Here, we compare two solutions to this imbalance problem: (1) over-expression of pyridine nucleotide transhydrogenase PntAB and (2) construction of an NADH-dependent pathway, using engineered enzymes. We demonstrate that an NADH-dependent pathway enables anaerobic isobutanol production at 100% theoretical yield and at higher titer and productivity than both the NADPH-dependent pathway and transhydrogenase over-expressing strain. Our results show how engineering cofactor dependence can overcome a critical obstacle to next-generation biofuel commercialization.

1. Introduction

Recent advances in biotechnology have made it possible to convert sugars into a wide array of renewable chemicals (Liu and Khosla, 2010; Rude and Schirmer, 2009; Shen and Liao, 2008; Yan and Liao, 2009). Of all these possibilities, isobutanol, an excellent gasoline blend stock and precursor to C4 petrochemical building blocks, is especially promising because of its potential for high-yield production and compatibility with an existing fuel infrastructure (Rude and Schirmer, 2009). Isobutanol is an energy-dense, low-vapor-pressure, high-octane hydrocarbon that burns in a combustion engine like conventional gasoline without adversely affecting the performance (Bruno et al., 2010; Szwaja and Naber, 2010). Isobutanol is also readily converted to butenes that can be used directly to produce hydrocarbon-based fuels, commodity chemicals, and materials (Connor and Liao, 2009; Taylor et al., 2010). For these renewables to be cost-competitive with conventional fuel and butenes, fermentation of sugars to isobutanol must be as efficient as bioethanol production, which reaches commercial viability through anaerobic, high-productivity, high-titer, and high-yield fermentation performance in low capital cost production facilities.

Although the Ehrlich pathway (Ehrlich, 1907), the catabolic process that converts amino acid precursors to fusel alcohols, has long been known in yeast (Hazelwood et al., 2008), industrially relevant production of higher alcohols in native microorganisms has not been demonstrated (Alper and Stephanopoulos, 2009). Isobutanol fermentation has become feasible via an implementation of a synthetic Ehrlich pathway in *Escherichia coli*, where 2-keto-isovalerate serves as precursor for isobutanol (Atsumi et al., 2008b) (Fig. 1). The use of engineered strains with deletions in pathways competing for carbon and cofactors (Atsumi et al., 2008a) has enabled isobutanol titers of >20 g/L to be reached aerobically or micro-aerobically (Atsumi et al., 2008b; Atsumi et al., 2010). However, anaerobic conditions are preferred for large-scale production due to lower operating costs and higher theoretical yield. We hypothesized that the limited isobutanol production observed under anaerobic conditions is caused by a cofactor imbalance in the engineered pathway. Two of the five pathway enzymes are nicotinamide adenine dinucleotide phosphate (NADPH)-dependent: (1) ketol-acid reductoisomerase

* Corresponding author. Fax: +1 626 568 8743.
E-mail address:
frances@cheme.caltech.edu (F.H. Arnold).
[1] Permanent address: Laboratory of Biocatalysis and Bioprocessing, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, PR China.

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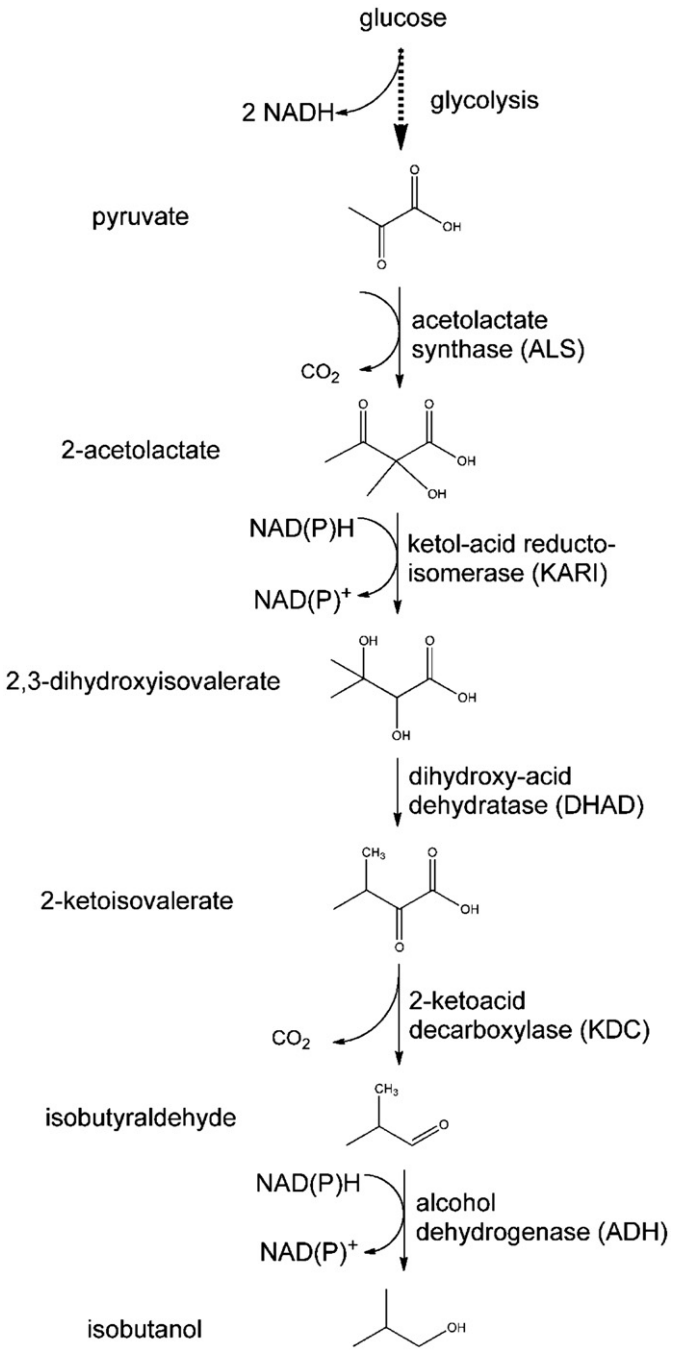

Fig. 1. Engineered pathway for isobutanol production (Atsumi et al., 2010). Acetolactate synthase (ALS), ketol-acid reductoisomerase (KARI), dihydroxy-acid dehydratase (DHAD), 2-ketoacid decarboxylase (KDC), and alcohol dehydrogenase (ADH).

(KARI, IlvC from *E. coli*; EC 1.1.1.86) catalyzes the two-step reaction from *S*-2-acetolactate (*S*-2-AL) to 2,3-dihydroxy-isovalerate (DHIV), involving a Mg^{2+}-dependent alkyl migration followed by ketone reduction (Chunduru et al., 1989), and (2) alcohol dehydrogenase (ADH, YqhD from *E. coli*; EC 1.1.1.2) reduces isobutyraldehyde to isobutanol (Atsumi et al., 2010) (Fig. 1). Thus, the conversion of glucose to isobutanol requires two equivalents of NADPH. Under anaerobic conditions, however, the only available reducing equivalent is nicotinamide adenine dinucleotide (NADH), produced through glycolysis. The cells cannot resolve this cofactor imbalance via the pentose phosphate pathway (PPP) or the tricarboxylic acid (TCA) cycle, because these are functional only in the presence of oxygen. NADPH-limited reactions have been previously noted in commercially relevant systems such as the production of xylitol (Chin et al., 2009) and the production of n-butanol (Steen et al., 2008).

One possible solution to a cofactor imbalance is to overexpress a transhydrogenase, such as *E. coli* PntAB (Weckbecker and Hummel, 2004), which catalyzes the reversible transfer of a hydride ion between NADH and $NADP^+$. This transfer is coupled to the proton motive force and uses one proton per hydride (Clarke and Bragg, 1985; Sauer et al., 2004). This energy requirement and the metabolic load associated with expression of the transhydrogenase are two drawbacks to this approach (Chin et al., 2009). As an alternative solution, Liao (Atsumi et al., 2010) and we propose to replace the NADPH-dependent enzymes of the pathway with NADH-utilizing homologs. Substituting the ADH with an NADH-dependent homolog is feasible due to the abundance of known NADH-dependent ADHs (Jornvall et al., 1987). For example, AdhA from *Lactococcus lactis* has been shown previously to be compatible with the isobutanol pathway (Atsumi et al., 2010). However, an NADH-dependent enzyme, catalyzing the conversion of *S*-2-AL to DHIV, has yet to be reported.

We therefore aimed to switch the cofactor dependence of the native *E. coli* IlvC from NADPH to NADH. Although there are literature examples of engineered changes in cofactor preference, reports of specificity reversals are rare (Ehsani et al., 2009). Examples in which the engineered enzyme is placed back into a pathway have shown only limited success in resolving the cofactor imbalance (Matsushika et al., 2008a, 2008b; Petschacher and Nidetzky, 2008). In this study, we describe the engineering of an NADH-dependent IlvC by directed evolution, using iterative, targeted mutagenesis. Upon recombining beneficial mutations, we found two variants with acetolactate (2-AL) reducing activity in the presence of NADH that is similar to the wild-type enzyme with NADPH. Utilizing the best IlvC variant and an engineered *L. lactis* AdhA variant in the isobutanol pathway improved the yield to 100% of the theoretical limit under anaerobic conditions. We demonstrate that (a) reversal of the cofactor preference of IlvC can be achieved, and (b) by engineering the cofactor dependence of the isobutanol metabolic pathway, the NADH-dependent pathway outperforms over-expression of a transhydrogenase for resolving the cofactor imbalance and the engineered pathway achieves the highest possible yield.

2. Materials and methods

2.1. General

Strain 1993 and plasmids pGV1662, pGV1777, pGV1925, pGV1947, pGV1711, and pGV1705 are to be found in Table A, Appendix A. Biological media were purchased from Research Products International (Mt. Prospect, IL, USA), NAD(P)H from Codexis, Inc. (Redwood City, CA, USA), oligonucleotides from Integrated DNA Technologies (San Diego, CA, USA), DNA polymerases, restriction enzymes, and T4 ligase from New England Biolabs (Ipswich, MA, USA), and ethyl 2-acetoxy-2-methylacetoacetate (EAMAA) from Sigma (St. Louis, MO, USA). (*R/S*)-2-acetolactate (2-AL) was prepared as described previously (Krampitz, 1957), using EAMAA as starting material. DNA sequencing was performed by Laragen (Los Angeles, CA, USA). Standard molecular biology methods were taken from Maniatis et al. (Sambrook et al., 1989).

2.2. Structure alignment

Mutation sites were selected following an inspection of the cofactor-binding site. To assess cofactor side-chain interactions, we used PyMOL Molecular Graphics System (Version 1.3,