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Minireview

Metabolic engineering for higher alcohol production

Nicole E. Nozzi [a], Shuchi H. Desai [a,b], Anna E. Case [a], Shota Atsumi [a,b,*]

[a] Department of Chemistry, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA
[b] Microbiology Graduate Group, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA

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ABSTRACT

Engineering microbial hosts for the production of higher alcohols looks to combine the benefits of renewable biological production with the useful chemical properties of larger alcohols. In this review we outline the array of metabolic engineering strategies employed for the efficient diversion of carbon flux from native biosynthetic pathways to the overproduction of a target alcohol. Strategies for pathway design from amino acid biosynthesis through 2-keto acids, from isoprenoid biosynthesis through pyrophosphate intermediates, from fatty acid biosynthesis and degradation by tailoring chain length specificity, and the use and expansion of natural solvent production pathways will be covered.

1. Introduction

As a part of a worldwide movement towards sustainability, the field of metabolic engineering looks to expand the scope of compounds produced from engineered biological systems. Biologically produced higher alcohols have received great interest as an engineering target for applications ranging from biofuels, flavors and fragrances to chemical feedstocks, many currently derived from petroleum (Atsumi et al., 2008b; Dellomonaco et al., 2011; Wang et al., 2010). The native production of higher alcohols from *Clostridium* strains initially generated interest as a potential source of bio-based production (Ezeji et al., 2007). However the intractability of *Clostridium* strains to large scale culturing necessitated the engineering of higher alcohol production in other hosts (Rabinovitch-Deere et al., 2013). Metabolic engineering strategies to facilitate non-natural production of higher alcohols have ranged from minor modifications to the *Clostridium* pathway expressed in a new host to newly designed non-fermentative strategies. Beyond alcohols targeted for their potential use as biofuels such as butanol, alcohols of greater complexity such as 2-phenylethanol and isoprenoid derived alcohols are of great interest to flavor and fragrance industries (Etschmann et al., 2002; Tokuhiro et al., 2009). These alcohols are primarily produced by plants in amounts too small to justify direct extraction. While more economically feasible, chemical synthesis often cannot match the purity of biological synthesis (Etschmann et al., 2002). These factors make flavor and fragrance alcohols prime targets for an engineered biological synthesis in a microbial host.

Most pathways are designed for expression in a well-characterized host such as *Escherichia coli* or *Saccharomyces cerevisiae*. However, other works have taken on the challenge of developing engineering tools for less commonly employed strains to take advantage of some of their desirable properties. Improving engineering strategies for *Clostridium* species, for example, would allow designs to take advantage of these strains' natural solvent producing ability (Lutke-Eversloh, 2014). The use of cyanobacteria would allow the input carbon source to be simply carbon dioxide (Machado and Atsumi, 2012). Pathway optimization for any chosen host must consider the following key engineering points: elimination of competing pathways, redox balance for necessary cofactors, optimal enzyme expression levels, forward driving force to best facilitate carbon flux to the desired end product, and finally methods to collect the produced product which goes hand-in-hand with avoiding product toxicity to the strain (Avalos et al., 2013; Nozzi et al., 2013; Stephanopoulos, 2007). We explore these points as they relate to engineered microbial production of higher alcohols, and how established strategies have been optimized for new hosts, carbon sources, and target chemicals.

2. The 2-keto acid pathway

The diversion of 2-keto acids to the production of branched chain alcohols has been a promising approach. The production of a number of higher chain alcohols in *E. coli* utilizing the cell's natural amino acid synthesis pathway has been demonstrated (Atsumi et al., 2008b).

* Corresponding author at: Department of Chemistry, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA.
E-mail address: satsumi@ucdavis.edu (S. Atsumi).

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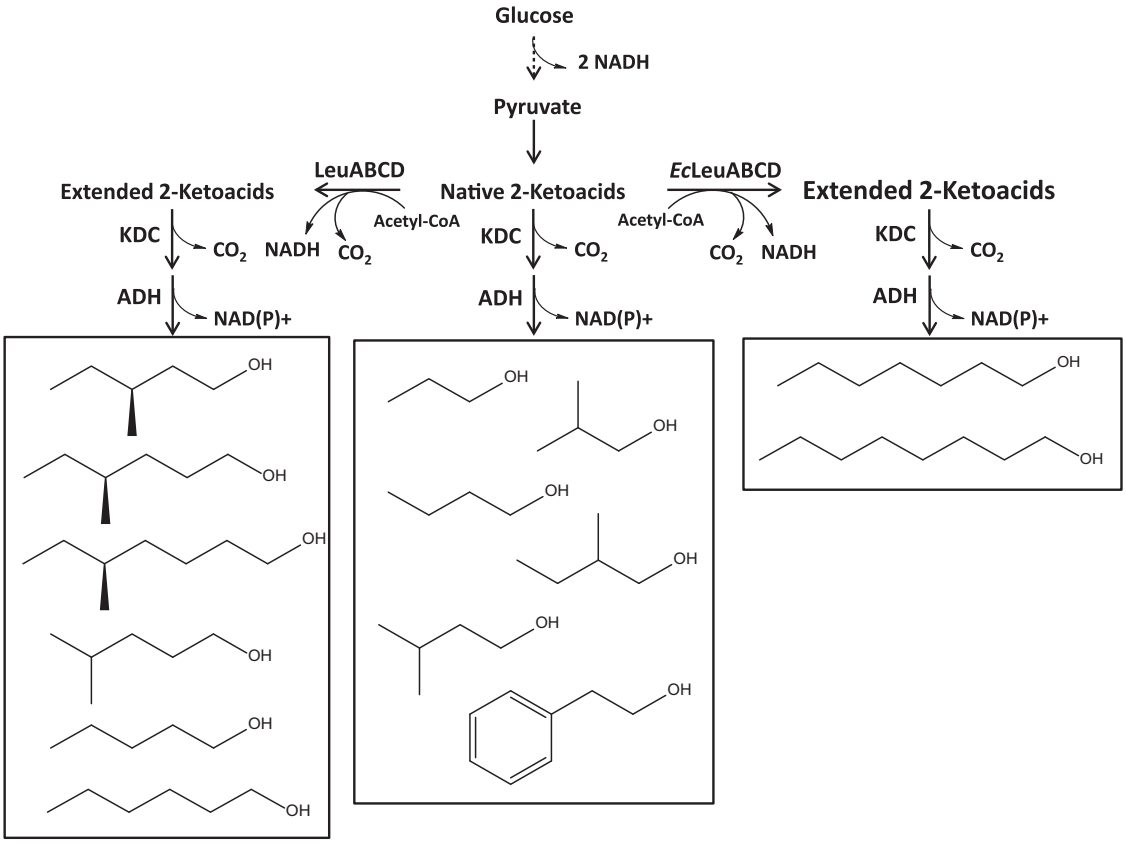

Fig. 1. Alcohols produced via 2-ketoacid pathway design. Higher alcohols which can be derived from 2-ketoacids both native and after further extension. KDC (ketoacid decarboxylase), ADH (alcohol dehydrogenase), LeuABCD (isopropylmalate synthase, isomerase, and dehydrogenase), *Ec*LeuABCD (including engineered LeuA for "+1" pathway in *E. coli*, "*E. coli* LeuA").

The 2-keto acid intermediate from the biosyntheses of branched chain amino acids was diverted to synthesize higher branched chain alcohols such as isobutanol, 1-butanol, 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol (Fig. 1). This opened up possibilities for production of these alcohols from a renewable source in high enough productivities to be considered a viable fuel candidate. By diverting the cell's natural production of 2-keto acids via amino acid catabolism, only two additional steps (keto-acid decarboxylase (KDC) and alcohol dehydrogenase (ADH)) taken from the Ehrlich pathway (Ehrlich, 1907), responsible for amino acid degradation in certain organisms, were necessary to produce the desired alcohol products. The Ehrlich pathway involves a functional group transfer between an amino acid and 2-oxoglutarate to form glutamate and a 2-ketoacid. The 2-ketoacid undergoes a decarboxylation followed by either a reduction or oxidation depending on culture conditions followed by cellular excretion (Rabinovitch-Deere et al., 2013).

2.1. Higher alcohol production from the 2-keto acid pathway

2.1.1. Isobutanol

Once production of various alcohols had been demonstrated an effort was made to improve production with isobutanol serving as the model target. Isobutanol is produced from pyruvate through valine biosynthesis (Atsumi et al., 2008b). To improve production the *ilvIHCD* genes were overexpressed in combination with the alcohol producing pathway. This improved isobutanol production up to 1.7 g/L, approximately a 5-fold increase. Genes that lead to the formation of by-products such as acetic acid and lactate were then deleted, slightly improving production to 2.2 g/L. Production was further improved by replacing *ilvIH* with *alsS* from *Bacillus subtilis*. Due to the higher affinity of AlsS for pyruvate over 2-ketobutyrate (Gollop et al., 1990), this increased production to 3.7 g/L. Finally the deletion of *pflB* decreased competition for pyruvate leading to a final production of 22.2 g/L isobutanol over 112 h. Production at 86% of the maximum theoretical yield was achieved for isobutanol via this pathway. This titer demonstrated the potential for scale up on an industrial level (Atsumi et al., 2008b).

Further improvements in production necessitated the resolution of cofactor imbalance. Engineered 2-keto acid pathways required NADPH as a cofactor for catalysis, however, under the anaerobic conditions that are generated in a large scale bioreactor, glycolysis produces only NADH. Thus, this cofactor imbalance presents a major hurdle towards commercialization. There are two possible approaches to resolving this problem (Bastian et al., 2011). The first is to overexpress pyridine nucleotide transhydrogenase (PntAB) which catalyzes the transfer of a hydride from NADH to $NADP^+$. This approach has been shown to successfully resolve cofactor imbalance; however the metabolic load and energy requirements of this process prevent it from being an ideal solution. An alternative approach for resolution of cofactor imbalance in the system is the use of engineered enzymes to create an NADH dependent pathway. There are two NADPH dependent enzymes in the pathway, keto-acid reductoisomerase (IlvC) and ADH. Previous work had compared three different ADH's and found that AdhA from *Lactococcus lactis*, a NADH dependent ADH, had the highest activity in *E. coli* (Atsumi et al., 2010). However, no NADH dependent IlvC was known so it was instead necessary to alter the cofactor dependence of the native IlvC in the pathway via directed evolution. After a series of iterative targeted mutagenesis and recombination, two variants with NADH reducing activity