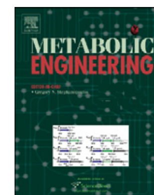
Metabolic Engineering 13 (2011) 674–681

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben



An evolutionary strategy for isobutanol production strain development in *Escherichia coli*

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ARTICLE INFO

Article history:
Received 26 April 2011
Received in revised form
30 July 2011
Accepted 29 August 2011
Available online 5 September 2011

Keywords:
Biofuel
Isobutanol
Metabolic engineering
E. coli

ABSTRACT

Higher alcohols such as isobutanol possess several physical characteristics that make them attractive as biofuels such as higher energy densities and infrastructure compatibility. Here we have developed a rapid evolutionary strategy for isolating strains of *Escherichia coli* that effectively produce isobutanol from glucose utilizing random mutagenesis and a growth selection scheme. By selecting for mutants with the ability to grow in the presence of the valine analog norvaline, we obtained *E. coli* NV3; a strain with improved 24-h isobutanol production (8.0 g/L) in comparison with a productivity of 5.3 g/L isobutanol obtained with the parental wild type strain. Genomic sequencing of NV3 identified the insertion of a stop codon in the C-terminus of the RNA polymerase σ^s-factor, RpoS. Upon repair of this inhibitory mutation (strain NV3r1), a final isobutanol titer of 21.2 g/L isobutanol was achieved in 99 h with a yield of 0.31 g isobutanol/g glucose or 76% of theoretical maximum. Furthermore, a mutation in *ldhA*, encoding D-lactate dehydrogenase, was identified in NV3; however, repair of LdhA in NV3r1 had no affect on LdhA activity detected from cell extracts or on isobutanol productivity. Further study of NV3r1 may identify novel genotypes that confer improved isobutanol production.

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1. Introduction

Efforts aimed at deriving energy and chemicals from biorenewable resources have intensified dramatically due to concerns of the diminishing supply of fossil fuel reserves and increasing environmental concerns (Stephanopoulos, 2007). Much of this attention has focused, in particular, on the production of ethanol by fermentation. For example, ethanol production reached 9.2 billion gallons in the United States in 2009, a 40% increase in comparison with 2008 (Seiferlein, 2009). However, several physical characteristics of ethanol make it less attractive as a biofuel in comparison with other higher alcohols (C3 or higher) such as its lower energy density and high hygroscopicity, making it incompatible with current infrastructure. Recently, the production of several higher alcohols that do not share these shortcomings has been demonstrated using *Escherichia coli* (Atsumi et al, 2008, 2009; Atsumi and Liao, 2008a; Cann and Liao, 2008; Connor and Liao, 2008; Hanai et al., 2007; Shen and Liao, 2008; Withers et al., 2007; Zhang et al., 2008). In addition to *E. coli*, isobutanol has also been produced using the well-known amino acid producer, *Corynebacterium glutamicum* (Smith et al., 2010), and with *Synechococcus elongatus* through CO_2 fixation via photosynthesis (Atsumi et al., 2009; Lan and Liao, 2011).

Metabolic engineering is a strategy often used to increase the microbial production of valuable products (Stephanolpoulos and Stafford, 2002). Typically, a rational design approach is employed to increase the production of a target compound by overexpression of the biosynthetic pathway and/or inactivation of other competitive pathways. Although this method has been proven to be useful for a wide variety of target compounds (Atsumi et al., 2008; Atsumi and Liao, 2008b; Causey et al., 2004; Lee et al., 2009; Lehmann and Lutke-Everslosh, 2011; Yazdani and Gonzalez, 2008; Yu et al., 2011), further improvement of production often defies biochemical reasoning and physiological insight.

On the other hand, random mutagenesis and selection have been proven to be powerful tools to rapidly isolate a strain with a desired phenotype such as improved amino acid production (Ikeda, 2003). This method allows one to consider an enormous number of genotypes if a proper selective pressure is applied. Using this method, strain development can be quite rapid, although unnecessary and/or inhibitory mutations are often introduced. Therefore, it is highly desirable to identify the beneficial mutations and introduce them into a strain with a clean background.

Random mutagenesis is often done using chemicals that introduce mutations throughout the entire genome such as N′-nitro-N-nitrosoguanidine (NTG) or ethylmethane sulfonate (EMS). Exposing the cell to an analog of the target amino acid

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doi:10.1016/j.ymben.2011.08.004

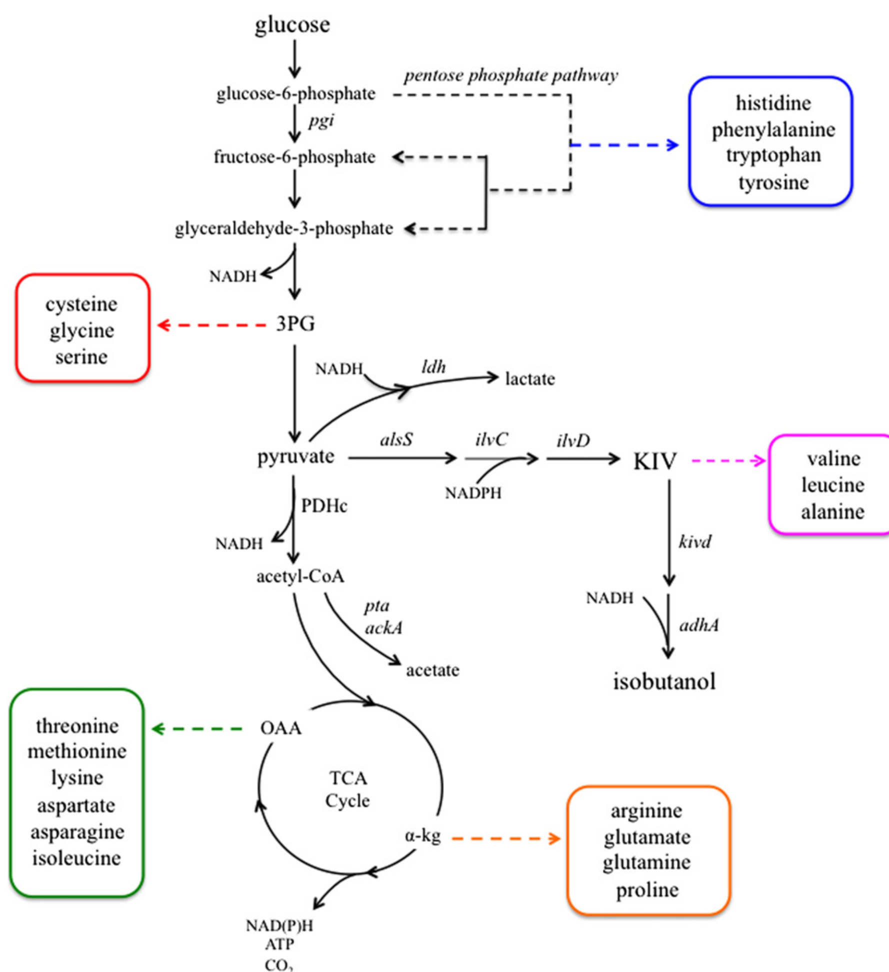

Fig. 1. The isobutanol and amino acid biosynthesis pathways from glucose in *E. coli*. KIV, 2-ketoisovalerate; 3PG, 3-phospho-D-glycerate; α-kg, alpha-ketoglutarate; OAA, oxaloacetate.

can be used as a selective pressure for amino acid strain development. After mutagenesis, mutants are selected for the ability to grow in the presence of these toxic analogs. A minimum concentration of the analog is used such that a cell must be harboring beneficial mutations to survive by deregulating natural biosynthesis or outcompeting the analog for incorporation into growing polypeptides. It is noteworthy to say, however, that better growth in the presence of the analog does not guarantee improved amino acid production. Various resistance mechanisms may be introduced (i.e. improved valine synthesis, improved norvaline efflux, removal of allosteric regulation in key enzymes) and so measuring amino acid production is required. This method has been shown to be successful for engineering strains of *corynebacteria* for valine and lysine production (Oh et al., 1993; Tsuchida and Yoshinaga, 1974), and in *E. coli* for increased valine synthesis (Uemura et al., 1972). This can also be applied towards isolating strains with improved higher alcohol production being that the higher alcohols are derived from the same 2-keto acid precursors of native amino acids. Indeed, the successful isolation of an *E. coli* strain with improved 3-methyl-1-butanol production has been demonstrated using random mutagenesis and selection for growth in the presence of a leucine analog, 4-aza-D,L-leucine (Connor et al., 2010).

Isobutanol production in *E. coli* has been successful using a strain engineered through rational design (Atsumi et al., 2008, 2009) by expression of the heterologous isobutanol pathway from plasmid (Fig. 1). Similarly, the strains presented in this study do not naturally produce isobutanol and are thus harboring plasmids for expression of the isobutanol pathway. This study demonstrates the application of an established strain construction method, commonly used to isolate improved amino acid producers, to generate an improved isobutanol production strain of *E. coli*. Isolation and sequencing of such a strain may then lead to the discovery of new genotypes related to improved higher chain alcohol production.

2. Materials and methods

2.1. Reagents

KOD DNA polymerase was purchased from EMD Chemicals (San Diego, CA). Oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). DL-norvaline was purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Strains and plasmids

All *E. coli* strains and plasmids used are listed in Table 1. *E. coli* XL1-Blue (Stratagene) was used to propagate all plasmids. The mutation identified in the *rpoS* gene of strain NV3 (C910T) was repaired by amplification of the C-terminus of *rpoS* from *E. coli* MG1655 genomic DNA (gDNA) using primers K902 (5′ CATACGCAACCTGGTGGATTCGC 3′)+K903 (5′ CAGATGCTTACTTACTCGCGGAACAG 3′) and fusing the resulting PCR fragment by SOE with

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