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Engineering *Escherichia coli* for the efficient conversion of glycerol to ethanol and co-products

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ABSTRACT

Given its availability, low prices, and high degree of reduction, glycerol has become an ideal feedstock for producing reduced compounds via anaerobic fermentation. We recently identified environmental conditions enabling the fermentative metabolism of glycerol in *E. coli*, along with the pathways and mechanisms mediating this metabolic process. In this work, we used the knowledge base created in previous studies to engineer *E. coli* for the efficient conversion of crude glycerol to ethanol. Our strategy capitalized on the high degree of reduction of carbon in glycerol, thus enabling the production of not only ethanol but also co-products hydrogen and formate. Two strains were created for the co-production of ethanol–hydrogen and ethanol–formate: SY03 and SY04, respectively. High ethanol yields were achieved in both strains by minimizing the synthesis of by-products succinate and acetate through mutations that inactivated fumarate reductase (Δ*frdA*) and phosphate acetyltransferase (Δ*pta*), respectively. Strain SY04, which produced ethanol–formate, also contained a mutation that inactivated formate–hydrogen lyase (Δ*fdhF*), thus preventing the conversion of formate to CO_2 and H_2. High rates of glycerol utilization and product synthesis were achieved by simultaneous overexpression of glycerol dehydrogenase (*gldA*) and dihydroxyacetone kinase (*dhaKLM*), which are the enzymes responsible for the conversion of glycerol to glycolytic intermediate dihydroxyacetone phosphate. The resulting strains, SY03 (pZSKLMgldA) and SY04 (pZSKLMgldA), produced ethanol–hydrogen and ethanol–formate from unrefined glycerol at yields exceeding 95% of the theoretical maximum and specific rates in the order of 15–30 mmol/gcell/h. These yields and productivities are superior to those reported for the conversion of glycerol to ethanol–H_2 or ethanol–formate by other organisms and equivalent to those achieved in the production of ethanol from sugars using *E. coli*.

1. Introduction

Glycerol has become an inexpensive and abundant carbon source due to its generation as inevitable by-product of biodiesel fuel production. With every 100 lbs of biodiesel produced by the transesterification of vegetable oils or animal fats, 10 lbs of crude glycerol are generated. The tremendous growth of the biodiesel industry has created a glycerol surplus that resulted in a dramatic decrease in crude glycerol prices (Yazdani and Gonzalez, 2007 and references cited therein). This decrease in prices poses a problem for the glycerol-producing and -refining industries, and the economic viability of the biodiesel industry itself has been greatly affected (McCoy, 2006, 2005). The conversion of low-priced glycerol streams to higher value products has been proposed as a path to economic viability for the biofuels industry (Yazdani and Gonzalez, 2007). Such technologies could be readily integrated into existing biodiesel facilities, thus establishing true biorefineries and revolutionizing the biodiesel industry by dramatically improving its economics. While availability and low prices make glycerol an attractive carbon source for fermentation processes, there is yet another advantage in using this compound: fuels and reduced chemicals can be produced from glycerol at yields higher than those obtained from common sugars (Yazdani and Gonzalez, 2007). The latter is possible because the degree of reduction per carbon, κ (Nielsen et al., 2003), of glycerol is significantly higher ($C_3H_8O_3$: $\kappa = 4.67$) than that of sugars such as glucose ($C_6H_{12}O_6$: $\kappa = 4$) or xylose ($C_5H_{10}O_5$: $\kappa = 4$). To fully realize the aforementioned advantages, the use of anaerobic fermentation is highly desirable.

While many microorganisms are able to metabolize glycerol in the presence of external electron acceptors (respiratory metabolism), few are able to do so fermentatively (i.e., in the

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absence of electron acceptors). Until recently, the fermentative metabolism of glycerol had been reported in species of the genera *Klebsiella*, *Citrobacter*, *Enterobacter*, *Clostridium*, *Lactobacillus*, *Bacillus*, *Propionibacterium*, and *Anaerobiospirillum* (Yazdani and Gonzalez, 2007 and references cited therein). However, the potential for using these organisms at the industrial level could be limited due to issues that include pathogenicity, the need for strict anaerobic conditions and supplementation with rich nutrients, and unavailability of the genetic tools and physiological knowledge necessary for their effective manipulation. The use of microbes such as *Escherichia coli*, an organism very amenable to industrial applications, could help overcome the aforementioned problems.

Although it was long thought that the metabolism of glycerol in *E. coli* required the presence of external electron acceptors (Booth, 2005; Bouvet et al., 1994, 1995; Lin, 1976; Quastel et al., 1925; Quastel and Stephenson, 1925), we recently discovered that *E. coli* can metabolize glycerol in a fermentative manner (Gonzalez et al., 2008; Murarka et al., 2008; Dharmadi et al., 2006). We identified environmental conditions that enable this metabolic process, along with the pathways and mechanisms responsible for it (Gonzalez et al., 2008) (Fig. 1). In the work reported here we used the knowledge base created by our previous studies to engineer *E. coli* for the efficient conversion of crude glycerol into ethanol. Our strategy took advantage of the high degree of reduction of carbon in glycerol, thus enabling the production of not only ethanol but also the co-products hydrogen and formate. Product yields and productivities in these strains were superior to those reported for the conversion of glycerol to ethanol–H_2 or ethanol–formate (Jarvis et al., 1997; Ito et al., 2005; Sakai and Yagishita, 2007) and similar to those reported for the production of ethanol from sugars using engineered *E. coli* strains (e.g., Underwood et al., 2002).

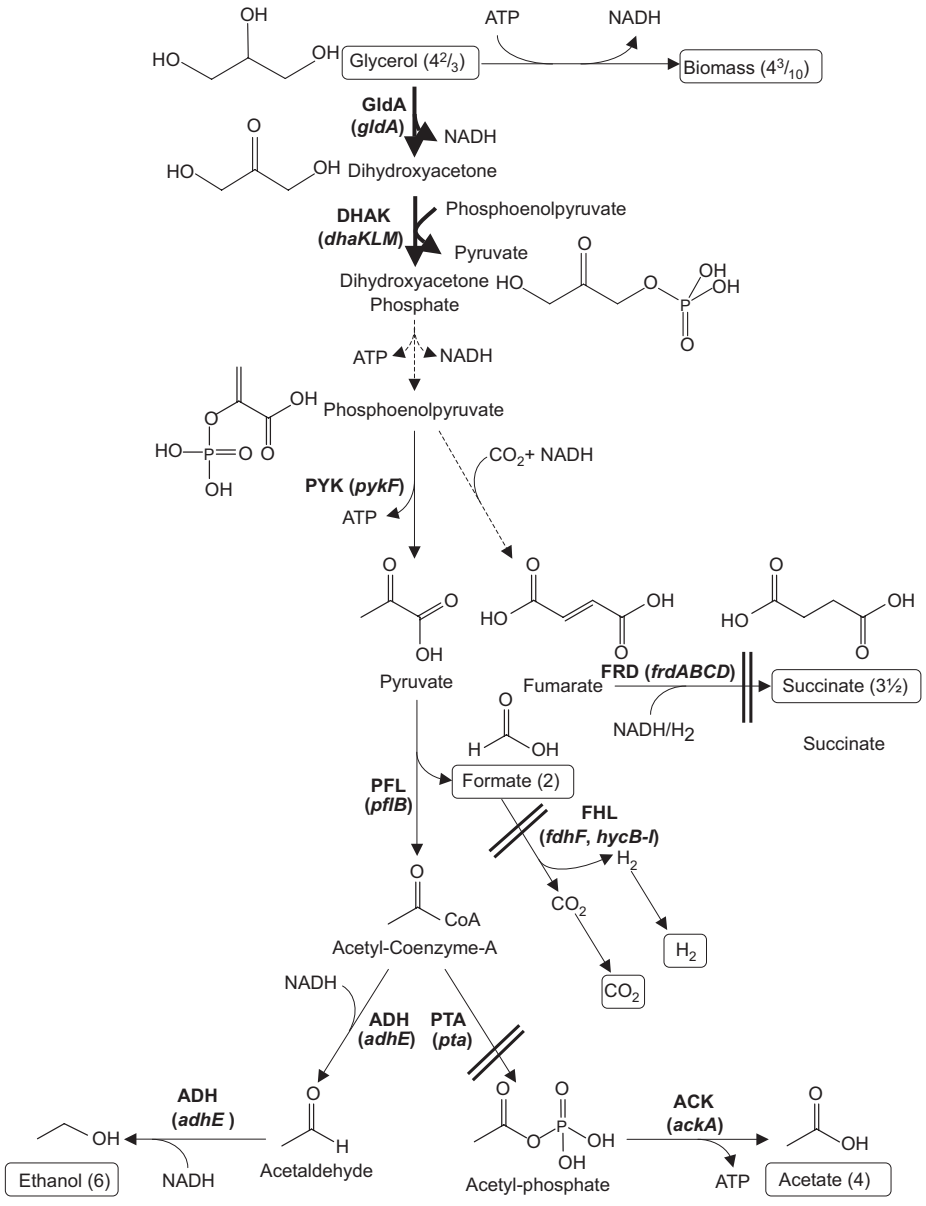

Fig. 1. Main fermentative pathways involved in the anaerobic fermentation of glycerol in *E. coli* (Murarka et al., 2008; Gonzalez et al., 2008). Relevant genes and corresponding enzymes are included. Glycerol dissimilation in the absence of electron acceptors is mediated by glycerol dehydrogenase and dihydroxyacetone kinase (Gonzalez et al., 2008). Ethanol, succinate, acetate, and formate are the main products of the fermentative utilization of glycerol (Dharmadi et al., 2006). Proposed genetic modifications are illustrated by thicker lines (overexpression of *gldA* and *dhaKLM*) or double bars (disruption of *frdA*, *pta*, and *fdhF*). Broken lines illustrate multiple steps. Substrates, products, and biomass are boxed. Abbreviations: ADH, acetaldehyde/alcohol dehydrogenase; ACK, acetate kinase; DHAK, dihydroxyacetone kinase; FHL, formate hydrogen-lyase; FRD, fumarate reductase; GldA, glycerol dehydrogenase; PFL, pyruvate formate-lyase; PTA, phosphate acetyltransferase; PYK, pyruvate kinase.