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Engineering *Escherichia coli* for renewable benzyl alcohol production

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ABSTRACT

Benzyl alcohol is an aromatic hydrocarbon used as a solvent and an intermediate chemical in the pharmaceutical, cosmetics, and flavor/fragrance industries. The *de novo* biosynthesis of benzyl alcohol directly from renewable glucose was herein explored using a non-natural pathway engineered in *Escherichia coli*. Benzaldehyde was first produced from endogenous phenylpyruvate via three heterologous steps, including hydroxymandelate synthase (encoded by *hmaS*) from *Amycolatopsis orientalis*, followed by (*S*)-mandelate dehydrogenase (encoded by *mdlB*) and phenylglyoxylate decarboxylase (encoded by *mdlC*) from *Pseudomonas putida* ATCC 12633. The subsequent rapid and efficient reduction of benzaldehyde to benzyl alcohol occurred by the combined activity and native regulation of multiple endogenous alcohol dehydrogenases and/or aldo-keto reductases. Through systematic deletion of competing aromatic amino acid biosynthesis pathways to promote endogenous phenylpyruvate availability, final benzyl alcohol titers as high as 114 ± 1 mg/L were realized, representing a yield of 7.6 ± 0.1 mg/g on glucose and a ~5-fold improvement over initial strains.

1. Introduction

Benzyl alcohol is a naturally occurring monoaromatic alcohol with a broad range of commercial applications and a current market price of $2000–2500 USD/ton. With both low volatility and toxicity yet strong polarity, benzyl alcohol is attractive as a safe and effective solvent, particularly for use with polymers and in applications including the production of inks, paints, glues, and hardening products (e.g., epoxy resins) (Ash and Ash, 2009; Stellman, 1998; Stoye and Freitag, 1998). Additionally, while benzyl alcohol itself confers a floral scent, it is more commonly employed as a precursor to synthesize a variety of other ester products with numerous flavor/fragrance uses, including in the manufacture of food products (Fenaroli and Burdock, 1995), as well as high value hygiene and cosmetic products. For example, prior reports have found benzyl alcohol to be used in 322 cosmetic formulations belonging to 43 product categories (Nair, 2001). Meanwhile, as it renders a bacteriostatic effect at even low concentrations (Marriott, 2010), benzyl alcohol is also commonly used as a topical agent and preservative in the pharmaceutical and healthcare industries (Felton, 2013; Meinking et al., 2010; Wilson and Martin, 1999).

Benzyl alcohol is naturally synthesized by many plants, notably accumulating in edible fruits and tea leaves, as well as in the essential oils of ylang–ylang, jasmine, and hyacinth (Budavari et al., 1989). In such cases, however, benzyl alcohol contents have rarely been found to surpass even ~30 mg/kg (COE, 1992) rendering these natural sources as unsuitable for supporting a commodity scale benzyl alcohol bioproduction efforts. Accordingly, conventional production of benzyl alcohol is achieved from petroleum-derived feedstocks. Most commonly this occurs from benzyl chloride (considered a 'probable carcinogen') via alkaline hydrolysis (e.g., with sodium hydroxide) (Yadav and Mehta, 1993). In addition to employing energy intensive and harsh reaction conditions, this process suffers from sustainability concerns as it involves the use of non-renewable feedstocks.

As an alternative and more sustainable approach, the *de novo* biosynthesis of benzyl alcohol directly from renewable glucose was herein explored through the systematic engineering of a non-natural biosynthetic pathway engineered in the bacterium *Escherichia coli*. The proposed pathway, which utilizes phenylpyruvate as its immediate endogenous precursor, is illustrated in Fig. 1. First, phenylpyruvate is converted to (*S*)-mandelate via expression of hydroxymandelate synthase (*hmaS*) from *Amycolatopsis orientalis*. Though its native substrate is 4-hydroxyphenylpyruvate, HmaS has also been shown to display activity on phenylpyruvate (Sun et al., 2011). (*S*)-Mandelate is subsequently converted to benzaldehyde by co-expression of two genes derived from the mandelate degradation pathway of *Pseudomonas putida* ATCC 12633 (Tsou et al., 1990). Specifically, conversion of (*S*)-mandelate to phenylglyoxylate by (*S*)-

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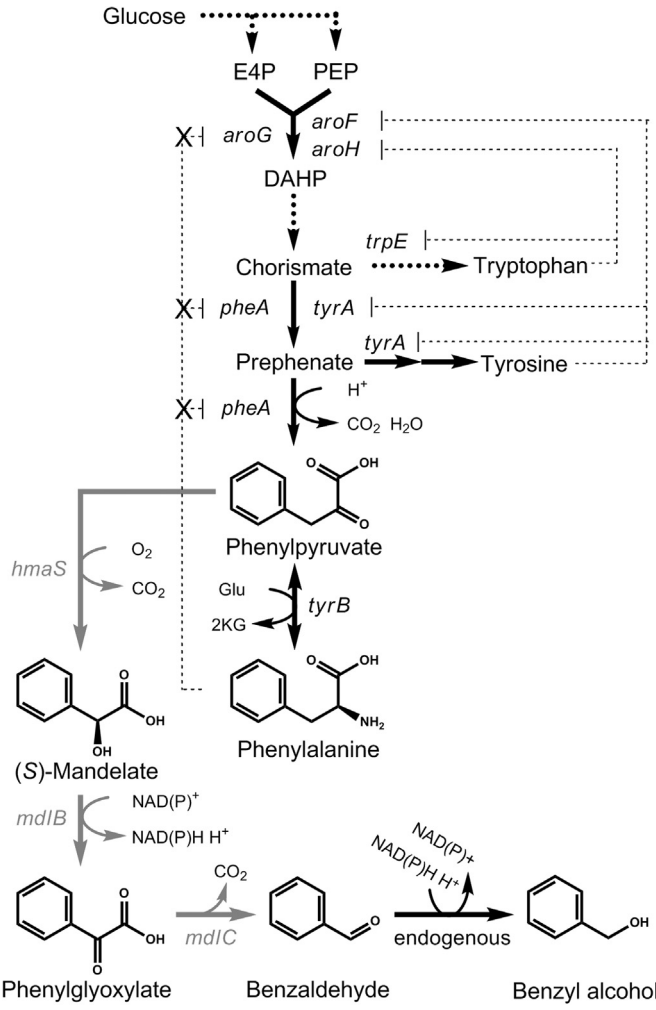

Fig. 1. Proposed pathway for benzaldehyde and benzyl alcohol biosynthesis from glucose by engineered *E. coli*. Dashed arrows indicate multiple steps. Black and gray arrows indicate native and heterologous pathway steps, respectively. Abbreviations: phosphoenolpyruvate (PEP), D-erythrose-4-phosphate (E4P), 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP).

mandelate dehydrogenase (*mdlB*) followed by decarboxylation of phenylglyoxylate to benzaldehyde by phenylglyoxylate decarboxylase (*mdlC*). The production of benzyl alcohol from benzaldehyde has been reported to occur naturally in *E. coli* as a result of the native function of multiple endogenous alcohol dehydrogenases (ADHs) and/or aldo–keto reductases (AKRs). For example, *E. coli yqhD* has been shown to display substantial activities with respect to the NADPH-dependent reduction of benzaldehyde (Sulzenbacher et al., 2004). Meanwhile, in another recent study it was demonstrated that the native regulation and activity of multiple ADHs/AKRs from *E. coli* (specifically, *yqhD, yjgB*, and *yahK*) was sufficient for the rapid and efficient *in vivo* reduction of 2-phenylacetaldehyde to 2-phenylethanol – a structurally similar aromatic substrate–product pair likewise synthesized via a heterologous pathway (Koma et al., 2012). This study outlines our recent progress towards the systematic engineering of the proposed benzyl alcohol pathway, along with preliminary efforts in host strain engineering to improve initial product titers and yields.

2. 2. Materials and methods

2.1. Bacterial strains and media

All strains constructed and used in this study are listed in Table 1. *E. coli* NEB10-Beta was obtained from New England Biolabs (NEB, Ipswich, MA) and was used for all cloning work and the plasmid propagation. *E. coli* NST74 (ATCC 31884) – a previously developed, feedback-deregulated phenylalanine overproducer (Tribe, 1987) – was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and used as the initial host platform for aromatics production. *P. putida* ATCC 12633 was also obtained from the ATCC and served as the genetic source of *mdlB* and *mdlC*. *E. coli* JW2581-1, JW4014-2, and JW0911-1 were all obtained from the Coli Genetic Stock Center at Yale University (CGSC, New Haven, CT) and used as the source of genetic materials for the chromosomal deletion of *tyrA*, *tyrB*, and *aspC*, respectively.

E. coli and *Pseudomonas* sp. were routinely cultured in Luria–Bertani (LB) broth supplemented with ampicillin (100 mg/L), chloramphenicol (34 mg/L), and kanamycin (40 mg/L), as appropriate. For aromatics production, strains were cultured in a phosphate-limited minimal media with 15 g/L glucose (herein referred to as "MM1"), previously adapted from that of Qi et al. (2007) and described by McKenna and Nielsen (2011). To compensate for auxotrophies introduced in mutant strains, MM1 media was supplemented with tyrosine (0.1 g/L) and aspartate (3 g/L), as appropriate.

Table 1
Strains and plasmids constructed and/or used in this study.

Strains	**Description**	**Source**
E. coli NEB10-Beta	*araD139 Δ(ara,leu)7697 fhuA lacX74 galK16 galE15 mcrA f80d(lacZΔM15)recA1 relA1 endA1 nupG rpsL rph spoT1Δ(mrr-hsdRMS-mcrBC)*	NEB
E. coli NST74	*aroH367, tyrR366, tna-2, lacY5, aroF394(fbr), malT384, pheA101(fbr), pheO352, aroG397(fbr)*	ATCC
P. putida ATCC12633	Source of *mdlB, mdlC, mdlD*	ATCC
E. coli JW2581-1	source of *tyrA*:FRT-Kan-FRT	CGSC
E. coli JW4014-2	source of *tyrB*:FRT-Kan-FRT	CGSC
E. coli JW0911-1	source of *aspC*:FRT-Kan-FRT	CGSC
E. coli NST74A	NST74 Δ*tyrA*:FRT	This study
E. coli NST74AB	NST74 Δ*tyrA*:FRT Δ*tyrB*:FRT	This study
E. coli NST74ABC	NST74 Δ*tyrA*:FRT Δ*tyrB*:FRT Δ*aspC*:FRT	This study
Plasmids	**Description**	**Source**
pTrc99A	Ptrc, pBR322 *ori*, *lacIq*, Amp^R	Prather Lab, MIT
pTrcCOLAK	Ptrc, ColA *ori*, *lacIq*, Kan^R	McKenna et al. (2013)
pUC57-HmaS	pMB1 *ori*, Amp^R, *hmaS* (codon optimized for *E. coli*)	Genscript
pHmaS	*hmaS* of pUC57-HmaS inserted into the NcoI and EcoRI sites of pTrcCOLAK	This study
pHmaS-MdlC	*mdlC* of *P. putida* ATCC 12633 inserted into the XbaI and HindIII sites of pHmaS with second Ptrc inserted ahead of *mdlC* between the BamHI and XbaI sites	This study
pMdlB	*mdlB* of *P. putida* ATCC 12633 inserted between the NcoI and EcoRI sites of pTrc99A	This study