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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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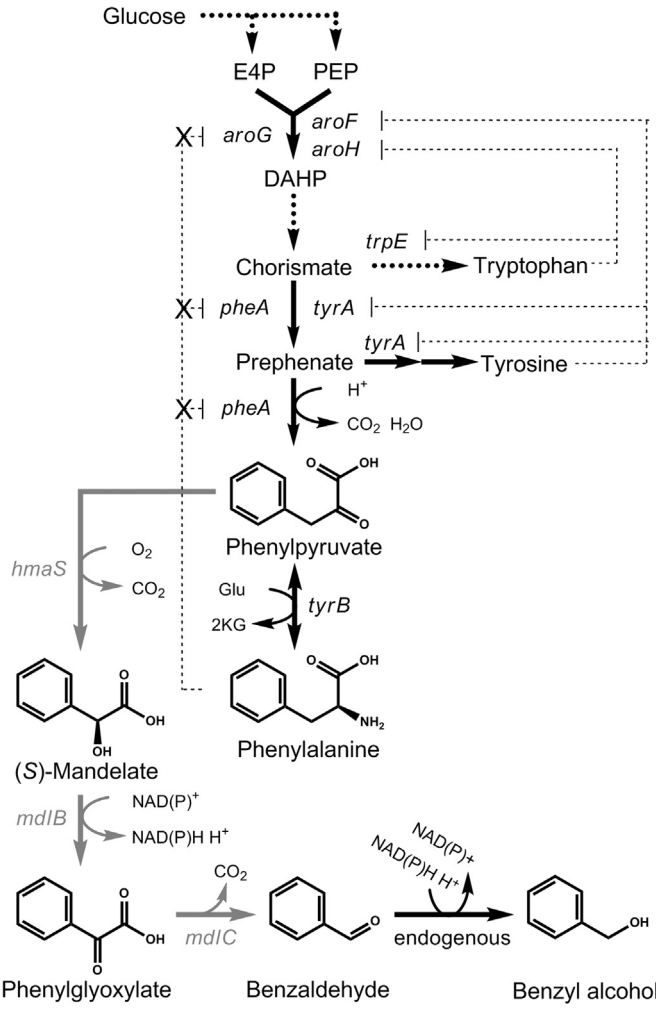

Fig. 1. Proposed pathway for benzaldehyde and benzyl alcohol biosynthesis from glucose by engineered *E. coli*. Dashed arrows indicate multiple steps. Black and gray arrows indicate native and heterologous pathway steps, respectively. Abbreviations: phosphoenolpyruvate (PEP), D-erythrose-4-phosphate (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	P*trc*, pBR322 *ori*, *lacIq*, Amp^R	Prather Lab, MIT
pTrcCOLAK	P*trc*, 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 P*trc* 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

2.2. Plasmid construction

All plasmids used in this study are also listed in Table 1. The plasmid pTrcCOLAK, a fusion of pTrc99A and pCOLADuet-1 (Invitrogen, Carlsbad, CA), was developed in house as previously described (McKenna et al., 2013). The hydroxymandelate synthase encoding gene, *hmaS* from *A. orientalis,* was synthesized to include codon optimization for high-level expression in *E. coli* by Genscript (Piscataway, NJ) and harbored in plasmid pUC57-HmaS. To construct pHmaS, codon optimized *hmaS* was PCR amplified from pUC57-HmaS and inserted between the NcoI and EcoRI sites of pTrcCOLAK. Subsequently, *mdlC* was PCR amplified from the gDNA of *P. putida* ATCC 12633 and inserted between the XbaI and HindIII sites of pHmaS. To ensure high-level expression of *mdlC,* a second P_{trc} promoter was inserted ahead of *mdlC* (between the BamHI and XbaI sites), resulting in pHmaS-MdlC. To construct pMdlB, *mdlB* was PCR amplified from *P. putida* ATCC 12633 gDNA and inserted between the NcoI and EcoRI sites of pTrc99A.

Custom DNA oligonucleotide primers were designed and synthesized by Integrated DNA Technologies (Coralville, IA). All oligonucleotide primers used in this study are listed in Table S1 (see Supplementary Information). Genomic DNA (gDNA) was prepared from cultures using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, Irvine, CA) according to vendor protocols. All genes were PCR amplified using Phusion High-Fidelity DNA Polymerase (NEB) according to standard protocols. Amplified linear DNA fragments were purified using the DNA Clean & Concentrator kit (Zymo Research) according to manufacturer protocols. Purified DNA was subsequently digested using appropriate restriction endonuclease enzymes (NEB) at 37 °C for 3 h. Digested fragments were gel purified using the Zymoclean Gel DNA Recovery kit (Zymo Research) and ligated using T4 DNA ligase (NEB) at 4 °C overnight. Ligation reactions were transformed into chemically competent *E. coli* NEB10-Beta (NEB) and selected by plating on LB solid agar containing appropriate antibiotics (as above). Subsequently, transformant pools were screened first by colony PCR (using the same primers as for cloning) and then by restriction digest mapping. Final plasmid constructs were verified by sequencing.

2.3. Construction of E. coli deletion mutants

Chromosomal in-frame gene deletions were accomplished via a protocol adapted from the one-step inactivation method of Datsenko and Wanner (2000). Deletion cassettes for all targeted loci, each of which harbored a kanamycin resistance gene flanked by FLP recognition target sites, were PCR amplified from the gDNA of appropriate Keio collection mutants (Table 1) (Baba et al., 2006). In each instance, primer pairs were designed to amplify 300 bp of homology both upstream and downstream of the target gene sequence. Subsequent recombination steps were performed as previously described (Datsenko and Wanner, 2000).

2.4. Assaying recombinant pathway function via whole cell biotransformation studies

E. coli NST74 was co-transformed with both pHmaS-MdlC and pMdlB. Single colonies were selected from the transformant pool and seed cultures were grown in 5 mL LB broth with appropriate antibiotics at 37 °C while shaking at 200 RPM overnight. Each seed culture was used to inoculate 50 mL LB supplemented with 20 g/L glucose and appropriate antibiotics in a 250 mL baffled shake flasks. Upon reaching an optical density at 600 nm (OD_{600}) of ~0.7, cultures were induced by addition of 0.25 mM IPTG. Culturing continued overnight before cells were harvested by centrifugation at 3000*g*, washed twice with pH7 phosphate buffered saline (PBS) solution, and re-suspended in 50 mL pH7 PBS with 5 g/L glucose and 1 g/L phenylpyruvate. Cultures were incubated at 37 °C while levels of each of phenylpyruvate, phenylalanine, (*S*)-mandelate, phenylglyoxylate, benzaldehyde, and benzyl alcohol were subsequently monitored in the media for the next 7 h by periodic sampling for high performance liquid chromatography (HPLC) analysis. This experiment was repeated in triplicate to provide estimates of standard error.

2.5. Benzyl alcohol production directly from glucose by engineered E. coli strains

To test for benzyl alcohol production directly from glucose, each of *E. coli* NST74, NST74A, NST74AB, and NST74ABC were co-transformed with pHmaS-MdlC and pMdlB. Seed cultures were prepared as above and used to inoculate 50 mL MM1 media in a 250 mL baffled shake flask supplemented with appropriate antibiotics and, as needed, required amino acids. Cultures were grown at 37 °C while shaking at 200 RPM for 10 h prior to induction by addition of 0.25 mM IPTG. Culturing continued for an additional 96 h, during which time media samples were routinely removed and prepared for analysis by HPLC. All cultures were performed in triplicate to provide estimates of standard error.

2.6. Assaying benzyl alcohol toxicity

Cursory estimates of benzyl alcohol toxicity against *E. coli* NST74 were obtained by monitoring for changes in growth rate and yield that occur following its exogenous addition to growing cultures at different concentrations. Cultures were grown in 50 mL MM1 media in 250 mL baffled glass shake flasks at 37 °C while shaking at 200 RPM. At an OD_{600} of ~0.5, benzyl alcohol was added to the cultures at final concentrations ranging from 0 to 2 g/L. Growth was then routinely monitored by measurement of OD_{600} for an additional 6 h. All cultures were performed in triplicate to provide estimates of standard error.

2.7. Metabolite analysis by HPLC

Samples were prepared by centrifuging 1 mL of culture at 10,000*g* for 3 min to pellet and remove cells. Supernatants were transferred to an HPLC vial with a Teflon-lined cap. HPLC analysis was performed using a Hewlett Packard 1100 series HPLC system (Agilent, USA). Metabolites were separated using a reverse-phase Hypersil Gold aQ polar end capped C18 column (4.6 mm × 150 mm; Thermo Fisher, USA) maintained at 45 °C and measured with a diode array detector operated at 215 nm (for phenylalanine, (*S*)-mandelate, phenylpyruvate, and benzyl alcohol) and 255 nm (for phenylglyoxylate and benzaldehyde). Samples (5 μL) were injected into a mobile phase with a constant total flow rate of 0.95 mL/min. The mobile phase consisted of 'solvent A' consisting of nanopure water, and 'solvent B' consisting of HPLC-grade methanol (99.8% pure). Beginning as a mixture (vol/vol) of 95% solvent A and 5% solvent B, a linear gradient was then applied over 8 min until reaching 20% solvent A and 80% solvent B. This condition was then held for 2 min before a second liner gradient was applied over 4 min until reaching 95% solvent A and 5% solvent B. Under these conditions, phenylalanine, phenylglyoxylate, (*S*)-mandelate, phenylpyruvate, benzyl alcohol, and benzaldehyde were eluted at 4.3, 4.6, 5.9, 6.2, 7.1, and 7.8 min, respectively. Standard solutions were developed for each species and used as external calibrations to determine concentrations.

2.8. Measurement of biomass growth

Optical density measurements at 600 nm (OD_{600}), performed with a DU800 spectrophotometer (Beckman Coulter, Brea, CA),

were used to determine biomass concentration. Dry cell weight (DCW) was then predicted using an established conversion factor (1 OD_{600} = 0.26 g/L) (Guo et al., 2012).

3. Results and discussion

3.1. Investigating heterologous enzyme and pathway function

Recombinant activity of all candidate enzymes as part of the composite pathway was first assayed by investigating the conversion of exogenously supplied phenylpyruvate by *E. coli* NST74 pHmaS-MdlC pMdlB resting cells. As illustrated in Fig. 2, the entire 1 g/L of initially added phenylpyruvate was consumed within the first 7 h of the experiment. In this time, phenylglyoxylate first accumulated before then being mostly consumed within ~1.5 h. As phenylglyoxylate was consumed, both benzaldehyde and benzyl alcohol began to accumulate, doing so at similar initial rates. After 1 h, however, benzaldehyde accumulation slowed, reaching a maximum titer of 60 ± 9 mg/L at 3 h before then gradually declining through the remainder of the experiment. Benzyl alcohol finally emerged as the major pathway metabolite, approaching a maximal titer of 203 ± 5 mg/L by 7 h (note: by 24 h, (*S*)-mandelate, phenylglyoxylate, and benzaldehyde were undetected or present only at trace levels while benzyl alcohol titers reached 222 ± 3 mg/L; data not shown). In addition, however, phenylalanine also accumulated, reaching a titer of 610 ± 30 mg/L after 7 h. At this level, the competing biosynthesis of phenylalanine was responsible for consuming 63% of supplied phenylpyruvate, with only 32% ultimately being converted to benzyl alcohol.

Several relevant insights were gained through this initial assay. First, the candidate heterologous enzymes and proposed pathway are functionally expressed in *E. coli* under the examined conditions. Second, a flux bottleneck emerged early on at MdlC, possibly as the result of a cofactor (i.e., NAD(P)$^+$) limitation that was later balanced and eliminated as flux proceed to benzyl alcohol via the NAD(P)H-dependent reduction of benzaldehyde. Third, despite only expressing *hmaS*, *mdlB*, and *mdlC* (which encode the three steps from phenylpyruvate to benzaldehyde; Fig. 1), metabolite flux continued efficiently through benzaldehyde to benzyl alcohol, confirming that one or more of *E. coli*'s native AKRs and/or ADHs with activity on benzaldehyde were expressed and functional under the conditions studied. Moreover, considering that cultures were not previously adapted with or even exposed to benzaldehyde, the native regulation of said gene(s) was not part of a benzaldehyde-inducible response, but rather was likely the subject to constitutive expression. Lastly, native phenylalanine biosynthesis clearly emerged as a significant competitor for phenylpyruvate availability (Fig. 1). This suggests that the engineered pathway, or at least its first committed step (i.e., HmaS), was poorly competitive against the native function of phenylalanine aminotransferase (i.e., TyrB).

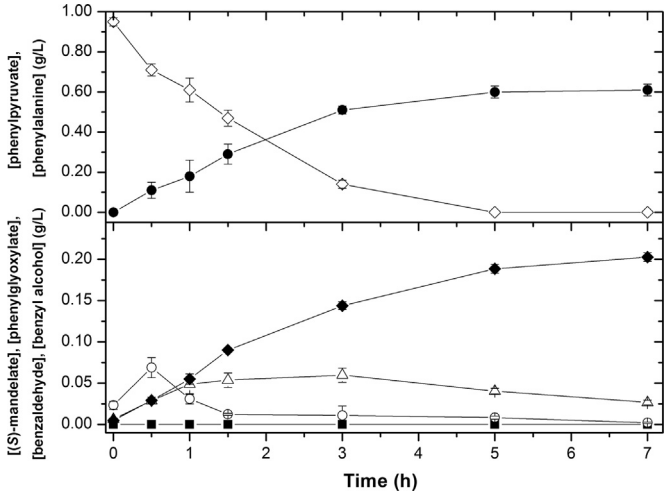

Fig. 2. Demonstrating pathway function and monitoring metabolite flux via a whole resting cell biotransformation assay using exogenous phenylpyruvate and E. coli NST74 pHmaS-MdlC pMdlB. The conversion of 1 g/L phenylpyruvate (open diamonds) to phenylalanine (solid circles), (S)-mandelate (solid squares), phenylglyoxylate (open circles), benzaldehyde (open triangles), and benzyl alcohol (solid diamonds) was monitored over the course of 7 h. Error bars reported at one standard deviation from triplicate experiments.

3.2. Benzyl alcohol biosynthesis from glucose by using E. coli NST74 as production host

Having demonstrated the initial and promising function of the engineered pathway, benzyl alcohol biosynthesis directly from glucose was next investigated. With *E. coli* NST74 as the initial production host, however, benzyl alcohol accumulation to a maximum titer of only 23 ± 2 mg/L was possible in 96 h. Instead, phenylalanine again accumulated as the major end product, reaching up to 900 ± 160 mg/L. This observation agrees well with the outcomes of the phenylpyruvate biotransformation study (Fig. 2), and was likely a result of the limited affinity of HmaS for phenylpyruvate as substrate. Although HmaS is known to demonstrate activity on phenylpyruvate, its natural and preferred substrate is 4-hydroxyphenylpyruvate. Accordingly, HmaS displays a nearly 70-fold lower affinity for phenylpyruvate (K_m 0.45 ± 0.04 mM) versus 4-hydroxyphenylpyruvate (K_m 6.5 ± 0.8 μM) (He et al., 2010). However, as no other isoenzymes specific for (*S*)-mandelate are presently known, HmaS remains the only suitable candidate at this time. Future isolation or engineering of a superior mandelate synthase will likely be required to achieve increased metabolite flux into the benzyl alcohol pathway, thereby improving achievable titers and yields. For now, the prospect of enhancing benzyl alcohol production by preserving phenylpyruvate availability was examined by eliminating competing aromatic amino acid biosynthesis pathways.

3.3. Host strain engineering to improve precursor availability and benzyl alcohol production

In addition to metabolite flux losses to phenylalanine (Fig. 2), the competing biosynthesis of tyrosine has also previously been found to detract from achievable titers and yields of other aromatic chemicals similarly derived from the phenylalanine biosynthesis pathway (McKenna et al., 2013). Accordingly, disruption of both tyrosine and phenylalanine biosynthesis was systematically examined in support of enhancing benzyl alcohol production. While deletion of *tyrA* (a bifunctional chorismate mutase/prephenate dehydratase) resulted in greater flux of prephenate through the phenylalanine branch of the pathway (leading to 53% higher phenylalanine titers; NST74A in Table 2), increased production of benzyl alcohol was not coincidentally observed (both titer and yield were reduced). To preserve phenylpyruvate, phenylalanine aminotransferase activity was next targeted for disruption. *E. coli* possesses three aminotransferases with reported activity on phenylpyruvate, including *tyrB*, *aspC*, and *ilvE* (the latter two are nominally functional on aspartate and branched-chain amino acids, respectively) (Keseler et al., 2005). Prior studies have shown that by deleting both *aspC* and *tyrB* (which possesses nearly 1000-fold higher activity than *aspC*) while leaving *ilvE* intact, flux of phenylpyruvate to phenylalanine can be reduced to all but the minimal level required to avoid generating a complete phenylalanine auxotroph (Keseler et al., 2005; Sun et al., 2011).

Table 2
Comparison of benzyl alcohol, phenylalanine, and biomass produced after 72 h by different *E. coli* host strains each harboring pHmaS-MdlC pMdlB.

Host strain	Benzyl alcohol Titer (mg/L)	Yield (mg/g)	Phenylalanine Titer (mg/L)	Yield (mg/g)	Biomass (DCW) Titer (g/L)	Yield (g/g)
NST74	23 ± 3	1.5 ± 0.2	900 ± 160	60 ± 10	2.2 ± 0.2	0.15 ± 0.02
NST74A	7 ± 1	0.5 ± 0.1	1380 ± 20	92 ± 1	1.7 ± 0.1	0.11 ± 0.01
NST74AB	45 ± 4	3.0 ± 0.2	550 ± 20	37 ± 1	1.1 ± 0.1	0.07 ± 0.01
NST74ABC	114 ± 1	7.6 ± 0.1	410 ± 20	27 ± 1	0.9 ± 0.1	0.06 ± 0.01

Accordingly, strains NST74AB and NST74ABC were next constructed and tested as benzyl alcohol production hosts. As illustrated in Table 2, benzyl alcohol titers were nearly doubled to 45 ± 4 mg/L using NST74AB, and further increased to 114 ± 1 mg/L (a ~5-fold increase) using NST74ABC. In both cases, increased benzyl alcohol production was met with corresponding decreases in both phenylalanine and net biomass accumulation, with the latter likely resulting from fitness losses due to reduced amino acid biosynthesis. It should also be noted that no benzaldehyde accumulation was observed at any time for any strains, again confirming that sufficient expression of *E. coli*'s associated AKRs and/or ADHs was achieved via native regulation alone. At its maximum achievable output, the current yield of benzyl alcohol on glucose reached 7.6 ± 0.1 mg/g, or just 3.2% of its theoretical maximum value (240 mg/g; note: the theoretical yield of phenylalanine on glucose has been reported as 0.4 mol/mol (Juminaga et al., 2012)).

3.4. Assaying the effect benzyl alcohol on E. coli growth

In addition to the potential fitness reducing effects caused by disrupting aromatic amino acid biosynthesis pathways (Table 2), it is possible that benzyl alcohol accumulation in the culture medium further contributed to the observed reduction in biomass growth. This was a particularly relevant concern in this study because, as discussed above, benzyl alcohol is known to possess and is often specifically utilized for its bacteriostatic properties (Marriott, 2010). To understand the potential toxic effects associated with benzyl alcohol accumulation, as well as to estimate future limits on achievable titers, a growth challenge assay was lastly performed using exogenous benzyl alcohol. Although exogenous addition does not fully represent the expected environment when benzyl alcohol is instead synthesized intracellularly, this approach has been to provide at least useful first approximations of toxicity for other aromatic products against *E. coli* (McKenna et al., 2013 ; Pugh et al., 2014). As seen in Fig. 3, the initial growth rate was reduced in the presence of 0.25 g/L benzyl alcohol, but not growth yield. However, as the benzyl alcohol concentration was increased to 0.5 g/L and beyond, both growth rates and yields continued to decline. In the presence of as much as 0.75 g/L benzyl alcohol growth was completely halted following exposure. From this, the toxicity limit of benzyl alcohol against *E. coli* was approximated to be ~0.75 g/L. Lucchini et al. (1990) reported a value of ~0.4 g/L, albeit with respect to a different strain of *E. coli* and under different culture conditions.

The toxicity of most aromatic hydrocarbons against *E. coli* and other Gram-negative bacteria has commonly been suggested to be non-specific in nature, occurring as a result of species lipophilicity which leads to accumulation within the cytoplasmic membrane, whereupon structural integrity and function become damaged (Meylan et al., 1999; Ramos et al., 2002). To this end, species with log $K_{O/W}$ values of 1–5 have been found to impose significant toxicity against bacteria (note: for benzyl alcohol, log $K_{O/W}$=1.10) (Ramos, 2004). At the levels observed here, however, benzyl alcohol toxicity against *E. coli* was interestingly found to be poorly represented by a general toxicity model that was previously

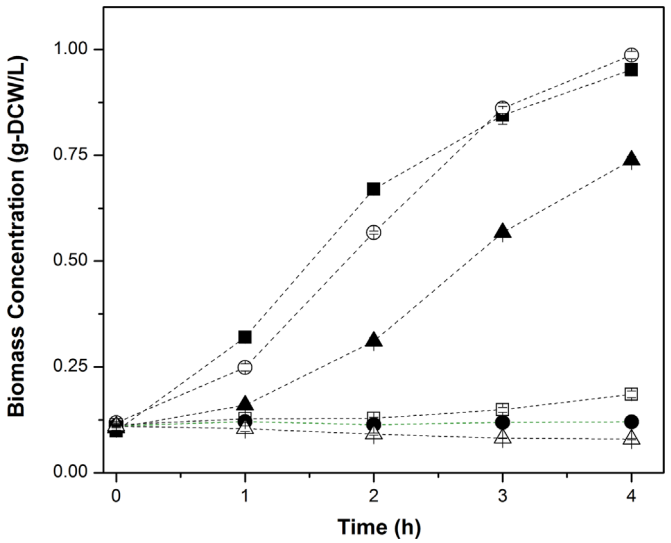

Fig. 3. Growth response of *E. coli* NST74 to benzyl alcohol addition at concentrations of 0 g/L (solid squares), 0.25 g/L (open circles), 0.5 g/L (solid triangles), 0.75 g/L (open squares), 1 g/L (solid circles), and 2 g/L (open triangles). Error bars reported at one standard deviation from triplicate experiments.

developed to relate the relative toxicity threshold of an aromatic hydrocarbon with its relative affinity for membrane accumulation (as quantified via the membrane–water partition coefficient, $K_{M/W}$, and predicted using a previously developed model (Sikkema et al., 1994)) (McKenna et al., 2013). Despite providing a strong linear correlation for several other monoaromatic solvents (i.e., styrene, (*S*)-styrene oxide, (*R*)-1,2-phenylethanediol, *trans*-cinnamate, *p*-hydroxystyrene), the apparent toxicity threshold of benzyl alcohol determined here was ~3-fold lower than its predicted level (~2–2.5 g/L). This implies that other, more specific stress factors may also be contributing to the overall toxicity of benzyl alcohol. Others have also found the toxicity of related aromatic alcohols to be due to both general and specific mechanisms (Lucchini et al., 1990). Although a causal relationship was not fully elucidated, exposure to the closely related aromatic alcohol 2-phenylethanol, for example, has been shown to lead to decreased rates of both DNA and RNA synthesis, on top of expected membrane stresses (Lucchini et al., 1993). Still, as current achievable benzyl alcohol titers remain well below the apparent toxicity threshold, it is unlikely that toxicity was a significant, productivity-limiting factor in this study. However, strategies to address this concern will ultimately be required as future strain and pathway engineering efforts lead to further improved benzyl alcohol production. One attractive approach to this end would involve the expression solvent efflux pumps to actively expel inhibitory products from the cell as they are produced (Dunlop, 2011; Dunlop et al., 2011). This strategy appears to be a particularly promising for aromatic products such as benzyl alcohol since several resistance-nodulation-cell division (RND) family efflux pumps from *Pseudomonas* sp. are known to display activity on aromatic species

(Kieboom et al., 1998; Ramos et al., 2002; Rojas et al., 2001).

3.5. Understanding and controlling the native reduction of benzaldehyde to benzyl alcohol

As discussed above, prior works have shown that several ADHs and/or AKRs are involved in the native ability of *E. coli* to reduce benzaldehyde to benzyl alcohol. As we began to explore this phenomenon in the context of *E. coli* benzyl alcohol production (for example, we found that initial rates of benzaldehyde reduction by NST74 Δ*yahK* Δ*yjgB* were nearly 5-fold lower than by NST74; Fig. S1, see Supplementary Information), however, a concurrent study by Kunjapur et al. reported an insightful and comprehensive investigation to this very same end (Kunjapur et al., 2014). Ultimately, an *E. coli* strain was engineered in that study with reduced aromatic aldehyde reduction (RARE) abilities. Lacking 3 AKRs (*dkgB*, *yeaE*, *dkgA*), 3 ADHs (*yqhD*, *yahK*, *yjgB*), and the transcriptional activator *yqhC*, *E. coli* RARE converted less than 12% of exogenous benzaldehyde to benzyl alcohol after 24 h. For comparison, despite lower initial rates of benzaldehyde reduction by NST74 Δ*yahK* Δ*yjgB*, all added benzaldehyde was ultimately converted to benzyl alcohol within 24 h (data not shown). In related works, meanwhile, Rodriguez and Atsumi also explored the engineering of an *E. coli* strain deficient in aldehyde reductase activity (Rodriguez and Atsumi, 2014). Although benzyaldehyde was not evaluated as a substrate, the related aromatic 2-phenylacetaldyde (produced endogenously from phenylpyruvate by Kivd) was investigated. In this case, a mutant lacking 12 aldehyde reductase encoding genes (*eutE, yahK, yqhE, gldA, ybbO, yghA, yqhD, adhP, eutG, yiaY, yjgB, fucO*) was incapable of reducing 2-phenylacetaldyde to 2-phenylethanol. The similarly observed importance of deleting *yqhD*, *yahK*, and *yjgB* further underscores their likely and general role in *E. coli*'s native ability to reduce aromatic aldehydes.

This insight is important not only for understanding how the host genotype influences flux through the engineered pathway, but also, if this terminal step can be predictably controlled the proposed pathway could furthermore be leveraged to explore the *de novo* biosynthesis of benzaldehyde as an alternative end product. With a global annual production exceeding 90,000 tons (second to only vanillin), benzaldehyde, is a particularly important flavor molecule in the food and fragrance industry (Culp and Noakes, 1990; Krings and Berger, 1998; Satrio and Doraiswamy, 2001), in addition to serving as a precursor to several fine chemicals and pharmaceutical precursors (e.g., (L)-phenylacetylcarbinol, or L-PAC) (Rosche et al., 2001). Such prospects will be the subject of future investigations.

4. Conclusions

A non-natural pathway to synthesize benzyl alcohol from glucose has been established in *E. coli*. Furthermore, through additional strain engineering to control the native reduction of benzaldehyde it is expected that the same materials could furthermore be employed to establish a biosynthetic route to benzaldehyde. As the pathway is currently limited by low activity at the first committed step, further efforts in enzyme engineering and/or bioprospecting along with the systematic optimization of expression conditions are needed to ultimately elevate key production metrics to viable levels.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.meteno.2015.06.002.

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