Metabolic Engineering 19 (2013) 33–41

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

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

Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production

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ARTICLE INFO

Article history:
Received 17 November 2012
Received in revised form
17 May 2013
Accepted 22 May 2013
Available online 29 May 2013

Keywords:
Limonene
Perillyl alcohol
Mevalonate pathway
Microbial production
Metabolic engineering
Escherichia coli

ABSTRACT

Limonene is a valuable monoterpene used in the production of several commodity chemicals and medicinal compounds. Among them, perillyl alcohol (POH) is a promising anti-cancer agent that can be produced by hydroxylation of limonene. We engineered *E. coli* with a heterologous mevalonate pathway and limonene synthase for production of limonene followed by coupling with a cytochrome P450, which specifically hydroxylates limonene to produce POH. A strain containing all mevalonate pathway genes in a single plasmid produced limonene at titers over 400 mg/L from glucose, substantially higher than has been achieved in the past. Incorporation of a cytochrome P450 to hydroxylate limonene yielded approximately 100 mg/L of POH. Further metabolic engineering of the pathway and in situ product recovery using anion exchange resins would make this engineered *E. coli* a potential production platform for any valuable limonene derivative.

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1. Introduction

Limonene, a monoterpene, is an important precursor to several pharmaceutical and commodity chemicals (Keasling, 2010) Traditionally, (+)-D-limonene has been obtained from plant biomass as a byproduct of orange juice production, but fluctuations in availability and cost limit its use as a biofuel and chemical feedstock, the demand for which are increasing (Duetz et al., 2003). The hydrogenated form of limonene has low freezing point and is immiscible with water, favorable properties for next generation jet-biofuels and fuel additives that enhance cold-weather performance (Ryder, 2009; Tracy et al., 2009). Limonene is also notable for its pleasant fragrance (orange scent) and its designation as a Generally Recognized As Safe (GRAS) compound has driven demand for inclusion of limonene in earth-friendly cleaning products (Bomgardner, 2011). There is also great market potential for oxygenated derivatives of limonene, such as α-terpineol, perillyl alcohol (POH), carveol, carvone, and menthol, which are important flavor and medicinal compounds (Duetz et al., 2003). For example, bulk prices for menthol and both enantiomers of carvone are in the range of U.S. $30–60/kg and prices for (−)- and (+)-POH are even higher (i.e., ~$4500/kg) due to their limited availability in nature and potential anti-carcinogenic properties (Gelb et al., 1995; Gould, 1997; Hohl, 1996; van Beilen et al., 2005; Wagner and Elmadfa, 2003). The regiospecific hydroxylation of limonene by traditional chemical oxidation usually produces mixtures of products due to the similar electronic properties of the target carbons on the limonene skeleton (Duetz et al., 2003). As a consequence, the biocatalytic conversion of limonene into a regioselectively hydroxylated product has great potential.

Previous efforts to biosynthesize limonene and its derivatives in *E. coli* were limited by the low intracellular levels of the isoprenoid precursors, especially geranyl pyrophosphate (GPP) provided by the native deoxyxylulose 5-phosphate (DXP) pathway (Carter et al., 2003). All isoprenoids are synthesized from isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), which are subsequently condensed into GPP, farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP), the precursors to monoterpenes, sesquiterpenes, and diterpenes, respectively (Fig. 1a). To produce IPP and DMAPP there are two major isoprenoid biosynthetic pathways: the mevalonate-dependent isoprenoid pathway (MEV pathway) and the mevalonate-independent or DXP pathway. Eukaryotes use the MEV pathway (with the exception of plants that

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1096-7176/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.ymben.2013.05.004

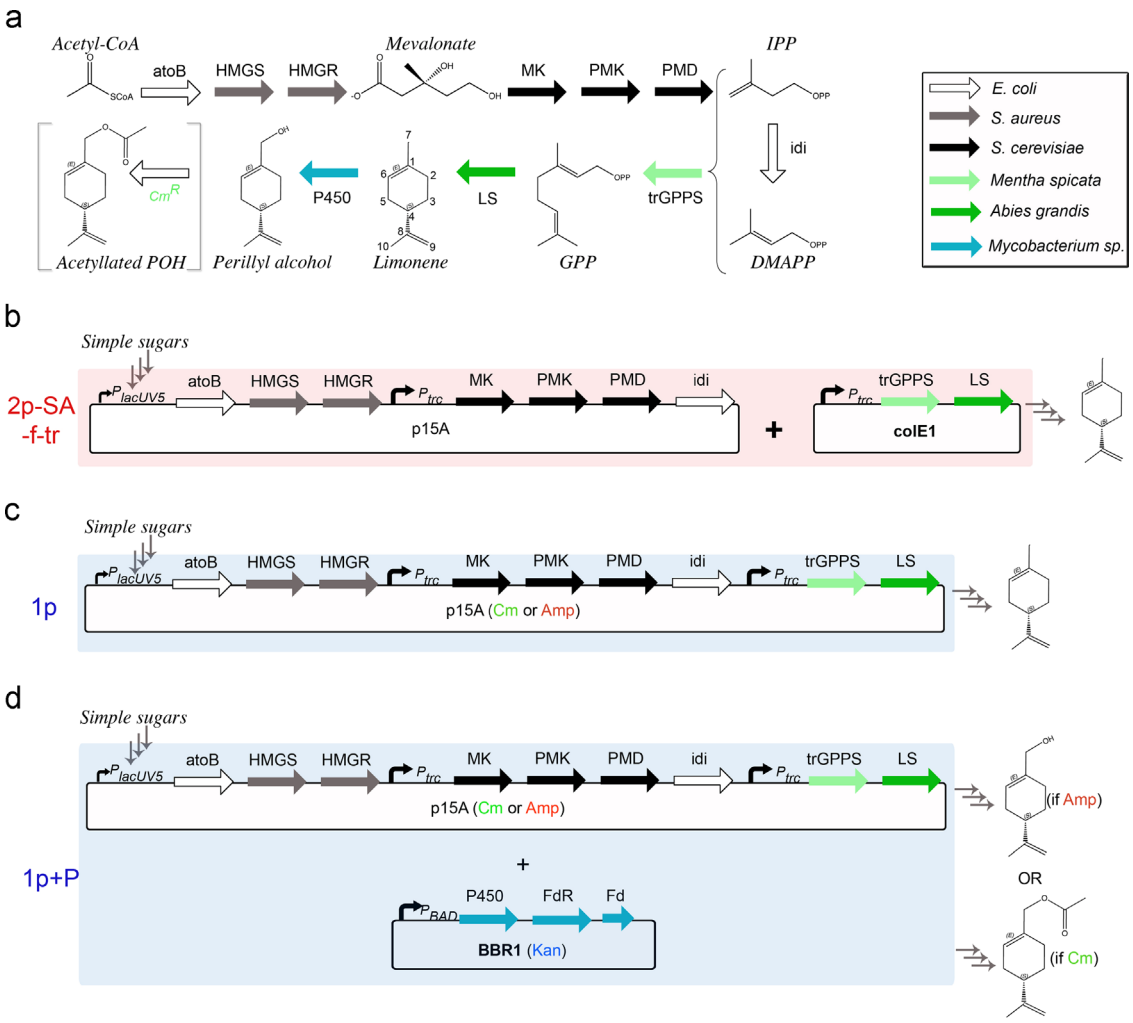

Fig. 1. The heterologous mevalonate (MEV) pathway introduced into *E. coli* for the production of limonene and perillyl alcohol (POH). (A) Enzymes and some of the reaction intermediates necessary for the production of limonene and POH through the MEV pathway. Coding sequences for the different enzymes were obtained from different organisms as depicted in the legend. In brackets is the reaction carried out by the chloramphenicol resistance gene (Cm^R), which is actually a promiscuous acetyltransferase (CAT) that acetylates chloramphenicol to inactivate it, but also uses POH as a substrate to produce the ester. (B) The engineered microbes (colored boxes) convert simple sugars into acetyl-CoA *via* primary metabolism that is further transformed to limonene or POH in high titers. The best set of gene operons to produce limonene were arranged either in two plasmids (strain 2p) or into a single plasmid (strain 1p), that was further co-transformed with a second plasmid containing the genes necessary for the expression of P450 system able to oxidize the limonene to POH (strain 1p+P). Depending on the antibiotic marker used on the limonene-producing plasmid (1p) the final product was either POH (with ampicillin resistant vector) or acetylated-POH (with chloramphenicol resistant vector). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

use both pathways) while most prokaryotes use the DXP pathway (Boucher and Doolittle, 2000; Connolly and Winkler, 1989). An engineered *E.coli* harboring a heterologous MEV pathway was the first platform to produce isoprenoids at titers greater than 100 mg/L (Martin et al., 2003). Subsequent metabolic engineering of both isoprenoid pathways resulted in higher titers of sesquiterpenes, diterpenes and tetraterpenes (Alper et al., 2005; Anthony et al., 2009; Dueber et al., 2009; Farmer and Liao, 2000; Ma et al., 2011; Martin et al., 2003; Peralta-Yahya et al., 2011; Pitera et al., 2007; Redding-Johanson et al., 2011; Ro et al., 2006; Tsuruta et al., 2009; Wang et al., 2011; Yoon et al., 2009).

Although IPP and DMAPP overproduction can be readily routed to produce any relevant terpene by changing the prenyl transferase and terpene synthase, microbial production of monoterpenes has been limited due to the toxicity and volatility of most monoterpenes (Dunlop et al., 2011) and the poor heterologous expression of geranyl pyrophosphate synthase (GPPS) and monoterpene synthases (Carter et al., 2003; Reiling et al., 2004). Even when the limonene was added exogenously to *E. coli* strains expressing modifying enzymes (e.g. P450 to oxidize limonene to carveol or perillic acid), the cultures produced very low levels of the desired derivatives (Carter et al., 2003; Mars et al., 2001) suggesting that limonene import is poor or that it is efficiently excreted by efflux pumps before being transformed by any enzyme.

In this study, we engineered *E. coli* to produce L-limonene and its derivative (−)-POH from a simple sugar by heterologous expression of the mevalonate pathway, a GPP synthase, a limonene synthase (LS), and a cytochrome P450 (Fig. 1). A series of gene modifications to improve the enzyme availability and activity lead to increases in limonene titers to 430 mg/L of L-limonene (72 h, 1% glucose), nearly 90-fold higher than previous reports (Carter et al., 2003; Misawa, 2011). The engineered *E. coli* strain reported here is able to produce limonene endogenously with titers high enough to surpass the transport problems into and out of the cell that greatly compromised the efficiency of downstream conversion of limonene in the previous report (Carter et al., 2003).

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