1 **Metabolic engineering of *Escherichia coli* for production of valerenadiene**

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8 **Abstract**

9 *Valeriana officinalis* is a medicinal herb which produces a suite of compounds in its root tissue useful for

10 treatment of anxiety and insomnia. The sesquiterpene components of the root extract, valerenic acid and

11 valerena-1,10-diene, are thought to contribute to most of the observed anxiolytic of Valerian root

12 preparations. However, valerenic acid and its biosynthetic intermediates are only produced in low

13 quantities in the roots of *V. officinalis.* Thus, in this report, *Escherichia coli* was metabolically engineered

14 to produce substantial quantities of valerena-1,10-diene in shake flask fermentations with decane overlay.

15 Expression of the wildtype valerenadiene synthase gene (pZE-*wvds*) resulted in production of 12 µg/mL

16 in LB cultures using endogenous FPP metabolism. Expression of a codon-optimized version of the

17 valerenadiene synthase gene (pZE-*cvds*) resulted in 3-fold higher titers of valerenadiene (32 µg/mL). Co-

18 expression of pZE-*cvds* with an engineered methyl erythritol phosphate (MEP) pathway improved

19 valerenadiene titers 65-fold to 2.09 mg/L valerenadiene. Optimization of the fermentation medium to

20 include glycerol supplementation enhanced yields by another 5.5-fold (11.0 mg/L valerenadiene). The

21 highest production of valerenadiene resulted from engineering the codon-optimized valerenadiene

22 synthase gene under strong P_{trc} and P_{T7} promoters and via co-expression of an exogenous mevalonate

23 (MVA) pathway. These efforts resulted in an *E. coli* production strain that produced 62.0 mg/L

24 valerenadiene (19.4 mg/L/OD_{600} specific productivity). This *E. coli* production platform will serve as the

25 foundation for the synthesis of novel valerenic acid analogues potentially useful for the treatment of

26 anxiety disorders.

27 Keywords: *Valeriana*, valerenic acid, metabolic engineering, sesquiterpene, *Escherichia coli*

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Introduction

29

30   *Valeriana officinalis* is a medicinal wild herb indigenous to many habitats, and the root of this
31   plant is used as a nutraceutical preparation (Valerian) that is currently used for the treatment of anxiety
32   and insomnia (Bent et al., 2006). The roots of *V. officinalis* produce a suite of compounds, including
33   valepotriate alkaloids and sesquiterpenes (Bos et al., 1996). Notably, the sesquiterpene components of
34   Valerian root extract are hypothesized to exhibit many of the beneficial anti-anxiety and anti-insomnia
35   effects. Of the sesquiterpenes, valerenic acid is the most potent GABA-A agonist in these extracts, while
36   valerenal, valerenol, and valerena-1,10-diene (valerenadiene) also modulate GABA-A activity to varying
37   extents in zebrafish and mouse models (Del Valle-Mojica and Ortíz, 2012; Takemoto et al., 2014). Most
38   importantly, the anxiolytic effect of valerian has been demonstrated in human clinical studies in recent
39   years (Anderson et al., 2005; Barton et al., 2011).

40   Valerenic acid in particular has demonstrated nanomolar binding affinity for the GABA-A receptor
41   (Benke et al., 2009). Recently, the putative binding site of valerenic acid has been determined via
42   docking studies and site-directed mutagenesis (Luger et al., 2015). Luger et al. modeled valerenic acid in
43   the GABA-A receptor in a distinct cleft near the β2/3N265 transmembrane residue. The valerenic acid C-
44   12 carboxyl group is predicted to have important hydrogen-bonding interactions with residues β3N265
45   and β1S265. Furthermore, the C-13, C-14, and C-15 methyl groups of the valerenane skeleton are
46   predicted to have significant hydrophobic interactions within the pocket at residues within this binding
47   pocket (Figure 1) (Luger et al., 2015). In summation, these observations have renewed interest in
48   development of novel valerenic acid analogues for structure activity relationship studies.

49   Despite these advances, valerenic acid is produced as a minor constituent in root tissue of
50   *Valeriana officinalis* (0.7-0.9% DW) (Bos et al., 1998). The low production of valerenic acid hinders further
51   attempts at structure activity relationship and biological activity studies. Synthetic routes toward valerenic
52   acid derivatives are also expensive and ecologically unsuitable. Recently, Ricigliano et al. have
53   engineered *V. officinalis* hairy roots for enhanced production of valerenic acid, which lends credence to a
54   metabolic engineering approach for availing these molecules (Ricigliano et al., 2016). Despite this
55   progress, the hairy root system still produces valerenic acid at very lower titers.

56   As an alternative approach, metabolic engineering of microbial organisms represents a green,
57   cost-effective approach for large-scale production of sesquiterpene pharmaceuticals (Zhang et al., 2011).
58   For example, *Escherichia coli* is one such model host that affords several advantages over plant-based
59   systems due to its fast growth kinetics and capacity to produce high-value chemicals via fermentation on
60   simple carbon sources (Lee, 1996). Furthermore, *E. coli* boasts considerable genetic tools, including
61   multiple promoters and expression vectors that establish it as an ideal host for metabolic engineering.
62   Additionally, terpenes are attractive molecules for metabolic engineering, due to their use as fragrances,
63   flavors, and advanced biofuels (Peralta-Yahya et al., 2011; Sowden et al., 2005). Subsequently, these

64   observations established *E. coli* as a suitable platform for engineering of valerenic acid in this present
65   study.

66   Two specialized isoprenoid biosynthetic pathways exist for production of C_5 isoprenyl phosphate
67   precursors, dimethyl allyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP). Many bacteria,
68   algae, and plant chloroplasts employ the methyl erythritol phosphate pathway (MEP), which fluxes
69   glyceraldehyde-3-phosphate and pyruvate towards IPP and DMAPP (Figure 1) (Rohmer, 1999). Fungi
70   and non-plant eukaryotes utilize the mevalonate pathway (MVA) to convert acetyl-coenzyme A (acetyl-
71   CoA) to IPP and DMAPP via eight enzymatic steps (Figure 1) (Martin et al., 2003). Subsequently, IPP
72   and DMAPP are concatenated into progressively longer C_{10}, C_{15}, or C_{20} molecules by prenyltransferase
73   enzymes. For sesquiterpene metabolism, farnesyl pyrophosphate synthase condenses 2 IPP units and 1
74   DMAPP unit to produce farnesyl pyrophosphate (FPP). Subsequently, FPP can be cyclized by a variety
75   of sesquiterpene hydrocarbons by cognate terpene synthases, such as valerenadiene synthase (VDS)
76   (Figure 1).

77   However, *E. coli* generates only a finite pool of FPP, and while introduction of a sesquiterpene
78   synthase results in detectable production of sesquiterpenes (Martin et al., 2001), overproduction of the
79   molecule requires redirecting substantial carbon flux to the limited substrate FPP. For example,
80   amorphadiene is a sesquiterpene intermediate in the biosynthesis of the antimalarial drug artemisinin.
81   Martin and co-workers discovered that heterologous expression of the mevalonate isoprenoid pathway
82   from yeast in *E. coli* leads to unregulated carbon flux towards FPP and amorphadiene, which can be
83   semi-synthetically converted to artemisinin (Martin et al., 2003). In using this substrate-engineering
84   approach, Keasling and co-workers have produced amorphadiene in *E. coli* at yields of 500 mg L^{-1} in
85   shake flask (Redding-Johanson et al., 2011) and 27 g L^{-1} in bioreactors (Tsuruta et al., 2009). This
86   generalized approach lends itself to the microbial synthesis of other sesquiterpenes via introduction of
87   variant terpene synthases.

88   In this report, a metabolic engineering platform was developed for synthesis of valerenadiene in
89   *E. coli* in three steps. Because plant terpene synthase genes are poorly expressed in *E. coli*, owing to
90   different codon usage, first an *E. coli* codon-optimized version of the valerenadiene synthase gene (*cvds*)
91   was synthesized. This lead to a three-fold higher production of valerenadiene over expression of the
92   wildtype plant gene (*wvds*). Secondly, we enhanced carbon flux to FPP by designing a construct that
93   overexpressed several rate-limiting steps of the native methyl erythritol phosphate (MEP) pathway.
94   Thirdly, we cloned *cvds* under the control of P_{trc} and P_{T7} promoters to further enhance terpene synthase
95   protein levels and we co-expressed these constructs with a heterologous mevalonate pathway from
96   baker's yeast. These efforts resulted in a high-level production strain that synthesized 62.0 mg L^{-1}
97   valerenadiene (19.4 mg/L/OD$_{600}$ specific productivity) in shake flask cultures.

98   **Methods and Materials**

3

99   *Bacterial strains and growth conditions*

100   *E. coli* JM109 (New England Biolabs) was used as the host for all routine cloning manipulations,

101   and *E. coli* DH5αZ1 (Expressys, Germany) and *E. coli* BL21(DE3) (ThermoFisher) were used as hosts for

102   sesquiterpene production (Supplementary Table 1). *E. coli* DH5αZ1 overexpresses a copy of the lacIq

103   repressor on the chromosome for efficient repression of the lac operator (Lutz and Bujard, 1997).

104   Chemically competent *E. coli* were generated with the *E. coli* Mix and Go Transformation Kit (ZYMO

105   Research) and were transformed using standard molecular methodologies (Sambrook and W Russell,

106   2001). *E. coli* strains were grown in LB agar or LB broth at 37 C for routine maintenance. For

107   production of sesquiterpenes, *E. coli* DH5αZ1 derivatives were grown in 2xYT with supplemented glycerol

108   at 30 C. for production of valerenadiene. For expression of the mevalonic acid pathway, bacterial

109   growth media was buffered with phosphate buffered saline using a stock solution of 10x PBS (Sambrook

110   and W Russell, 2001). Strains were supplemented with ampicillin (100 µg mL^{-1}), chloramphenicol (35 µg

111   mL^{-1}), and kanamycin (50 µg mL^{-1}) as necessary. When multiple plasmids were co-expressed, the

112   chloramphenicol and kanamycin concentrations were adjusted to one-half these amounts.

113   *Cloning of vds and engineered MEP pathway constructs*

114   Oligonucleotide primers were synthesized by IDT-DNA (Supplementary Table 2), and sequences

115   were verified by sequencing analysis (ACGT, Inc.). Polymerase chain reaction was carried out using

116   Primestar® HS Polymerase (Takara Bio.) by following the manufacturer's protocols. The wildtype

117   valerenadiene synthase gene (*wvds*) gene was amplified via polymerase chain reaction from the

118   pET28a(+)-VoTPS1 (hereafter referred to as pET-*wvds*) construct as described previously (Yeo et al.,

119   2013). The *cvds* gene was codon-optimized for expression in *E. coli*, synthesized, and spliced into

120   cloning vector pUC57-*cvds* (GenScript). The *cvds* gene was amplified via polymerase chain reaction, and

121   both the *wvds* and *cvds* genes were cut and spliced into the *Eco*RI/*Bam*HI restriction sites of pZE12MCS

122   under the control of the intermediate strength P$_{LlacO1}$ promoter (Expressys, Germany) to afford constructs

123   pZE-wVDS and pZE-cVDS, respectively (Lutz and Bujard, 1997). For insertion under the stronger P$_{trc}$

124   promoter, *cvds* was PCR amplified, cut, and spliced into the *Bam*HI/*Eco*RI sites of pTrcHisA (Thermo

125   Fisher Scientific) to afford pTrcHis-*cvds*. In this construct, *cvds* is fused to the *N*-terminal hexahistidine

126   and Xpress™ epitope tags. For comparisons involving the P$_{T7}$ promoter, the *cvds* gene was cloned into

127   the *Nco*I and *Eco*RI sites of pET28a(+) to afford pET-*cvds*. pET-*wvds* features *wvds* fused to the P$_{T7}$

128   promoter and was included in experiments to reflect baseline production of valerenadiene from T7-

129   polymerase driven expression of the wildtype gene.

130   The *dxs-idi-fps* genes were synthesized as a polycistron as previously described in vector

131   pUC57-operon 3 (Genscript) (Bell et al., n.d.). The *dxs-idi-fps* genes were digested with *Eco*RI/*Bam*HI

132   and were spliced into the *Eco*RI/*Bam*HI sites of pSTV28 to afford pSTV-*dxs-idi-fps*.

133   *Cloning and expression of mevalonate pathways*

134   pBbA5c-MevT(CO)-MBIS(CO, ispA) was a gift from Jay Keasling & Taek Soon Lee (Addgene

135   plasmid # 35151). For co-expression of valerenadiene synthase and the mevalonate pathway, *E. coli*

136   competent cells were co-transformed with the relevant constructs and plated on LB agar supplemented

137   with antibiotics. For terminal endpoint assays, *E. coli* strains were grown in 1 mL of LB supplemented

138   with antibiotics for 12-16 hours until the strains were in stationary phase growth, and then they were

139   inoculated into 5 mL 2x YT medium containing 3% glycerol with an overlay of 1 mL of decane. For time

140   course assays, *E. coli* strains were grown in 50 mL 2x YT medium containing 3% glycerol with an overlay

141   of 10 mL of decane. Time points were collected at 12, 24, 36, 48, 72, and 96 hours of 100 microliters of

142   culture broth (for OD_{600} measurements) and 100 microliters of decane overlay for valerenadiene

143   determination.

GC-MS analysis of valerenadiene

145   Valerenadiene production by the various strains was measured by GC-MS via selective ion

146   monitoring for the molecular ion (204 *m/z*), the 189 *m/z* ion, and the 69 *m/z* ion, as described previously

147   (Martin et al., 2003). Cells were grown in LB or 2x YT medium with 3% glycerol and induced with 200

148   micromolar IPTG to express the *wvds* or *cvds* gene and either the engineered MEP operon or the

149   mevalonate pathways. Cultures were covered with a 20% (v/v) decane overlay to trap volatile

150   sesquiterpenes. 100 microliters of decane overlay was diluted in 900 microliters of hexane, and 1

151   microliter of sample was subjected to GC-MS analysis. Samples were analyzed at the Ferris State

152   University Shimadzu Core Laboratory for Academic Research Excellence (FSU-SCLARE) on a Shimadzu

153   QP-2010 Ultra GC mass spectrometer. Valerenadiene in experimental samples was compared to

154   authentic valerenadiene standard. Valerenadiene concentration was converted to beta caryophyllene

155   equivalents using a caryophyllene standard curve and the relative abundance of 69 *m/z*, 189 *m/z*, and

156   204 *m/z* mass fragments. Valerenadiene standard was isolated as described previously (Yeo et al.,

157   2013).

Results

Valerenadiene production in E. coli and gene optimization

160   To evaluate the production of valerenadiene synthase in *E. coli*, the gene encoding wildtype

161   valerenadiene synthase (*wvds*) from *V. officinalis* was cloned under the control of the IPTG-inducible

162   P_{LlacO1} promoter of high copy number expression plasmid pZE12MCS to afford pZE-wVDS. Previously,

163   other groups have reported that sesquiterpene production in *E. coli* is limited by poor expression of

164   cognate plant terpene synthases (Martin et al., 2001; Wang et al., 2011). We surmised that the *wvds*

165   gene may encode several rare codons that inhibit optimal translation in *E. coli*. Therefore, we

166   synthesized a codon-optimized version of the valerenadiene synthase gene for expression in *E. coli*

167   (GenScript) and fused it to the P_{LlacO1} to afford pZE-cVDS. pZE-wVDS, pZE-cVDS, and empty

168   pZE12MCS vector were transformed in *E. coli*. The *E. coli* lines were grown in 5 mL of LB medium

169   overlaid with decane for 48 hours and induced with IPTG. A major valerenadiene peak was detected in

170   extracts of pZE-wVDS and pZE-cVDS cultures that eluted at the same retention time and exhibited

171   identical mass fragmentation pattern to valerenadiene standard (Supplementary Figure 1). This peak was

172   not detected in pZE12MCS control strains, which confirmed low production of valerenadiene in the VDS-

173   engineered lines. *E. coli* pZE-wVDS produced 8 μg/L and 12 μg/L valerenadiene at 24 and 48 hours,

174   respectively, whereas *E. coli* pZE-cVDS produced 16 and 32 μg/L valerenadiene at 24 and 48 hours,

175   respectively (Figure 2). This results suggests that the codon-optimized valerenadiene synthase is more

176   efficiently expressed than the wildtype version, which results in a 3-fold improvement in valerenadiene

177   titer.

178   However, the yields of valerenadiene achieved were quite low, and we surmised this was likely

179   due to low levels of prenyl phosphate precursors (IPP, DMAPP, and FPP). The endogenous MEP

180   pathway of *E. coli* produces a small pool of FPP that is the precursor for *trans*-octaprenyl diphosphate

181   (ODP) in production of ubiquinone and *cis, trans*-undecaprenyl diphosphate (UDP) for production of

182   peptidoglycan (Asai et al 1994, Bouhss et al 2008, Okada et al 1997). However, the production of FPP

183   by the endogenous pathway is limited due to regulation of several key steps (Estévez et al., 2001).

184   Therefore, we designed a construct that would overexpress rate-limiting steps of the MEP pathway to

185   augment greater carbon flux from primary metabolism to FPP. In our previous work, we engineered

186   *Rhodobacter capsulatus* to produce high levels of the triterpene botryococcene, which is biosynthesized

187   from two molecules of FPP via squalene synthase-like enzymes (SSL-1 and SSL-3), via expression of the

188   deoxy-xylulose phosphate synthase (*dxs*), isopentenyl diphosphate isomerase (*idi*) genes from *E. coli*,

189   and farnesyl pyrophosphate synthase (*fps*) gene from *Gallus gallus* (Khan et al., 2015). Fusion of the

190   *dxs-idi-fps* genes to the SSL-1+3 chimeric botryococcene synthase and expression of the resulting

191   construct generated 5 mg/gDW production of botryococcene in the *R. capsulatus* host. In a similar

192   fashion, we fused the *dxs-idi-fps* genes under the control of the IPTG-inducible P_{lacUV5} promoter of

193   pSTV28 to generate construct pSTV-*dxs-idi-fps*. This construct was co-transformed with pZE-*wvds* and

194   pZE-*cvds*, and the lines were grown in 5 mL LB medium with decane overlay at 30°C for 48 hours and

195   induced with IPTG. Co-expression of pSTV-*dxs-idi-fps* with pZE-wVDS and pZE-cVDS resulted in a 14-

196   fold increase (0.17 mg/L) and a 65-fold increase (2.09 mg/L) in production of valerenadiene, respectively

197   (Figure 2). These results demonstrate that expression of the engineered MEP pathway greatly

198   augmented carbon flux to FPP and valerenadiene.

199   *Effect of glycerol supplementation on valerenadiene production*

200   With the engineered MEP pathway construct in hand, we hypothesized that optimization of the

201   production medium could further enhance sesquiterpene yield. In fact, Zhang et al. reported that glycerol

202   supplementation of culture medium increased sabinene production to >40 mg/L in *E. coli* engineered with

203   sabinene synthase and a heterologous mevalonate pathway (Zhang et al., 2014). Furthermore, Morrone

204   and co-workers engineered higher levels of C_{20} abietadiene production and increased biomass when
205   glycerol supplemented media was used (e.g. increasing abietadiene specific productivity from 1
206   mg/L/OD$_{600}$ without glycerol supplementation to 2.5 mg/L/OD$_{600}$ with glycerol supplementation) (Morrone
207   et al., 2010). *E. coli* pZE-wVDS/pSTV-*dxs-idi-fps* and *E. coli* pZE-cVDS/pSTV-*dxs-idi-fps* were grown in 5
208   mL of 2x YT medium supplemented with 0 to 5% glycerol. The *E. coli* pZE-wVDS/pSTV-*dxs-idi-fps*
209   valerenadiene production increased 15-fold from 0.11 mg/L to 1.5 mg/L when supplemented with glycerol,
210   however, further production was possibly stymied by poor heterologous expression of valerenadiene
211   synthase. In contrast, *E. coli* pZE-cVDS/pSTV-*dxs-idi-fps* demonstrated better responsiveness to the
212   glycerol supplementation, as valerenadiene productivity increased from 5.1 mg/L to 11.0 mg/L when
213   supplemented with 3% glycerol (Figure 3). Therefore, 3% glycerol was used for all further experiments.

214   To observe production of valerenadiene over time, we conducted a time course of *E. coli* pZE-
215   wVDS/pSTV-*dxs-idi-fps* and *E. coli* pZE-cVDS/pSTV-*dxs-idi-fps* in 2x YT+3% glycerol media over 72
216   hours. The results of the time course replicated the observations in the glycerol supplementation
217   experiment. *E. coli* co-expressing the engineered MEP pathway and the *wvds* gene reached maximal
218   growth (OD$_{600}$= 9.0) and valerenadiene production (3 mg/L) at 24 hours (Figure 4). The *E. coli* line co-
219   expressing the engineered MEP pathway and *cvds* gene reached also reached both its greatest level of
220   growth (OD$_{600}$ =9.0) and valerenadiene production (10.6 mg/L) at 24 hours. Valerenadiene concentration
221   decreased in both lines from 48 to 72 hours, likely due to volatilization of the sesquiterpene.

222   *Engineering of vds under the P$_{trc}$ promoter*

223   We hypothesized that low-level expression of the terpene synthase from the P$_{LlacO1}$ promoter
224   might be a metabolic rate-limiting factor in the turnover of FPP substrate. Based on the previous
225   experiment, expression of the *cvds* gene resulted in 5-10-fold higher quantities of valerenadiene than
226   expression of *wvds*, depending on glycerol supplementation, which was inferred to be the direct result of
227   higher concentration of terpene synthase catalyst in the *E. coli* cell. Therefore, the *cvds* gene was fused
228   to the strong P$_{trc}$ promoter to afford construct pTrcHis-*cvds*. This construct was expressed alone and co-
229   expressed with pSTV-*dxs-idi-fps* in *E. coli* DH5αZ1 cells in triplicate 5 mL 2xYT+3% glycerol
230   fermentations for 48 hours. The strain harboring pTrcHis-*cvds* produced 0.046 mg/L valerenadiene,
231   whereas the strain harboring pTrcHis-*cvds* and pSTV-*dxs-idi-fps* produced approximately 20.3 mg/L
232   (Supplementary Figure 2). This represents a 440-fold improvement over the pTrcHis-*cvds* only
233   expression line. Furthermore, expression of the *cvds* gene by the P$_{trc}$ promoter (e.g. by strain *E. coli*
234   DH5αZ1/(pTrcHis-*cvds*)/(pSTV-*dxs-idi-fps*)) represented a 40% increase in valerenadiene titers as
235   compared to expression from P$_{LlacO1}$ (e.g. by strain *E. coli* DHαZ1/(pZE-*cvds*)/(pSTV-*dxs-idi-fps*)).

236   Recently, the MEP pathway has been demonstrated to be subject to endogenous regulation by *E.*
237   *coli*, possibly due to feedback inhibition of the metabolite methylerythritol cyclodiphosphate (MEcDP)
238   (Banerjee and Sharkey, 2014). We rationalized that FPP enhancement might be limited via the MEP

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239　pathway, therefore we co-expressed an exogenous MVA pathway to greatly augment FPP precursor

240　levels along with pTrcHis-*cvds*. In a previous study, Peralta-Yahya and co-workers engineered a

241　heterologous MVA pathway for production of the sesquiterpene biofuel bisabolene (Peralta-Yahya et al.,

242　2011). Expression of the construct pBbA5c-MevT(CO)-MBIS(CO, ispA) along with the codon-optimized

243　AgBis gene resulted in production of 586±65 mg/L bisabolene. pBbA5c-MevT(CO)-MBIS(CO, ispA)

244　features a codon-optimized, eight gene MVA pathway driven by the P_{trc} promoter on a medium copy

245　number plasmid (Supplementary Table 1). The three upstream gene products (*atoB*, *hmgs*, *thmgr*)

246　convert acetyl-CoA to mevalonic acid, and the five downstream gene products (*erg12*, *erg8*, *mvd1*, *idi*,

247　*ispA*) convert mevalonic acid to farnesyl pyrophosphate (Figure 1). Similarly, *E. coli* DH5αZ1 was

248　transformed with pTrcHis-*cvds* and pBbA5c-MevT(CO)-MBIS(CO, ispA) and grown in triplicate 5 mL

249　2xYT+3% glycerol fermentations for 48 hours. This strain produced 42.5 mg/L valerenadiene, a 1000-fold

250　increase over the *cvds*-expressing line and a 2-fold increase over the engineered MEP pathway

251　(Supplementary Figure 2). This result demonstrated that the mevalonic acid pathway efficiently coupled

252　the microbial synthesis of FPP to valerenadiene synthase to produce significant levels of valerenadiene.

253　Thus, the mevalonic acid pathway was used for further experiments. These results were further confirmed

254　in a time course experiment of the same strain, which achieved a titer of 35 mg/L valerenadiene at 24

255　hours (Figure 5).

256　*Co-expression of vds with the T7 RNA polymerase promoter and MVA pathway*

257　　　We observed that the P_{trc}-*cvds* gene constructs demonstrated more robust production of

258　valerenadiene than the comparatively weaker P_{LacO1}-*cvds* gene constructs, and we inferred that levels of

259　VDS catalyst may be rate-limiting in this latter system. Therefore, we decided to compare the expression

260　of the *wvds* and *cvds* genes from a "weak" promoter system (P_{LacO1}), an "intermediate/strong" promoter

261　system (P_{trc}), and a strong promoter system (P_{T7}) along with the mevalonic acid pathway. We

262　hypothesized that the P_{LacO1} promoter might not be able to drive sufficient expression of VDS to efficiently

263　turnover the abundant FPP substrate synthesized via the mevalonic acid pathway. *E. coli* DH5αZ1 pZE-

264　*wvds* and *E. coli* DH5αZ1 pZE-*cvds* were grown in triplicate cultures as before and produced minimal

265　amounts of valerenadiene (Supplementary Figure 3. When pBbA5c-MevT(CO)-MBIS(CO, ispA) was co-

266　expressed in these lines, they produced 2.8 mg/L (0.497 mg/L/OD$_{600}$) and 7.3 mg/L (1.03 mg/L/OD$_{600}$),

267　respectively, which strongly suggested that the amount of VDS catalyst was limited (Supplementary

268　Figure 3). Expression of *cvds* from the P_{trc} promoter with the mevalonic acid pathway increased

269　production 6-fold to 42.5 mg/L (6.07 mg/L/OD$_{600}$) (Supplementary Figure 3).

270　　　Subsequently, the *wvds* and *cvds* genes were cloned into pET28a for expression under the strong

271　T7 RNA polymerase promoter. The resulting constructs, pET-*wvds* and pET-*cvds* were introduced into *E.*

272　*coli* BL21(DE3) cells to utilize the IPTG-inducible T7 RNA polymerase system. These plasmids were

273　transformed alone or in combination with the mevalonic acid pathway and grown in triplicate 2xYT + 3%

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274   glycerol cultures for 48 hours. The pET-*wvds*-only line produced 0.345 mg/L valerenadiene, whereas the

275   pET-*cvds*-only line produced 1.09 mg/L valerenadiene (Figure 6). Co-expression of pET-*wvds* and

276   pBbA5c-MevT(CO)-MBIS(CO, ispA) resulted in production of 10.5 mg/L valerenadiene (3.27 mg/L/OD$_{600}$)

277   (Figure 7). Most importantly, when pET-*cvds* was introduced with the mevalonic acid pathway, the

278   highest-yielding valerenadiene strain was achieved with production of 62.0 mg/L valerenadiene (19.4

279   mg/L/OD$_{600}$ specific productivity). These experiments demonstrated that the amount of VDS catalyst and

280   FPP substrate *in vivo* are both limiting factors for production of valerenadiene, which can be remedied via

281   expression of *cvds* from a strong P$_{trc}$/P$_{T7}$ promoter and heterologous expression of the mevalonic acid

282   pathway.

283   **Discussion**

284   Valerenic acid is the most potent sesquiterpenoid component produced in the roots of *Valeriana*

285   *officinalis* that exhibits GABA-A activity. Development of this compound as a novel anxiolytic drug is

286   hindered by the low production of valerenic acid by the native plant. As a first step towards the

287   development of a production platform for valerenic acid analogues, we have employed *E. coli* as a

288   workhorse metabolic host for engineering of terpene chemicals. In this study, valerenadiene was

289   produced by introducing a codon-optimized valerenadiene synthase gene into *Escherichia coli* along with

290   an engineered MEP pathway or *Saccharomyces cerevisiae*-based mevalonic acid pathway. The codon-

291   optimized valerenadiene synthase gene was found to be a critical component for engineering high

292   valerenadiene titers, as it resulted in 3-fold higher production of valerenadiene as compared to the

293   wildtype sequence. This very likely indicates that translation of the codon-optimized form of the gene is

294   very efficient. Further improvements in productivity were realized by the overexpression of the *dxs*, *idi*,

295   and *fps* genes to augment carbon flux through the MEP pathway. This strategy resulted in a 65-fold

296   increase in production of valerenadiene (2.09 mg/L). This was improved a further 6-fold by optimizing the

297   fermentation medium to 2xYT with 3% glycerol supplementation (11.0 mg/L). Next, the *cvds* gene was

298   fused to the stronger P$_{trc}$ promoter, which resulted in approximately 40% increase in production of

299   valerenadiene when pTrcHis-*cvds* was co-expressed with the engineered MEP pathway (20 mg/L). This

300   result demonstrated the importance of driving expression of valerenadiene synthase from strong

301   promoters to ensure ample availability of terpene synthase catalyst.

302   Notably, the valerenadiene synthase exhibits similar kinetic properties (e.g. K$_m$ for FPP = 7.2 μM,

303   k_{cat} = 5.7 x 10^{-3} s^{-1}) to other published terpene synthases (Yeo et al., 2013). For example, the well-

304   characterized amorphadiene synthase exhibits a two-fold lower K$_m$ for FPP (i.e. K$_m$ = 3.3 μM and k_{cat} =

305   6.8 x 10^{-3} s^{-1}) (Picaud et al., 2005). Further efforts to improve the catalytic functionality of valerenadiene

306   synthase could incorporate rationale modifications to the enzyme. Alternatively, further improvements in

307   terpene synthase activity have been realized via expression of a terpene synthase as a gene fusion with

308   farnesyl pyrophosphate synthase to achieve substrate channeling of FPP to the active site of VDS. Wang

9

309 and co-workers reported that expression of *ispA* and apple farnesene synthase (*aFS*) from the same

310 construct with the mevalonic acid pathway resulted in production of 57.6 mg/L of α-farnesene in *E. coli*

311 (Wang et al., 2011). Fusing of *ispA-aFS* as a single gene construct with a (GGGGS)$_2$ amino acid linker

312 increased production to 87.8 mg/L of α-farnesene. Additionally, Niehaus et al. achieved substrate

313 channeling by fusing the triterpene synthases SSL-1 and SSL-3 genes to form a single gene fusion, SSL-

314 1+SSL-3, which increased production from 20 mg/L botryococcene to 50 mg/L botryococcene in yeast

315 (Niehaus et al., 2011).

316 However, the greatest increases in valerenadiene yield involved heterologous expression of the

317 *Saccharomyces cerevisiae* mevalonic acid pathway. Expression of the MVA pathway resulted in an

318 unregulated metabolic flux towards FPP, which could be efficiently coupled to valerenadiene synthase for

319 production of 62.0 mg/L valerenadiene in culture tubes with a specific productivity of 19.4 mg/L/OD$_{600}$.

320 This study showed that *E. coli* is capable of high-level production of valerenadiene with relatively minimal

321 optimization of the production media and commercially available expression vectors, such as pBBa5c-

322 MevT-MBIS(CO,IspA). Further efforts to improve valerenadiene yield will require optimization of the

323 upstream and downstream genetic components of the MVA pathway and subsequent scale-up

324 fermentation in bioreactors. In addition, we are conducting rational attempts to engineer CYP450s to

325 oxidize and functionalize the valerenadiene skeleton to generate a library of potential GABA-A ligands.

Acknowledgements

Funding

335

336   **Figure 1** Biosynthesis of valerenadiene via the methyl erythritol phosphate pathway (MEP, pathway A)
337   and the mevalonic acid pathway (MVA, pathway B). IPP and DMAPP are synthesized via the MEP or
338   MVA pathways via different upstream routes. The MEP pathway is initiated via condensation of
339   glyceraldehyde 3-phosphate and pyruvate from glycolysis via 1-deoxy-D-xylulose 5-phosphate synthase
340   (DXS), and the MVA pathway is initiated via condensation of two molecules of acetyl-CoA. Two
341   molecules of IPP and one molecule of DMAPP are condensed into C_{15} farnesyl pyrophosphate (FPP).
342   FPP is cyclized into valerenadiene via valerenadiene synthase (VDS). The abbreviations of the enzymes
343   are as follows: DXS, 1-deoxy-D-xylulose 5-phosphate synthase; atoB, thiolase; HMGS, hydroxy-
344   methylglutaryl-CoA synthase; HMGR, truncated hydroxy-methylglutaryl-CoA reductase; ERG12,
345   mevalonate kinase; ERG8, phosphomevalonate kinase; MVD1, diphosphomevalonate decarboxylase;
346   IDI, isopentenyl diphosphate isomerase; FPS, farnesyl pyrophosphate synthase from *Gallus gallus*; IspA,
347   farnesyl pyrophosphate synthase from *Escherichia coli*; VDS, valerenadiene synthase.
348

349   **Figure 2** Engineering of wildtype (pZE-*wvds*) and codon-optimized (pZE-*cvds*) valerenadiene synthase.
350   **(A)** *E. coli* engineered with empty vector control, wildtype, and codon optimized VDS genes was grown
351   for 24 (white bars) and 48 hours (black bars) and yield of valerenadiene was determined. **(B)** Growth of
352   engineered *E. coli* lines was determined by measuring optical density (OD_{600}) at 24 (white bars) and 48
353   hours (black bars), respectively. **(C)** *E. coli* strains were co-transformed with empty vector control,
354   wildtype, or codon-optimized *vds* and pSTV-*dxs-idi-fps* for enhancement of the endogenous MEP
355   pathway. (**D**) Growth of engineered *E. coli* lines was determined by measuring optical density (OD_{600}).
356   Strains were inoculated in 5 mL cultures of LB media with 1 mL decane overlay and experiments were
357   carried out in triplicate. Error bars indicate standard error of the mean.
358

359   **Figure 3** Effect of glycerol conditions on production of valerenadiene. *E. coli* strains engineered with
360   pZE-*wvds* and pSTV-*dxs-idi-fps* or pZE-*cvds* and pSTV-*dxs-idi-fps* were grown in 5 mL 2xYT media with
361   0%-4% glycerol and 1 mL decane overlay for 48 hours and valerenadiene yield was determined.
362   Experiments were carried out in triplicate and error bars indicate standard error of the mean.
363

364   **Figure 4** Time course of *E. coli* expressing an engineered MEP pathway and valerenadiene synthase.
365   *E. coli* DH5αZ1 strains harboring the engineered MEP construct pSTV-*dxs-idi-fps* were co-expressed with
366   the wildtype (pZE-*wvds,* solid line) or codon-optimized (pZE-*cvds*, dotted line) valerenadiene synthase
367   gene. Cultures were grown in 50 mL 2x YT+3% glycerol with 10 mL decane overlay for 72 hours. (Left
368   panel) Valerenadiene yield (mg L^{-1}) and (right panel) biomass (OD_{600}) were determined from double
369   triplicate experiments. Error bars indicate the standard error of the mean.
370

371   **Figure 5** Time course experiment of *E. coli* co-expressing pTrcHis-*cvds* and the *Saccharomyces*
372   *cerevisiae* MVA pathway. *E. coli* DH5αZ1 strains harboring the pBBa5c-MevT+MBIS(CO,IspA) and
373   pTrcHis-*cvds* constructs were grown in 50 mL 2x YT+3% glycerol with 10 mL decane overlay for 72
374   hours. (Left panel) Valerenadiene yield (mg L^{-1}) and (right panel) biomass (OD_{600}) were determined from
375   double triplicate experiments. Error bars indicate the standard error of the mean.
376

377   **Figure 6** Production of valerenadiene using the T7 RNA polymerase to drive expression of *vds*. *E. coli*
378   BL21(DE3) strains harboring empty vector control, pET-*wvds*, and pET-*cvds* were expressed with and
379   without the *Saccharomyces cerevisiae* MVA pathway (pBBa5c-MevT-MBIS(CO, IspA)). Strains were
380   grown in triplicate 5 mL 2xYT media + 3% glycerol fermentations with 1 mL decane overlay for 48 hours.
381   (Left panel) valerenadiene volumetric production (mg/L) was determined after 48 hours. (Right panel)
382   Valerenadiene production was normalized by dividing volumetric production by the biomass after 48
383   hours (mg/L/OD_{600}).

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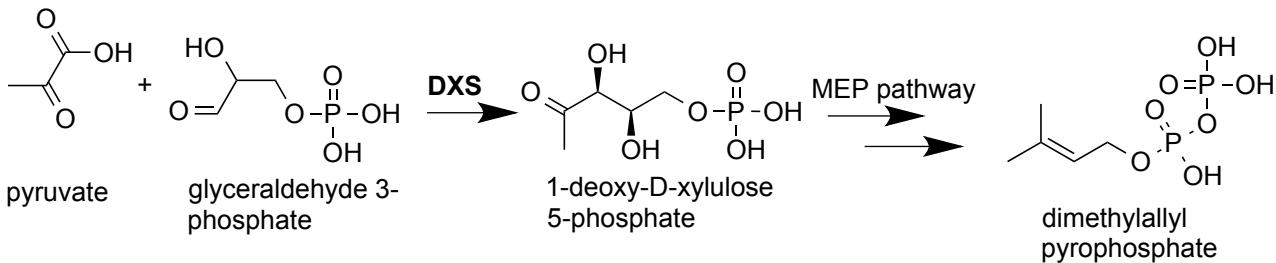
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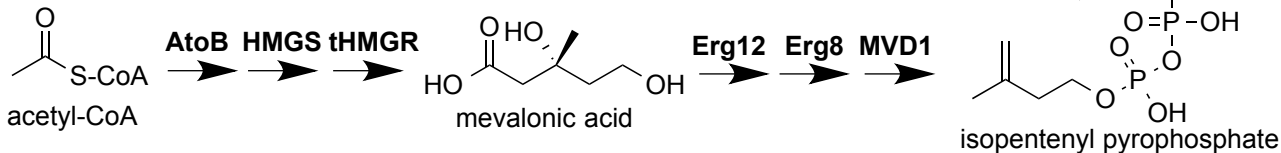487   20. doi:10.1186/1475-2859-13-20

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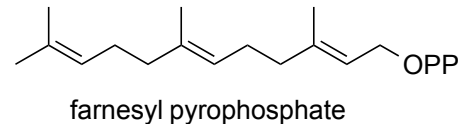489

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Methyl erythritol phosphate (MEP)

A

pyruvate + glyceraldehyde 3-phosphate → (DXS) → 1-deoxy-D-xylulose 5-phosphate → (MEP pathway) → dimethylallyl pyrophosphate

B

acetyl-CoA → (AtoB HMGS tHMGR) → mevalonic acid → (Erg12 Erg8 MVD1) → isopentenyl pyrophosphate

IDI

(FPS IspA) → farnesyl pyrophosphate → (VDS) → valerena-1,10-diene

Mevalonic acid pathway (MVA)