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Biosynthesis of pinene from glucose using metabolically-engineered *Corynebacterium glutamicum*

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Abstract Pinene is a monoterpenes (C_{10}) that is produced in a genetically-engineered microbial host for its industrial applications in fragrances, flavoring agents, pharmaceuticals, and biofuels. Herein, we have metabolically-engineered *Corynebacterium glutamicum*, to produce pinene and studied its toxicity in *C. glutamicum*. Geranyl diphosphate synthases (GPPS) and pinene synthases (PS), obtained from *Pinus taeda* and *Abies grandis*, were co-expressed with over-expressed native 1-deoxy-d-xylulose-5-phosphate synthase (Dxs) and isopentenyl diphosphate isomerase (Idi) from *C. glutamicum* using CoryneBrick vector. Most strains expressing PS-GPPSs produced detectable amounts of pinene, but co-expression of DXS and IDI with PS (*P. taeda*) and GPPS (*A. grandis*) resulted in 27 μg ± 7 α-pinene g^{-1} cell dry weight, which is the first report in *C. glutamicum*. Further engineering of PS and GPPS in the *C. glutamicum* strain may increase pinene production.

Keywords *Corynebacterium Glutamicum* · Metabolic Engineering · Monoterpene · Pinene · Toxicity

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Introduction

Terpenes are derived from C_5 isoprene units, namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). They are synthesized by two distinct pathways: the mevalonate (MVA) pathway, found in yeast, plant, and animal cytoplasm, and the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway found in most bacteria and plastids in plants (Phillips et al. 2008). Terpenes have a broad range of functions and are categorized by the number of carbon atoms they contain, such as monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}). Prenyl intermediates are synthesized by condensation of DMAPP and

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IPP (isoprene units) and terpenes are synthesized from these intermediates by corresponding terpene synthases (Kirby and Keasling, 2009). Since terpenes and their derivatives are generally recognized as safe (GRAS) compounds (Russo, 2011), there is a high demand for commercial applications widely used in manufacture of fragrances, flavoring agents, and pharmaceuticals (Kirby and Keasling, 2009) as well as potential biofuel application (Sarria et al. 2014).

Pinene is a natural and active monoterpenes. For its biosynthesis, the key intermediates, IPP and DMAPP, are condensed via geranyl diphosphate synthase (GPPS) to form geranyl diphosphate (GPP), which is then cyclized by pinene synthase (PS) to form pinene (Fig. 1A). α-Pinene has been produced (5.4 mg l^{-1}) under shake-flask conditions in an engineered *Escherichia coli* through introduction of an heterologous MVA pathway and an α-pinene synthase (Pt30) (Yang et al. 2013). In addition, pinene toxicity can be eliminated in yeast cultures by in situ extraction with solvent (Brennan et al. 2012) or by engineering an AcrB efflux pump in *E. coli* (Foo and Leong, 2013). Recently, combinatorial gene expression of PS, GPPS, and their fusion proteins have enhanced pinene production (32 mg l^{-1}) in *E. coli* (Sarria et al. 2014).

Corynebacterium glutamicum, also classified as GRAS, is a Gram-positive bacterium, primarily used in industrial amino acid production, particularly for the flavor enhancer L-glutamate and the feed additive L-lysine (Kalinowski et al. 2003). *C. glutamicum* can be used as a microbial cell factory to produce other commercially relevant chemicals such as succinate, isobutanol, cadaverine, and ethanol (Woo and Park, 2014). *C. glutamicum crtEb* deletion mutant has been engineered with over-expression of *crtE*, *crtB*, and *crtI* genes that code for prenyl transferase, phytoene synthase, and phytoene desaturase, respectively, to produce the C_{40} carotenoid terpenoid, lycopene, at 2.4 mg g^{-1} cell dry weight (CDW) (Heider et al. 2012).

To metabolically-engineer pinene production in *C. glutamicum* for an expansion of the capabilities of *C. glutamicum* as microbial cell factory, we introduced a heterologous pinene biosynthetic pathway and combinatorially overexpressed key genes from the MEP pathway to increase the pool of intermediate IPP available for pinene biosynthesis (Fig. 1B). In addition, the toxicity of pinene in *C. glutamicum* was studied for in situ extraction.

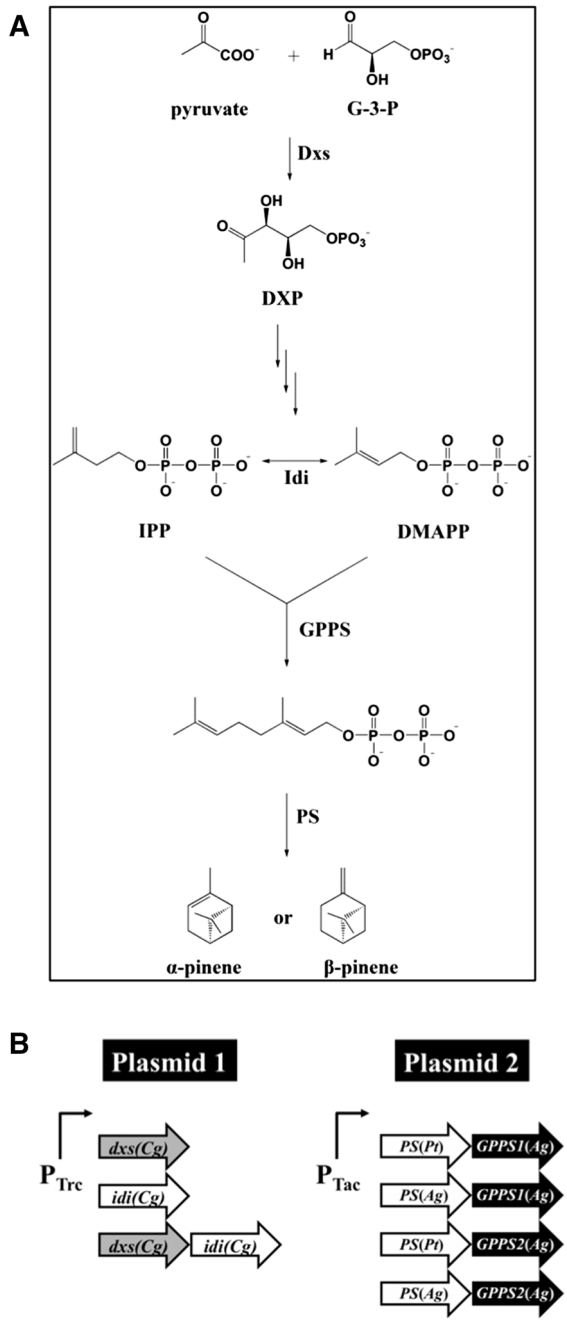


Fig. 1 Scheme of pinene production in engineered *C. glutamicum*. **a** The heterologous pinene can be synthesized from intermediate isopentenyl diphosphate (IPP) by expressing geranyl diphosphate synthase (GPPS) and pinene synthase (PS) in *C. glutamicum*. **b** Combinatorial gene expressions of heterologous PS and GPPS with native DXS and IDI were designed in two expression plasmids in *C. glutamicum*. *G-3-P* glyceraldehyde 3-phosphate, *Dxs* 1-deoxy-*D*-xylulose 5-phosphate synthase, *Idi* isopentenyl diphosphate isomerase, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPPS* geranyl diphosphate synthase, *PS* pinene synthase

Table 1 Bacteria strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli HIT-DH5α	F-(80d *lac*Z M15) (*lac*ZYA-*arg*F) U169 *hsd*R17($r^- m^+$) *rec*A1 *end*A1 *rel*A1 *deo*R96	RBC Bioscience
C. glutamicum	Wild type ATCC 13032	ATCC
Cg-pD/pPPG1	*C. glutamicum* strain harboring pD and pPPG1	This study
Cg-pD/pAPG1	*C. glutamicum* strain harboring pD and pAPG1	This study
Cg-pD/pPPG2	*C. glutamicum* strain harboring pD and pPPG2	This study
Cg-pD/pAPG2	*C. glutamicum* strain harboring pD and pAPG2	This study
Cg-pI/pPPG1	*C. glutamicum* strain harboring pI and pPPG1	This study
Cg-pI/pAPG1	*C. glutamicum* strain harboring pI and pAPG1	This study
Cg-pI/pPPG2	*C. glutamicum* strain harboring pI and pPPG2	This study
Cg-pI/pAPG2	*C. glutamicum* strain harboring pI and pAPG2	This study
Cg-pDI/pPPG1	*C. glutamicum* strain harboring pDI and pPPG1	This study
Cg-pDI/pAPG1	*C. glutamicum* strain harboring pDI and pAPG1	This study
Cg-pDI/pPPG2	*C. glutamicum* strain harboring pDI and pPPG2	This study
Cg-pDI/pAPG2	*C. glutamicum* strain harboring pDI and pAPG2	This study
Plasmids		
pZ8-1	pHM1519, Km^r, *E. coli-C. glutamicum* shuttle vector	(Dusch et al. 1999)
pBbEB1c-RFP	ColE1 (Ec), pBL1 (Cg), Cm^r, P_{trc},BglBrick sites, *rfp* encoding for red fluorescent protein, CoryneBrick vector	(Kang et al. 2014)
pD	pBbEB1c vector carrying the native *C. glutamicum dxs* gene encoding for1-deoxy-*D*-xylulose 5-phosphate synthase	This study
pI	pBbEB1c vector carrying the native *C. glutamicum idi* gene encoding for isopentenyl diphosphate isomerase	This study
pDI	pBbEB1c vector carrying the native *C. glutamicum dxs* and *idi* genes	This study
pPPG1	pZ8-1 vector carrying *P. taeda* pinene synthase (PS) and *A. grandis GPPS1* (geranyl diphosphate synthases) genes codon-optimized for *C. glutamicum*	This study
pAPG1	pZ8-1 vector carrying *A. grandis PS* and *A. grandis GPPS1* genes codon-optimized for *C. glutamicum*	This study
pPPG2	pZ8-1 vector carrying *P. taeda PS* and *A. grandis GPPS2* genes codon-optimized for *C. glutamicum*	This study
pAPG2	pZ8-1 vector carrying *A. grandis PS* and *A. grandis GPPS2* genes codon-optimized for *C. glutamicum*	This study

Materials and methods

Bacterial strains and growth conditions

E. coli strains used for cloning were grown in Lysogeny broth (LB) medium at 37 °C. *C. glutamicum* strains (Table 1) were grown at 30 °C and 200 rpm. Pre-cultures of *C. glutamicum* strains were carried out in Brain–Heart Infusion medium supplemented with 45.5 g sorbitol l^{-1} (BHIS medium). For production of pinene and toxicity analysis in *C. glutamicum*, CGXII minimal medium (Eggeling and Bott, 2010) was used containing 2 % (w/v) glucose in a 250 ml Erlenmeyer flask without baffles. If necessary, appropriate antibiotics (50 μg kanamycin ml^{-1} and 25 μg chloramphenicol ml^{-1} for *E. coli*; 25 kanamycin μg ml^{-1} and 7.5 μg chloramphenicol ml^{-1} for *C. glutamicum*) were added in the media.

Plasmid construction

Plasmid constructs were generated using *E. coli* DH5α and transformation was performed according to the manufacturer's protocol. All plasmids used in this study are listed in Table 1. For construction of pD and pI plasmids, *dxs* and *idi* genes coding for deoxy-D-xylulose-5-phosphate synthase (Dxs) and isopentenyl diphosphate isomerase (Idi) respectively were amplified with PCR from *C. glutamicum* genomic DNA. Primers (Supplementary Table 1) included ribosomal binding sites and enzyme sites. The *dxs* and *idi* genes were inserted at *Bgl*II and *Xho*I (for pD plasmid), or *Bgl*II and *Pst*I (for pI plasmid) sites of the vector pBbEB1c-RFP (Kang et al. 2014). For construction of the pDI plasmid, a portion of the pI plasmid was amplified using CPEC-pBbEB1c-Cgidi FW/RV primers (Supplementary Table 1). The *dxs* gene was amplified from the pD plasmid using CPEC-Cgdxs FW/RV primers (Supplementary Table 1) and the two fragments were ligated using circular polymerase extension cloning (CPEC) assembly (Quan and Tian, 2011). For introduction of the heterologous pinene biosynthesis pathway, genes coding for PS from *Pinus taeda* and *Abies grandis,* as well as two geranyl diphosphate synthases (GPPS1 and GPPS2) from *A. grandis,* were codon-optimized using Gene Designer 2.0 software (DNA2.0, Menlo Park, CA, USA) and were synthesized (Genscript Inc., Piscataway, NJ, USA). The gene accession numbers are followings: PS of *Pt1* from *P. taeda* (AF543527), PS of *AG 3.18* from *A. grandis* (U87909), GPPS1 from *A. grandis* (AF513111) and GPPS2 genes from *A. grandis* (AF513112). Subsequently, PS-GPPS constructs were synthesized with BglBrick formatting (Lee et al. 2011) and the constructs were finally inserted into pZ8-1 vector (Dusch et al. 1999). The final plasmid constructs were confirmed by DNA sequencing. *C. glutamicum* transformation procedures were followed as previously described (Van der Rest et al. 1999) with some modifications (less than 1 μg plasmid was used). These manipulations yielded the plasmids pPPG1 (pZ8-1 vector carrying *P. taeda PS* and *A. grandis GPPS1* genes codon-optimized for *C. glutamicum*; NCBI accession number KJ814974), pAPG1 (pZ8-1 vector carrying *A. grandis PS* and *A. grandis GPPS1* genes codon-optimized for *C. glutamicum*; NCBI accession number KJ814975), pPPG2 (pZ8-1 vector carrying *P. taeda PS* and *A. grandis GPPS2* genes codon-optimized for *C. glutamicum*; NCBI accession number KJ814973), and pAPG2 (pZ8-1 vector carrying *A. grandis PS* and *A. grandis GPPS2* genes codon-optimized for *C. glutamicum*; NCBI accession number KJ814972).

Toxicity analysis of dodecane and pinene in *C. glutamicum*

Dodecane, α-, and β-pinenes were purchased from Sigma-Aldrich. For the toxicity tests, cell suspensions of *C. glutamicum* strains were adjusted to an initial OD_{600} of 1 in 50 ml CGXII medium (Eggeling and Bott, 2010) containing 2 % (w/v) glucose after the preculture of cells in BHIS medium supplemented with 2 % (w/v) glucose for overnight (OD_{600} 10). When an OD_{600} of 4 was reached in the exponential phase, 20 % (v/v) dodecane was added to investigate solvent toxicity of dodecane. Pinene toxicity was analyzed by exposure to 0.25, 0.5, and 1 % (v/v) α-pinene, 30 min after addition of dodecane.

Pinene biosynthesis and extraction

Engineered *C. glutamicum* strains were grown for 2 h in the CGXII medium. After induction with 1 mM IPTG, 20 % (v/v) dodecane was added to the culture media after 4 h. Samples were collected from the dodecane layer of each culture at 24, 48, and 72 h intervals. The samples were diluted with ethyl acetate containing 5 μg caryophyllene ml^{-1} for GC–MS analysis.

Pinene analysis and quantification

To quantify pinene, 1 μl was injected into the GC–MS system, using Agilent 6890N series GC equipped with a TOF–MS (LECO), injector temperature of 250 °C, flow rate of 1.2 ml min^{-1}, split ratio of 2:1, oven initially at 60 °C for 5 min followed by 4 °C min^{-1} increase up to 160 °C and 15 °C min^{-1} increase up to 240 °C, He carrier gas, and HP-Ultra2 (25 m × 0.2 mm in diameter; 0.11 μm film thickness). The peak area was converted to calculate pinene concentration and normalized with caryophyllene as internal standard. The standard curve for pinene quantification was shown in the supplementary figure S1 (7 μg α-pinene ml^{-1} and 8.2 μg β-pinene ml^{-1} for limit of detection).

Results and discussion

In situ extraction for pinene production in *C. glutamicum*

Monoterpenes, including pinene, are toxic to the cells due to phase toxicity other than membrane damage (Brennan et al. 2012; Uribe and Pena, 1990). This is due to the occurrence of the second phase when the aqueous phase is fully saturated. As several organic solvents as a product sink, including dodecane, have been used to eliminate toxicity from pinene and other monoterpenes in yeast without losing cell viability (Brennan et al. 2012), we selected dodecane as the overlay solvent to investigate its feasibility as an organic solvent for in situ pinene production by *C. glutamicum*. Various concentrations [5, 10, and 20 % (v/v)] of dodecane were added to an exponentially growing *C. glutamicum* culture (Fig. 2a). No effect was observed on growth at any dodecane concentrations. This result confirmed that *C. glutamicum* can tolerate dodecane as an organic solvent, similar to *E. coli* and yeasts.

Minimal inhibitory concentration of the positive pinene isomers showed 117 μg to 4,150 μg ml^{-1} against fungi and bacteria but no antimicrobial activities of the negative enantiomers (Rivas da Silva et al. 2012). The theoretical yield of pinene is 0.25 g pinene g^{-1} glucose as sole carbon and it will be 5.1 g pinene l^{-1} using 20 g glucose l^{-1} as sole carbon source in this study. Thus, addition of 0.25, 0.5, or 1 % (v/v) pinene (or 2.1 g pinene l^{-1}, 4.2 g pinene l^{-1}, 8.4 g pinene l^{-1}, respectively) into the CGXII culture medium without dodecane overlay resulted in a decrease to 20 % of cell growth rates as compared to that without pinene ($\mu_{max} = 0.26 \pm 0.01\ h^{-1}$) (Fig. 2b). However, the growth rates were normal as compared to the control (1 h^{-1}/h^{-1}), in the presence of 20 % (v/v) dodecane overlay. Thus, we developed a two-phase culture system with in situ extraction using dodecane overlay in order to eliminate the toxicity of pinene added to *C. glutamicum*.

Construction of metabolically-engineered *C. glutamicum*

To enable *C. glutamicum* to produce pinene, we reconstructed a heterologous pathway by expressing PS and GPPS. Different PSs produce different

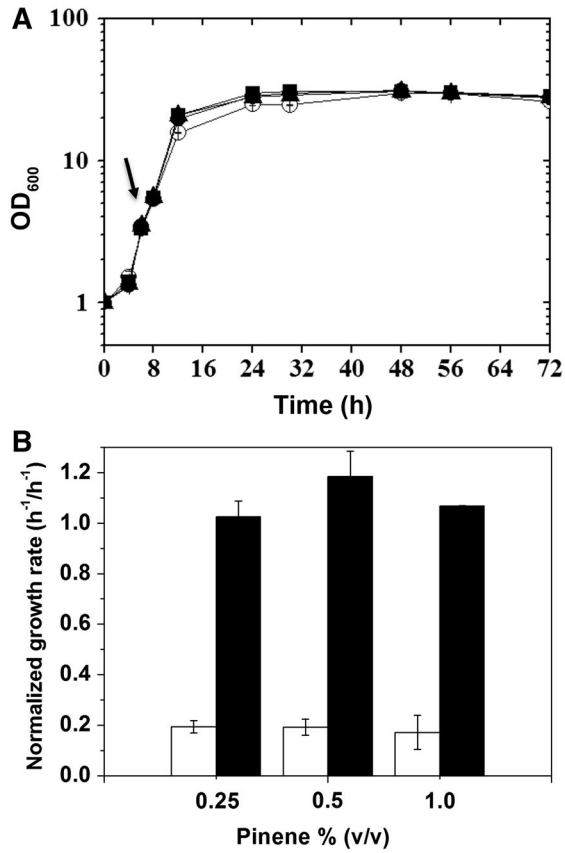


Fig. 2 Toxicity tests of dodecane and pinene. Growth of *C. glutamicum* under various concentration of dodecane, *empty circle* 0 % (v/v), *filled circle* 5 % (v/v), *filled triangle* 10 % (v/v), *filled square* 20 % (v/v) (**a**). *C. glutamicum* wild type strain showed no growth inhibitions under dodecane overlays. *Arrow* indicates the dodecane addition to the culture medium. Pinene toxicities were also tested with *C. glutamicum* wild type strain with 0.25, 0.5, or 1 % (v/v) pinene spiked into the medium [normalized growth rate (h^{-1}/h^{-1}); *white bar*] without dodecane and in situ extraction of pinene were tested with 20 % (v/v) dodecane overlay *black bar* (**b**). Mean values and standard deviations (SD) of triplicate cultures are shown for experiments in (**a**) and (**b**)

mixtures of α- and β-pinene (Bohlmann et al. 1997; Phillips et al. 2003). We tested codon-optimized *Pt1* PS from *P. taeda* and *AG 3.18* PS from *A. grandis*. In addition, to increase GPP availability, GPPS genes were examined. We tested GPPS1 and GPPS2 genes from *A. grandis* that showed high enzyme activity previously (Burke and Croteau, 2002). We first constructed *C. glutamicum* strain harboring either only plasmids with combinations PS and GPPS in them (pPPG1, pAPG1, PPG2, or pAPG2). No pinene could be detected in these strains (data not shown).

Table 2 Quantification of α-/β-pinene production in engineered *C. glutamicum* strains

Strains[a]	α-Pinene (μg l^{-1})			β-Pinene (μg l^{-1})		
	24 h	48 h	72 h	24 h	48 h	72 h
Cg-pDI/pPPG1	141±34.7	122±19.9	135.3±8.5	<8.2	<8.2	<8.2
Cg-pDI/pAPG1	<7	<7	<7	<8.2	<8.2	165
Cg-pDI/pPPG2	172.7±47.3	176±60	140.7±84.9	<8.2	<8.2	<8.2
Cg-pD/pAPG1	<35	<35	<35	<41,165	<41	<41
Cg-pD/pPPG2	48,173	<35,68	<35,175	<41,117	<41	<41
Cg-pD/pAPG2	<35	<35	<35	157,<41	153,<41	<41
Cg-pI/pPPG1	<35,75	<35,46	<35	<41	<41	<41
Cg-pI/pPPG2	56,62	<35	<35,74	<41	<41	<41
Cg-pI/pAPG2	<35	<35	<35	<41	<41	144,<41

The pinene production is presented as μg l^{-1} of culture. Limit of detection (LOD) is 7 μg α-pinene l^{-1}, limit of quantification (LOQ) of 35 μg α-pinene l^{-1}, LOD of 8.2 μg β-pinene l^{-1}, LOQ of 41 μg β-pinene l^{-1}

[a] Some strains below LOQ of both α-/β-pinene are not listed in the Table 2 as followings: *Cg-pDI/pAPG2, Cg-pD/pPPG1* and *Cg-pI/pAPG1*. For some quantifiable data, mean values and standard deviations of biological triplicate or at duplicate experiments were shown except (*Cg-pDI/pAPG1*)

Therefore, to increase levels of available IPP using metabolic engineering strategies that were used previously to enhance isoprenoid production in *E. coli* (Alper et al. 2005; Choi et al. 2010), we overexpressed the native *dxs* and *idi* genes. Each MEP gene was overexpressed individually or in combination with the other from a plasmid, and these three plasmids were combined with the four PS:GPPS plasmids. Thus, the strategy of using combinatorial gene expression of key enzymes, namely Dxs and Idi along with heterologous pinene metabolic pathways required the construction of 12 engineered *C. glutamicum* strains (Table 1).

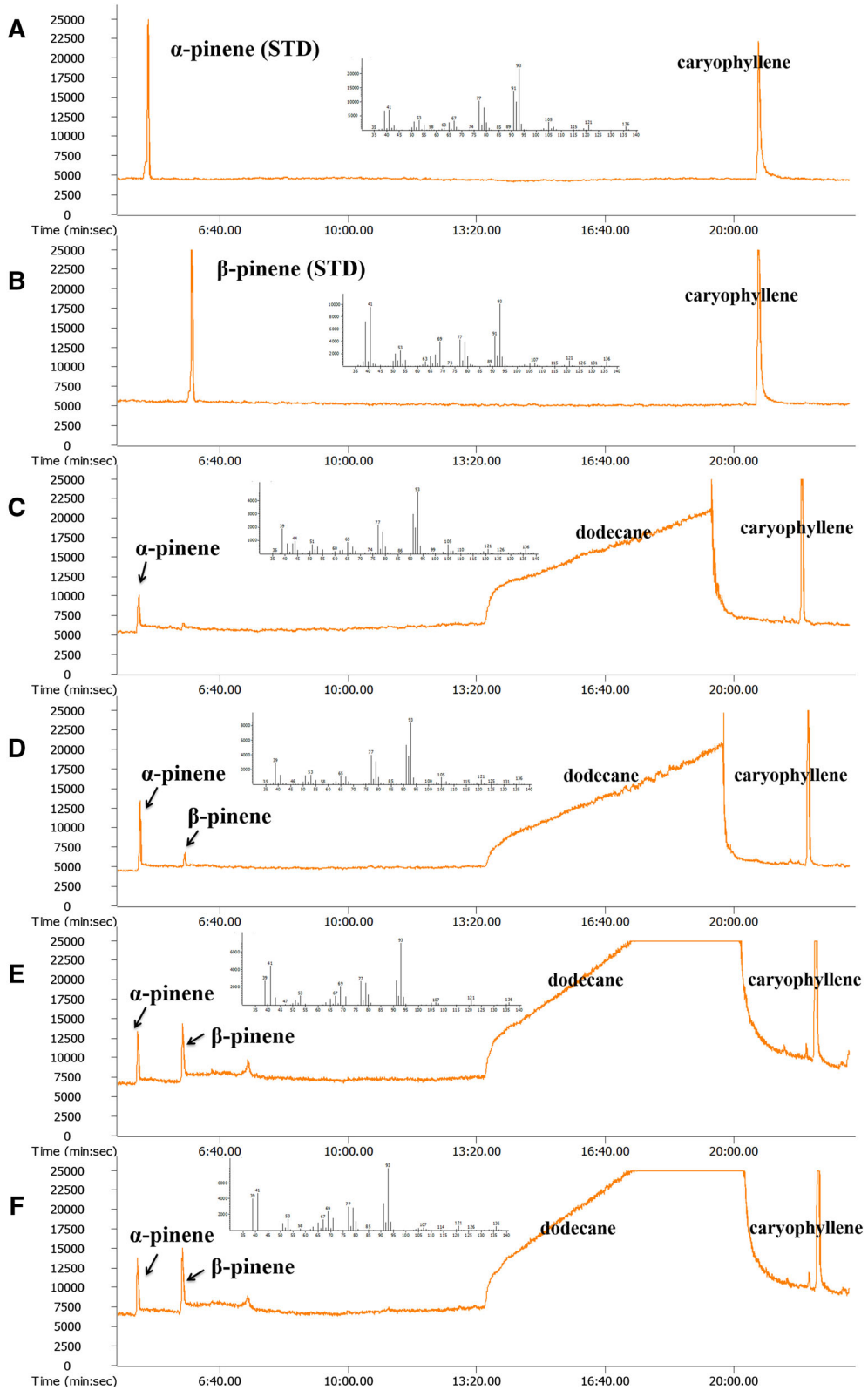
Biosynthesis of pinene in engineered *C. glutamicum*

Engineered *C. glutamicum* strains produced two forms of pinenes in vivo (Fig. 3). When PS encoded by the *Pt1* gene was used in *C. glutamicum*, a strong α-pinene peak was observed; however, the β-pinene peak and other minor terpene peaks were below the limit of quantification. This has been reported previously as α-pinene (79 %) and to a lesser extent, β-pinene (4.2 %) in vitro (Phillips et al. 2003). On the other hand, PS from *AG 3.18* produces similar levels of both pinenes (48 % α-pinene and 52 % β-pinene) in vitro (Bohlmann et al. 1997). In our study, *C. glutamicum* strains with PS from *AG 3.18* produced more β-pinene than α-pinene (Fig. 3) in vivo.

Fig. 3 GC-MS analysis of dodecane fractions from the▶ engineered *C. glutamicum* strains *Cg-pDI/pPPG1* and *Cg-pDI/pPPG2*. Standard α-pinene (**a**), standard β-pinene (**b**), extracted samples of *Cg-pDI/pPPG1* (**a**), *Cg-pDI/pPPG2* (**d**), *Cg-pD/pAPG1* (**e**), and *Cg-pD/pAPG2* (**f**). Dodecane was used for in situ extraction of pinene produced in the culture and it was diluted with ethyl acetate for the measurement. Caryophyllene was used as internal standard

We conclude that in *C. glutamicum*, pinene synthase determines the ratio of pinenes in vivo when only AgGPPS1 or 2 were used. Similar results were also found in production of pinenes from engineered *E. coli,* however the ratios of α/β-pinene were altered when different GPPSs were used with the corresponding PSs due to that GPP concentration regulates the product specificity of PSs (Sarria et al. 2014). For biosynthesis of pure α-pinene, previous in vitro activity of *Pt30* showed much higher ratio of α-pinene (97 %) (Phillips et al. 2003) compared to the activity of *Pt1*, which could be another targets for PS gene expression to produce pure α-pinene.

Although the pinene production from engineered strain was very low, combinatorial gene expression of Dxs and Idi together, showed the quantifiable measurement of α-pinene in *Cg-pDI/pPPG2 and Cg-pDI/pPPG1* strains (Table 2). The *Cg-pDI/pPPG2 and Cg-pDI/pPPG1* strains produced at 26.9 μg ± 7 and 20.4 μg ± 7.3 α-pinene g^{-1} CDW at 48 h, respectively. Since engineered *C. glutamicum* produced lycopene at


A
α-pinene (STD)
caryophyllene
B
β-pinene (STD)
caryophyllene
C
α-pinene
dodecane
caryophyllene
D
α-pinene
β-pinene
dodecane
caryophyllene
E
α-pinene
β-pinene
dodecane
caryophyllene
F
α-pinene
β-pinene
dodecane
caryophyllene
Time (min:sec)
6:40.00
10:00.00
13:20.00
16:40.00
20:00.00

2.4 mg g^{-1} CDW (Heider et al. 2012), there could be a potential to engineer *C. glutamicum* to produce more pinene. Despite the low levels of pinene produced in this study, this is the first report of pinene biosynthesis in *C. glutamicum* using glucose as the sole carbon source.

Additionally, redirection of metabolic carbon flux for IPP supply was attempted in *C. glutamicum*. Particularly, glutamate dehydrogenase deletion mutant strain of *E. coli* showed a significantly higher lycopene yield (Alper et al. 2005). However, when we examined strain LN ΔGDH in which the *gdh* gene coding for glutamate dehydrogenase was deleted in *C. glutamicum* (Müller et al. 2006), no growth was observed for LN ΔGDH harboring pDI/pPPG2 in CGXII medium after pre-cultivation in BHIS medium (data not shown). The reason for cell death is unclear.

Previous studies have reported that truncations of limonene synthase and GPPS have resulted in high titer of limonene and its derivative in *E. coli* (Alonso-Gutierrez et al. 2013). Further engineering of *C. glutamicum* strain with structural truncations of PS (Pt30) and GPPS2 for enhanced pinene production could be useful to ensure high enzyme activity of PS and GPPS in *C. glutamicum* and to prevent misfolding proteins of PS and GPPS. Additionally, PS-GPPS fusion protein (Sarria et al. 2014) could play an important role increasing the activity of PS and GPPS in *C. glutamicum*.

Conclusion

We present the first report of the development of a two-phase, in situ extraction system for *C. glutamicum* and engineered *C. glutamicum* strains for production of pinene as another target chemicals that can be produced from glucose as microbial cell factory. Further protein engineering of PS and GPPS based on direct evolution through random mutagenesis or protein structures could be useful to increase the activities to enhance the carbon flux toward biosynthesis of pinene in *C. glutamicum* in addition to the modification of central metabolism to increase the pool of key intermediates. OMICS-based System biology also could be applied to understand the cellular metabolism of the pinene-producing *C. glutamicum* to identify the bottlenecks. Considering its GRAS status like yeasts, further engineering of *C. glutamicum* is required for the production of monoterpenes in manufacture of fragrances, flavors, and pharmaceuticals. Besides monoterpenes, other terpenes including sesquiterpenes (Paddon et al. 2013) and diterpenes (Ajikumar et al. 2010) could be good targets for *C. glutamicum* as other industrial applications combined with chemical process.

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Conflict of interests The authors declare that they have no conflicts of interest.

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