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CO-EXPRESSION OF TERPENE CYCLASE AND CYTOCHROME P450 FROM ACTINOMYCETES FOR TERPENE PRODUCTION IN *Escherichia coli*

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Abstract

Terpenes are one of the many metabolites produced by actinomycetes, ranging from essential metabolites such as sterols and hormones to unique secondary metabolites. Most secondary metabolites are isolated from plants and fungi due to the responsible bacterial genes that are silent in their parent microorganisms. With the development of genome mining and heterologous expression system, potential bacterial gene sequences can be identified and heterologously expressed in host microorganisms. The cytochrome P450 (CYP450) is an enzyme in bacteria that has numerous biotechnology applications. In this study, we heterologously co-expressed actinomycetes terpene cyclase and CYP450 in *Escherichia coli*. We demonstrated the use of synthetic DNA fragments for terpene production in *E. coli*. Terpene cyclase genes were synthesized based on sequences from *Streptomyces clavuligerus* and *Streptomyces griseus*, whereas CYP450 genes from *S. griseus*, *S. griseus* NBRC13350, and *Streptomyces coelicolor*, respectively. The genes were recombined in *Saccharomyces cerevisiae* and expressed in *E. coli*. Co-expression of terpene cyclase from *S. clavuligerus* and CYP450 from *S. coelicolor* produced monoterpenes, and a higher expression level was observed.

INTRODUCTION

Terpenes are one of the many metabolites produced by actinomycetes, ranging from essential metabolites such as steroids and hormones to unique secondary metabolites [1-3]. They are available in the market as fragrances, flavoring substances, pharmaceuticals, fuels, bulk chemicals, and nutrients [4]. The actinomycetes have arisen as fertile producers for bacterial terpenes. Numerous studies report that volatile terpenes have been detected in actinomycetes [1, 5, 6, 7]. 2-methylisoborneol and geosmin are two odoriferous terpenoids that have been identified to represent degraded methylated monoterpene and sesquiterpene. The characterization of terpene cyclases from numerous actinomycetes species confirmed that these enzymes are not membrane-bound and can be overexpressed as functional recombinant proteins in *Escherichia coli* [1, 8]. Some of these secondary metabolites have proved to be a valuable source of bioactive compounds for use in the agrochemical and pharmaceutical industries.

Using recombinant deoxyribonucleic acid (DNA) technology, these secondary metabolites can be produced heterologously. The first artificially-produced hybrid DNA was developed with bacterial plasmids, and countless remarkable advances and discoveries relied on microbial systems. With an emerging number of bacterial genome sequences publicly available, it further provides a powerful platform for the identification and characterization of new terpene metabolites. Heterologous expression in *E. coli* proved to be an effective method for producing novel terpenes [9, 10]. CYP450 can catalyze a diverse range of reactions, often with specificity and selectivity, making it

attractive for broad applications in biotechnology [11]. Understanding the bacterial functions of CYP450 will provide new opportunities to characterize these enzymes and to engineer their biosynthetic machinery for natural product structural diversity.

Previous research suggests that gene encoding such as terpene cyclase exists extensively in bacteria, and this rich source can be manipulated to discover novel natural products [12]. These presumptive terpene cyclase genes appear to be dormant in their parent microorganisms and are waiting to be discovered. The expression of these genes in an engineered heterologous host has allowed for the identification of the encoded terpene cyclase's biochemical function. *E. coli* is an ideal heterologous hosts since they are fast growers, need little resources, express protein at exceptional levels, and have an abundance of genetic tools available for engineering. Direct experiments between terpene cyclase and CYP450 co-expressions are necessary to uncover the potentials of this enzyme, subsequently diversifying terpenes from their biosynthetic contexts. This research was carried out to co-express actinomycetes terpene cyclases and CYP450 in *E. coli* and subsequently to biosynthesize, screen, and identify terpene metabolites produced by the engineered *E. coli.*

MATERIALS AND METHODS

Actinomycetes Synthetic Gene Fragments

Five gBlocks® Gene Fragments were synthesized based on the DNA sequence of selected genes from the National Center for Biotechnology Information (NCBI) by the Basic Local Alignment Search Tool (BLAST); Two synthetic genes encoded for terpene cyclase were from *S. clavuligerus* (NCBI accession number: WP_009998474.1) and *Streptomyces griseus* (NCBI accession number: WP_042496076.1), whereas three synthetic genes encoded for CYP450 were from *S. griseus* (NCBI accession number: WP_115068281.1), *S. griseus* NBRC13350 (NCBI accession number: WP_012378290.1), and *S. coelicolor* (NCBI accession number: NC_003888.3). The genes were synthesized by Integrated DNA Technologies, Inc (Singapore), and purchased through a local distributor, Apical Scientific Sdn. Bhd (Malaysia).

Insertion of Terpene Cyclase and Cytochrome P450 Genes into the Expression Vector, pET2U

pET2U expression vector which is replicable in both *S. cerevisiae* YPH499 and *E. coli* (Pahirulzaman KAK, unpublished data), was linearized by digestion with *Eco*RV. The synthetic genes encoded for terpene cyclase and the CYP450 genes were amplified using primers in Table 1. All primers had 30-base 5' extensions corresponding to sequences flanking the targeted insertion site in vector pET2U. PCR amplification was carried out with the program; pre-denaturation at 95 °C, 30 seconds (1 cycle), denaturation at 95 °C, 30 seconds; annealing at 55 °C, 45 seconds; extension at 68 °C, 1 minute (30 cycles) and a final extension at 68 °C, 5 minutes (1 cycle). The extension time (68 °C) was set at 1 minute/kb of DNA. The gene of interest(s) was then assembled between the T7 promoter and terminator by homologous recombination in yeast. Transformants were selected on SM-ura plates. The resultant plasmid was transformed into *E. coli* cells by electroporation and selected on LB agar supplemented with 100 µg/mL ampicillin. The restriction of enzyme digestions confirmed transformants; plasmids carrying the terpene cyclase gene were digested with *Eco*RV, whereas plasmids carrying the terpene cyclase and cytochrome P450 genes were digested with *Eco*RV plus *Pvu*II, respectively.

Screening of *Escherichia coli* Transformants

E. coli transformants were screened to determine whether the gene of interest (GOI) was successfully inserted into the plasmid vector. Colony lysate PCR was used to screen the *E. coli* transformants. Cells were picked from the single colonies that grew on LBamp agar using sterile toothpicks and were suspended into a 25 µL of 50 µg/mL Proteinase K in TE buffer. The cells were heated at 55 °C for 15 minutes, followed by heating at 80 °C for 15 minutes. The suspension was then centrifuged at 13200 rpm for 3 minutes. 2.5 µL of the supernatant was used as the template DNA for PCR amplification using the program, as previously described. PCR products were analyzed by electrophoresis on 1% agarose gel in 1X TAE buffer at 100V for 30 minutes. Positive colonies were streaked onto LBamp agar and incubated at 37 °C for 24 hours. Using the High-Speed Plasmid Mini Kit (Geneaid, Taiwan), and following the manufacturer's protocol, the plasmid was extracted from positive *E. coli* transformants. Digestion was done in Thermo Scientific Fast Digest Restriction Enzymes kit with 2 µL (analytical) or 15 µL (preparative) plasmid miniprep DNA, according to the manufacturer's protocol. After 1 hour at 37 °C digestion, the digested plasmid was viewed on 1% agarose gel in 1X TAE buffer at 100V for 30 minutes. The insertion of the gene(s) into the expression vector was also confirmed with DNA sequencing carried out by Apical Scientific Sdn. Bhd (Malaysia), and were analyzed using the BLASTN 2.10.0+ program from the NCBI database.

Table 1. Primers used in this work. Primers with 'exp' were used for RT-qPCR studies.

Primer name	DNA Sequence (5'→ 3')	Gene
Terp1_F	AATACGACTCACTATAGGGGAATTGTGAGATGCCTCACGCAGAATTCCA	Terpene cyclase from *S. clavuligerus*
Terp1_R	CCCGTTTAGAGGCCCCAAGGGGTTATGCTA TCAGGGTTTTAAATGCTGGA	
Terp2_F	AATACGACTCACTATAGGGGAATTGTGAGA TGTCTCAGATCACGTTGCC	Terpene cyclase from *S. griseus*
Terp2_R	CCCGTTTACACCCCCCAAGGGGTTATGCTATCACGCGAAGTGGCGGG	
T1P450Sc_F	CTGTCGTATATCCAGCATTTAAAACCCTGAATGACAGAGGAGACGATCTC	CYP450 from *S. coelicolor*
T1P450Sc_R	CCCGTTTAGAGGCCCCAAGGGGTTATGCTATCACCAAGTGACTGGTAAGG	
T1P450SgNB_ F	CTGTCGTATATCCAGCATTTAAAACCCTGAATGGTCCAAGATTTAGACGG	CYP450 from *S. griseus* NBRC13350
T1P450SgNB_ R	CCCGTTTAGAGGCCCCAAGGGGTTATGCTATCAACCCAACAATACAGGGA	
T1P450Sg_F	CTGTCGTATATCCAGCATTTAAAACCCTGAATGACGACCTCGCCTGGCCC	CYP450 from *S. griseus*
T1P450Sg_R	CCCGTTTAGAGGCCCCAAGGGGTTATGCTACTACCAACGTACTGGCAACT	
T2P450Sc_F	GATTCTTTATCCCGCCACTTCGCGGCGTGAATGACAGAGGAGACGATCTC	CYP450 from *S. coelicolor*
T2P450Sc_R	CCCGTTTAGAGGCCCCAAGGGGTTATGCTATCACCAAGTGACTGGTAAGG	
T2P450SgNB_ F	GATTCTTTATCCCGCCACTTCGCGGCGTGAATGGTCCAAGATTTAGACGG	CYP450 from *S. griseus* NBRC13350
T2P450SgNB_ R	CCCGTTTAGAGGCCCCAAGGGGTTATGCTATCAACCCAACAATACAGGGA	
T2P450Sg_F	GATTCTTTATCCCGCCACTTCGCGGCGTGAATGACGACCTCGCCTGGCCC	CYP450 from *S. griseus*
T2P450Sg_ R	CCCGTTTAGAGGCCCCAAGGGGTTATGCTACTACCAACGTACTGGCAACT	
T1P450_F	GCCTGGCAGACTATT	Terpene cyclase and CYP450
T2P450_F	ATTTGCTAAGCATCG	
Tub-F	TTGCTTCCGACAACGCGACT	β-tubulin from *E. coli*
Tub-R	TTGTCCATGCCGATACCTGT	
T1-exp-F	CTGGCAGACTATTTGCGCAC	Terpene cyclase from *S. clavuligerus*
T1-exp-R	CTGTAACCGTGTCTGGCACA	
T2-exp-F	ATTTGCTAAGCATCGCCGCG	Terpene cyclase from *S. griseus*
T2-exp-R	TAAAGAGCGAGCCGAATCCG	
P450Sc-exp-F	GGCAGATGTTTCACTTGTTG	CYP450 from *S. coelicolor*
P450Sc-exp-R	CAGCGCAGTAACTCATCGAT	
P450Sg-exp-F	GCTGGTCAAACGATTCGCAA	CYP450 from *S. griseus*
P450Sg-exp-R	TTCAGTACGCTCTGGGTTGA	

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

The expression level of each *E. coli* transformants were determined in duplicate by RT-qPCR using primers in Table 1. The levels of expression for each gene were quantified using the comparative method. Data output is expressed as expression level fold-change or fold-difference compared to an internal standard. The amplification of the target genes, which were terpene cyclase and CYP450, was measured and compared with an appropriate endogenous control (housekeeping gene), a β-tubulin gene from *E. coli*. Ribonucleic acid (RNA) was extracted from *E. coli* transformants using FavorPrep Blood/Cultured Cell Total RNA Mini Kit (Favorgen, Taiwan) followed by RNA transcription into complementary DNA (cDNA) using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Japan). The cDNA is then used as the template for the RT-qPCR reaction using the THUNDERBIRD SYBR qPCR mix kit (Toyobo, Japan). Procedures were carried out according to the manufacturer's protocol. RT-qPCR was carried out in an Mx3000P PCR system (Stratagene) with the program; pre-denaturation at 95 °C, 1 minute (1 cycle), denaturation at 95 °C, 15 seconds, annealing at 60 °C, 1 minute and extension at 68 °C, 5 minutes (40 cycles).

Terpene Extraction and Analysis by GC-MS

A single colony of the *E. coli* transformant was inoculated in a 50 mL LBamp medium and incubated at 37 °C with a 150rpm rotary agitation. The cells were induced with

isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.4 mM when OD600 of the bacterial culture reached 0.6-0.9. 5 mL of induced bacterial culture was transferred into a sterile GC-MS bottle and further incubated at 37 °C for 24 hours. The volatile terpenes produced by the transformant were analyzed using headspace GC-MS. Product characterization was carried out by capillary GC-MS using Thermo Scientific™ TSQ™ 9000 triple quadrupole GC-MS/MS system. A TG-5MS capillary column (15 m× 0.25 mm× 0.25 µm, Thermo Scientific) was used, with helium as the carrier gas at a flow rate of 1 mL/minutes. The following oven temperature program was carried out: 60 °C for 5 minutes, with an increase of 5 °C/minute to 200 °C for 5 minutes, then programmed from 200 °C to 300 °C at 10 °C/minute, where it was held for 2 minutes. The injector temperature was maintained at 150 °C; ion source temperature 230 °C; EI 70 eV; mass range 30-600 m/z. Nitrogen is the purging gas, with 1 mL of samples injected in split/splitless injection mode with a 1:10 split ratio. Peak identification was based on the full mass spectra searched against the National Institute of Standard and Technology (NIST) mass spectral database.

RESULTS AND DISCUSSION

E. coli is an ideal heterologous host since they are fast grower, need little resources, able to express protein at exceptional levels and abundant genetic tools available for engineering. Furthermore, *E. coli* naturally constitute the basic building block of terpenoids which are isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Despite the enormous structural differences between terpenoids, they are all resultant of five-carbon isoprene units. IPP and DMAPP are two precursors involved in terpenoids synthesis by means of a different number of repetitions, rearrangement and cyclization reactions. There are two metabolic pathways for biosynthesis of terpenes [13, 14, 15], which are methylerythritol 4-phosphate (MEP) and mevalonate acid (MVA) pathway. Various precursors and enzymes are needed for each pathway, and different species use one or both pathways. The MVA pathway occur in eukaryotes and it was shown that bacteria also used this pathway. However, the first and second enzymes in the MEP pathway; 1-deoxy-D-xylulose 5-phosphate (DXP) synthase and DXP reductoisomerase, were discovered in *E. coli* in the year 1990. Consequently, it has been demonstrated that most bacteria use the MEP pathway, while only few use the MVA pathway [14].

In this study, three synthetic genes encoded for terpene cyclase genes from *S. griseus*, *S. griseus* NBRC13350 and, *S. coelicolor* were synthesized (1182, 1188, and 1215 bp respectively). The sequences were selected based on previous findings by Pahirulzaman, KAK (unpublished data) that discovered terpene cyclase genes in isolated *S. clavuligerus* and *S. griseus*. Whereas, for the CYP450, it was randomly selected based on predicted functions as on the NCBI database.

In vivo vector construction via homologous recombination in *S. cerevisiae* strain YPH499 resulted in five expression vectors: pET2U-T1; pET2U-T2; pET2U-T1Sc; pET2U-T2Sc and pET2U-T2Sg (Table 2). The expression vectors were individually transformed in *E. coli* strain BL21 (DE3). The gene of interest arrangement was confirmed by DNA sequencing and restriction digestion analysis. Altogether, 56 transformants harboring either the terpene cyclase gene alone and both the terpene cyclase gene and CYP450 were recovered. Only pET2U-T1 and pET2U-T2 (digested with *Eco*RV), pET2U-T1Sc, pET2U-T2Sc, pET2U-T2Sg (digested with *Eco*RV and *Pvu*II) showed the predicted fragments (Figure 1) as shown in the map (Figure 2). These results correspond with the DNA sequencing (using forward primers T1P450_F and T2P450_F), which confirmed the correct insertion of the gene(s) of interest downstream the T7 promoter in pET2U vector.

Table 2. *E. coli* transformants.

Transformant name	Description
pET2U-T1	pET2U inserted with terpene cyclase from *S. clavuligerus*
pET2U-T2	pET2U inserted with terpene cyclase from *S. griseus*
pET2U-T1Sc	pET2U inserted with terpene cyclase from *S. clavuligerus* and CYP450 from *S. coelicolor*
pET2U-T2Sc	pET2U inserted with terpene cyclase from *S. griseus* and CYP450 from *S. coelicolor*
pET2U-T2Sg	pET2U inserted with terpene cyclase from *S. griseus* and CYP450 from *S. griseus*

The RT-qPCR analysis is fundamental to determine the expression level of recombinants in this study. It is most extensively used for computing gene expression levels due to its high specificity, sensitivity, reproducibility, and high-throughput capacity [16]. Results demonstrated that the expression level of the terpene cyclase gene is higher compared to the control gene, β-tubulin, in the pET2U-T1Sc transformant. Interestingly, pET2U-T1Sc expressed the terpene cyclase gene much higher in the presence of CYP450 compared to the pET2U-T1 transformant that is lacking in CYP450 with 21027 and 160-fold difference, respectively (Table 3). The gene expression level of other recombinants is not significantly high. A similar trend was reported, showing engineered *E. coli* strains can improve taxol biosynthesis, a CYP450-mediated 5α-oxidation of taxadiene to taxadien-5α-ol production by 2400-fold [17]. Terpene

cyclase production by recombinant *E. coli* was screened and analyzed using GC-MS with a headspace program. pET2U-T1Sc is the only transformant which is able to biosynthesize two monoterpene compounds, terpinen-4-ol, and α–terpineol.

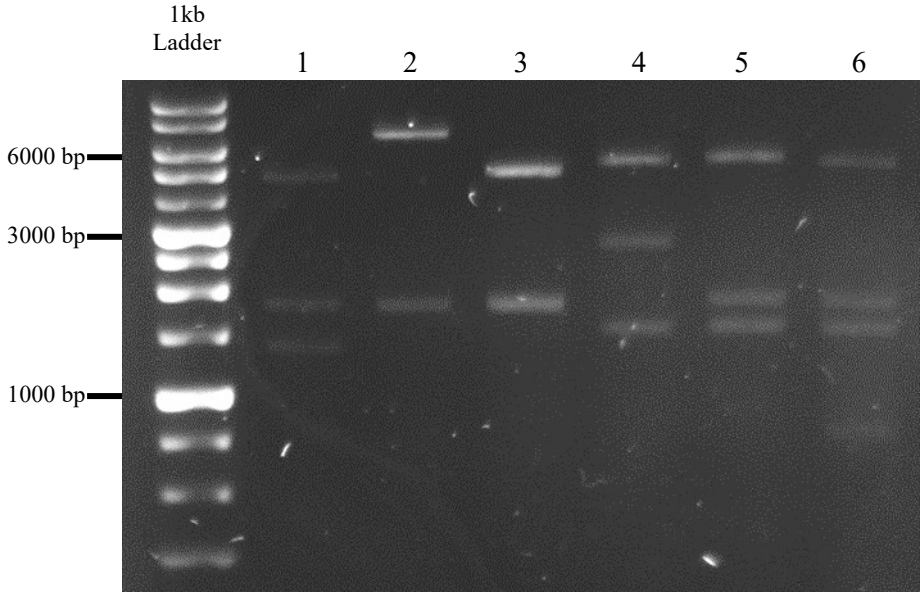

Figure 1. The restriction patterns of plasmid constructs are consistent with the predicted sizes as shown in the plasmid map. Digested products of pET2U – 4756, 1795 and 1347 bp (lane 1); pET2U-T1 – 6815 and 1795 bp (lane 2); pET2U-T2 – 4956, 1880 and 1795 bp (lane 3); pET2U-T1Sc – 5198, 2832, and 1552 bp (lane 4); pET2U-T2Sc – 5198, 1880 and 1552 bp (lane 5); pET2U-T2Sg – 5372, 1880, 1552 and 766 bp (lane 6).

Table 3. The expression level of terpene cyclase and CYP450 genes in the transformants compared to the control gene, calculated using comparative method.

Transformant	Transformant average Ct	β-tubulin average Ct	ΔCt[a]	ΔΔCt[b]	Fold difference[c]
pET2U-T1	24.18	31.50	24.18	-7.32	160
pET2U-T2	33.41	31.50	33.41	1.91	0.26
pET2U-T1Sc	17.14	31.50	17.14	-14.36	21027
pET2U-T2Sc	34.90	31.50	34.90	3.4	0.09
pET2U-T2Sg	32.14	31.50	32.14	0.64	0.64

[a] The ΔCt was determined by the average C_t cycle.
[b] The $\Delta\Delta C_t$ was determined by subtracting the ΔC_t value of tubulin gene from the ΔC_t value of a transformant.
[c] Assuming the efficiency is 100%, the $\Delta\Delta C_t$ was described as $2^{-\Delta\Delta Ct}$

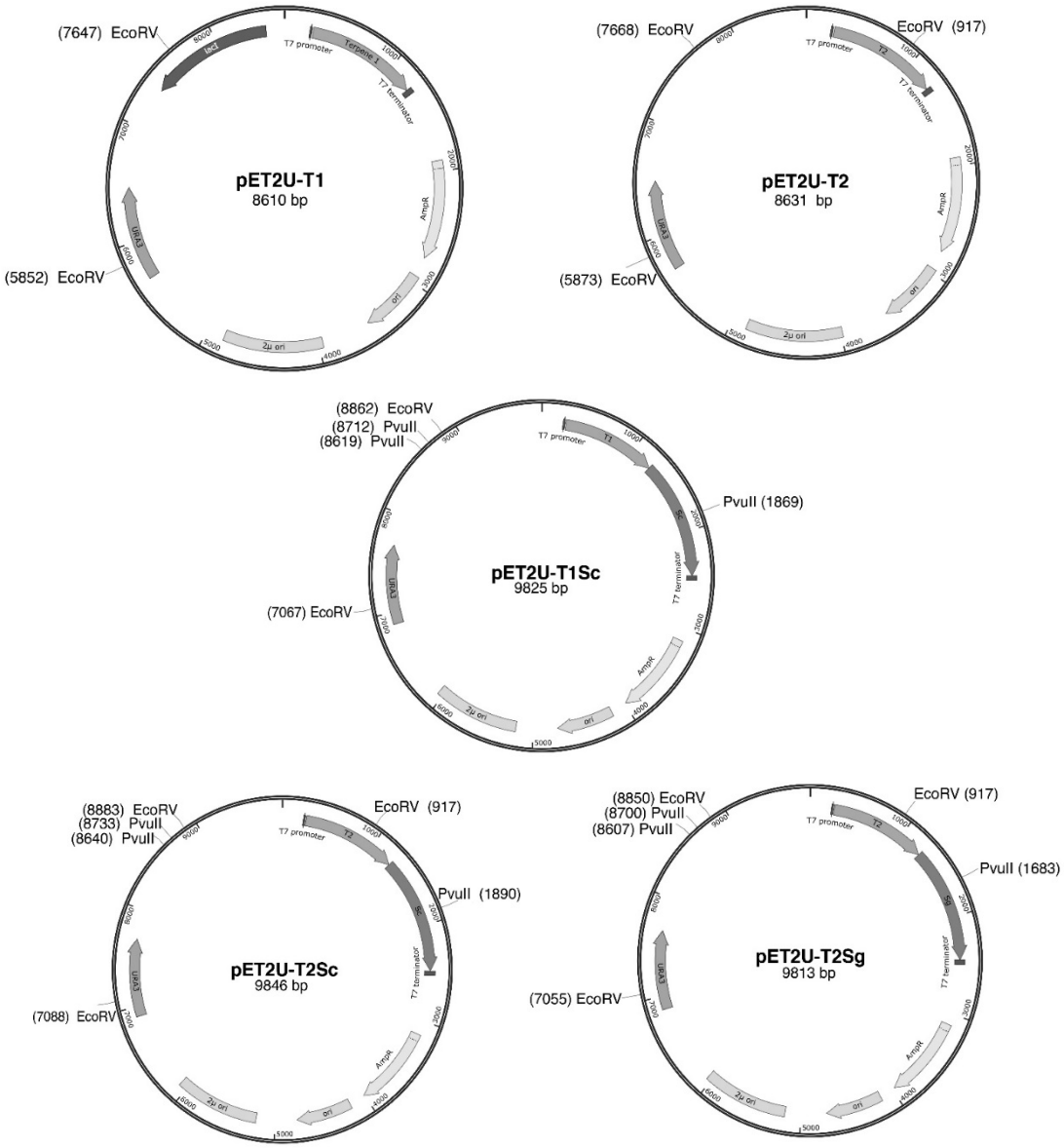

Figure 2. Plasmid map with predicted restriction enzyme sites. pET2U-T1 and pET2U-T2 were digested with *Eco*RV, whereas pET2U-T1Sc, pET2U-T2Sc, pET2U-T2Sg were digested with *Eco*RV and *Pvu*II, respectively.

GC-MS analyzed the volatile compounds trapped from the headspace of the sealed cultures. As expected, the indole peak was observed in all the transformants at retention times, between 13 to 14 minutes. GC-MS spectra (Figure 3) proved that pET2U-T1Sc is the only transformant able to produce two monoterpenes, which are terpinen-4-ol and α–terpineol. Peak identification was based on the full mass spectra searched against the NIST mass spectral database. This finding supports the huge 21027-fold difference of the RT-qPCR analysis. Indole is a signaling molecule that is secreted by more than 85 bacterial species, including *E. coli*. It is formed by the tryptophanase enzyme (TnaA), which converts tryptophan to indole, ammonia, and pyruvate [19].

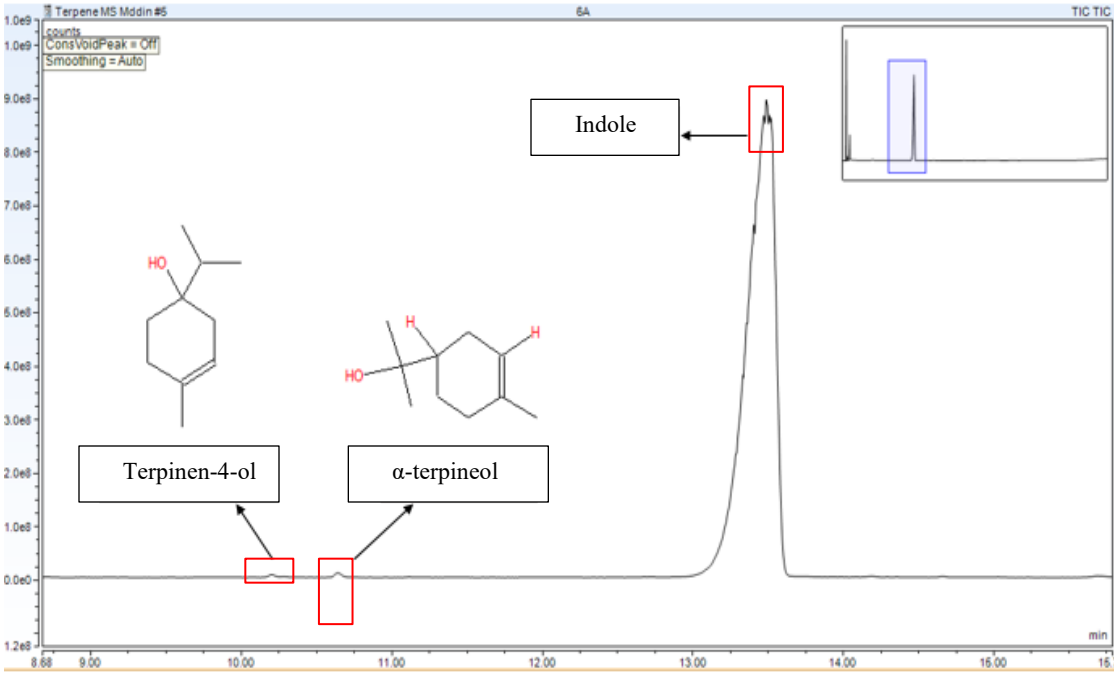

Figure 3. GC-MS spectra showing monoterpenes and indole (red boxes) produced by pET2U-T1Sc transformant.

In this study, we managed to produce monoterpenes, which are terpinen-4-ol and α–terpineol. The monoterpenes are a valuable class of terpenes and are essential to industries that use flavors and the perfume industry. Terpineol is a monoterpene alcohol derived from various sources such as petitgrain oil, cajuput oil, tea tree oil, and pine oil. There are five common isomers of terpineols; gamma-, beta-, delta-, alpha- and terpinen-4-ol. The most common terpineols found in nature are α-terpineol and its isomer terpinen-4-ol. Terpineol is currently derived from plants, which is ineffective and needs substantial natural resources due to low yields [20]. A low titer of terpinen-4-ol and α–terpineol were detected from the volatile analysis. This is due to indole acting as an inhibitory compound. While the biochemical mechanism of the indole interaction with the isoprenoid pathway is currently unknown, it does suggest a potential synergistic effect between the indole and terpenoid compounds of the isoprenoid pathway inhibiting cell growth [17]. Utilizing genes of rate-limiting enzymes from different organisms is an effective method to improve pathway efficiency. One of the rate-limiting enzymes in the *E. coli* terpene biosynthesis is the GPP synthase [15, 21]. Engineering a biosynthetic pathway using either an MEP or MVA pathway combined with the GPP and terpene cyclase genes in an engineered *E. coli* strain has proved effective in increasing the titer. Another possibility of increasing the yield is by directly adding GPP precursor to the culture medium [5]. The terpene yields can be improved by increasing the concentrations of the precursor [22]. However, this is the least favorable approach as GPP is quite expensive and is therefore not cost-effective for biosynthesis scale up.

Native strains *S. griseus*, *S. clavuligerus*, and *S. coelicolor* are known as terpene cyclase producers for germacradienol/geosmin synthase, 2-methylisoborneol synthase, (+)-caryolan-1-ol, epi-isozizaene synthase, 1, 8-cineole synthase, (-)-δ-cadinene synthase, (+)-T-muurolol synthase and linalool synthase [23, 24]. Those terpene cyclase genes in their parent microorganisms appear silent. The manipulation of these genes in an engineered heterologous *E. coli* host has enabled the biochemical function of the encoded terpene cyclase to be established [3]. As described earlier, CYP450 can catalyze a diverse range of reactions at non-activated C–H bonds with exceptional selectivity, which are useful for the production of high-value compounds [25]. Furthermore, CYP450 is not substrate-specific; thus, non-natural substrates can be modified but will maintain their high stereo- and regiochemical selectivity observed from the transformation of their natural substrate [26]. Recently, co-expression of commercially available monoterpenes with CYP450 resulted in 27 novel unreported terpenoids [27]. These promiscuous CYP450 do not necessarily co-localize with terpene cyclase encoding genes in the genomes, suggesting their potential to diversify terpenes out of their biosynthetic contexts.

The structural diversity of terpenoids is predicated on the orientation of their substrate within the site of their correlated terpene synthase which then undergo a series of cyclizations and/or rearrangements to produce specific terpenoid [28]. In this study, some of the constructs could not

produce terpenoids even though the plasmids harbored similar enzyme/homologs. Despite the general structural similarity of terpene synthases, the identification of individual amino acids that are associated with certain mechanistic steps is a difficult task. Indiscriminate activity in terpenoid biosynthesis is based on the mildness of the enzyme template. It involves malleable substrate and intermediate product conformation through a multi-step reaction to product formation [28]. Generally, product profile of a given enzyme cannot be determined from sequence similarity. Sometimes adequate expression may not be observed due to proteolysis or insufficient translation (mRNA may remain in the secondary structure and translation is impeded) [29].

CONCLUSION

In summary, we have managed to heterologously co-expressed actinomycetes terpene cyclase and CYP450 in *E. coli*. Homologous recombination in the yeast makes it possible to assemble and express terpene cyclase genes and CYP450 simply and rapidly way. Monoterpenes expressed by the transformants showed an interesting correlation between the functional CYP450 and the terpene cyclase genes. Screening procedures for an enhanced terpenoid synthesis would greatly facilitate the terpenoid production by engineered bacteria, as currently, only a limited number of high-throughput techniques are available for selected compounds.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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