# Generation and Functional Evaluation of Designer Monoterpene Synthases

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## ABSTRACT

Monoterpene synthases are highly versatile enzymes that catalyze the first committed step in the pathways toward terpenoids, the structurally most diverse class of plant natural products. Recent advancements in our understanding of the reaction mechanism have enabled engineering approaches to develop mutant monoterpene synthases that produce specific monoterpenes. In this article we are describing protocols to introduce targeted mutations, express mutant enzyme catalysts in heterologous hosts, and assess their catalytic properties. Mutant monoterpene synthases have the potential to contribute significantly to synthetic biology efforts aimed at producing larger amounts of commercially attractive monoterpenes.

## 1. INTRODUCTION

Terpenoids are a structurally diverse class of natural products (also called secondary or specialized metabolites) with numerous applications in the flavor, fragrance, nutraceutical, and pharmaceutical industries (Lange & Ahkami, 2013). The first committed step in the biosynthesis of a specific terpenoid class is generally catalyzed by a terpene synthase with high affinity for a prenyl diphosphate substrate of a certain chain length. For example, those accepting a C10 precursor (geranyl diphosphate or neryl diphosphate) are termed monoterpene synthases. Based on currently available experimental evidence plant genomes harbor large families of terpene synthase genes (Chen, Tholl, Bohlmann & Pichersky, 2011). Terpene synthases are also notorious for producing multiple products, which further increases the capacity to generate terpenoid diversity. Synthetic biology efforts by various laboratories have resulted in the development of microbial platform strains for the assembly of different classes of terpenoids. Prominent examples are the production of artemisinic acid, a precursor to the antimalarial sesquiterpene artemisinin in yeast (Paddon et al., 2013) and of diverse pharmaceutically relevant diterpene skeletons in *E. coli* (Morrone et al., 2010; Zerbe et al., 2013). In most cases, the formation of a particular high value terpenoid is desired and enzyme engineering has the potential to provide enzymes with high catalytic rates and specificity. A critical challenge is presented by the scarcity of structure-function data that would enable the knowledge-based engineering of terpene synthases. In this article, we describe how to generate and functionally characterize monoterpene synthase mutants engineered to produce specific monoterpenoid end products.

We chose 4*S*-limonene synthase from spearmint (*Mentha spicata*) as a model monoterpene synthase (Fig. 1) because of the availability of substantial resources. A crystal structure of the pseudomature form of the enzyme (termed R58; with the plastidial targeting sequence removed) and a bound substrate analogue, obtained at 2.7 Å resolution (Hyatt et al., 2007), was used to determine the distance between amino acid residues and the substrate. All amino acid residues that line the active site of the enzyme (and therefore have a high probability of being involved in catalysis) were exchanged with L-alanine and functionally characterized (Srividya, Davis, Croteau & Lange, 2015). Mutants where a single amino acid exchange resulted in a dramatic loss of enzyme fidelity (measured as percentage of (-)-limonene among all monoterpenes in the enzyme reaction; as reference, the R58 enzyme generates 96 % (-)-limonene) were considered for further mutagenesis. In particular, we were studying mutations that resulted in the release of a novel skeleton (acyclic (e.g., (+)-linalool and/or (-)-linalool) or bicyclic (e.g., (+)-sabinene) products) rather than the monocyclic (-)-limonene produced by R58 (Fig. 1).

To understand the implications of generating new products with R58 mutants, it is important to briefly mention the postulated catalytic mechanism of 4*S*-limonene synthase (Rajaonarivony, Gershenzon & Croteau, 1992; Pyun, Coates, Wagschal, McGeady & Croteau, 1993; Coates et al., 1997). The reaction involves the initial migration of the diphosphate moiety from GPP to form linalyl diphosphate (via a carbocation intermediate). Isomerization and reionization generate the linalyl cation, which then undergoes cyclization to the terpinyl cation (Fig. 1). Deprotonation yields (-)-limonene as the primary product of R58. Mutants that release acyclic alcohols (e.g., (+)-linalool and/or (-)-linalool) are not capable of stabilizing the terpinyl cation and water capture instead results in a premature termination of the enzymatic reaction (Fig. 1). In contrast, bicyclic monoterpenes (e.g., (+)-sabinene) are generated when the formation of the terpinyl cation is followed by a second cyclization prior to deprotonation (Fig. 1). The successful development of (-)-limonene mutants that catalyze novel reactions indicates the potential for enzyme engineering toward the production of specific high value monoterpenes.

## 2. EQUIPMENT

Analytical Balance (XS105, Mettler Toledo)
Centrifuge (RC-5B, Sorvall)

Electrophoresis System for DNA Gels (Thermo Scientific)

Electrophoresis System for Protein Gels (Bio-Rad)

Gas Chromatograph (7890, Agilent Technologies)

Gel Imager (MultiImage II, Alpha Innotech)

Incubator Shaker (Series 25, New Brunswick)

Micro-Centrifuge (Z180M, Hermle)

Multi-Tube Rotator (Barnstead/Thermolyne)

Orbital Shaker (Lab-Line)

pH Meter (430, Corning)

SDS-PAGE Apparatus (Mini-PROTEAN, Bio-Rad)

Single Tube Shaker (Vortex Genie 2, IKA)

Microplate Reader (Synergy H1, BioTek)

Thermocycler (Mastercycler Pro, Eppendorf)

Thermomixer (5437, Eppendorf)

Ultrasonic Cell Disruptor (475, Virsonic)

Water Bath (Lab-Line)

# 3. MATERIALS

## 3.1 Molecular Biology

*TE Stock Solution*

Add 1,214 mg tris(hydroxymethyl)aminomethane (Tris) and 372 mg ethylenediaminetetraacetic acid (EDTA) into a graduated cylinder. Fill to the 90 ml mark deionized water, adjust pH to 8.0, and add more deionized water to a final volume of 100 ml. Transfer the solution to a 250 ml glass bottle and sterilize in an autoclave. To prepare a working solution (1 x TE), add 9 ml of sterilized water to 1 ml of the TE stock solution.

*DNA Gel Loading Dye*

In a graduated cylinder, mix 25 mg bromophenol blue, 25 mg xylene cyanol and 3 ml glycerol, and then add deionized water to a final volume of 10 ml. Prepare 1 ml aliquots and store these at 4ºC until further use.

*IPTG Solution*

Dissolve 2.38 g isopropyl-β-D-1-thiogalactopyranoside (IPTG) in 10 ml sterilized water. Pass solution through a 0.45 µm filter and store at -20ºC until further use.

## 3.2 Recombinant Enzyme Purification

*MOPSO Buffer*

Dissolve 1,126 mg 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO) in 50 ml deionized water, adjust pH to 7.0, add 20 ml glycerol, and then add deionized water to a final volume of 100 ml. Add 200 µl 0.5 M dithiothreitol (DTT) just before use.

*Phosphate Buffer*

Dissolve 136.09 g monobasic potassium phosphate ($KH_2PO_4$) and 174.2 g dibasic potassium phosphate ($K_2HPO_4$) in 900 ml deionized water. Adjust pH to 7.0 and add deionized water to a

final volume of 1 l (1 M stock solution). To prepare a working solution (100 mM), add 9 ml deionized water to 1 ml phosphate buffer stock solution.

### 3.3 Protein Gel Electrophoresis

*Protein Gel Loading Dye*

Dissolve 1,214 mg Tris in 9 ml deionized water, adjust pH to 6.8 with hydrochloric acid (HCl), and add deionized water to a final volume of 10 ml (10 x stock solution). Dissolve 10 mg bromophenol blue in 10 ml deionized water (12.5 x stock solution).

Mix 2.5 ml Tris stock solution, 0.8 ml bromophenol blue stock solution, 1.0 g sodium dodecyl sulfate (SDS) and 4 ml glycerol, and then add deionized water for a final volume of 10 ml. Prepare 1 ml aliquots and store these at -20ºC until further use.

*Protein Gel Running Buffer*

In a graduated cylinder mix 30 g of Tris, 144 g glycine and 10 g of SDS and add deionized water to a final volume of 1 l (10 x stock solution). To prepare a working solution, add 900 ml deionized water to 100 ml Tris-glycine stock solution.

*Protein Staining Dye*

Dissolve 3.2 ml phosphoric acid ($H_3PO_4$) and 16 g ammonium sulfate ($(NH_4)_2SO_4$) in 16 ml deionized water. Add 160 mg Coomassie Brilliant Blue G-250 and 40 ml methanol and mix well until the dye dissolves. Add deionized water to a final volume of 200 ml and allow to stand at 23°C for 12 h before use.

*Protein Gel Destaining Solution*

Mix 200 ml methanol and 600 ml deionized water, add 70 ml acetic acid, and then add deionized water to a final volume of 1 l.

*Protein Gel (optional; no protocol provided here; for details see Brunelle & Green, 2014)*

Acrylamide

1.5 M Tris buffer, pH 8.8

0.5 M Tris buffer, pH 6.8

10 % (w/v) Sodium dodecyl sulfate (SDS)

10 % (w/v) Ammonium persulfate (APS)

N,N,N',N'-tetramethylethane-1,2-diamine (TEMED)

### 3.4 Enzyme Assays

*Geranyl Diphosphate Stock Solution*

Geranyl diphosphate (GPP) was synthesized as described elsewhere (Davisson et al., 1986; Woodside, Huang & Poulter, 1988). A GPP Stock Solution (10 mM) was prepared by dissolving 3.14 mg GPP in 1 ml deionized water. Aliquots (100 µl each) were stored at -80ºC until further use.

*Enzyme Assay Buffer*

Dissolve 1.126 g MOPSO in 50 ml deionized water, adjust pH to 7.0, add 1.016 g $MgCl_2$ x 6 $H_2O$ and 20 ml glycerol, and add deionized water to a final volume of 100 ml. Filter-sterilize the solution before use.

## 4. STEP 1 – GENERATION OF EXPRESSION CONSTRUCTS

The introduction of point mutations was achieved using one of two approaches. When possible, the fast and cost-effective Quick Change PCR method (based on Fisher & Pei (1997) and modified from the Strategene protocol) was employed. However, for unknown reasons, this method sometimes failed and in those cases a traditional Overlap Extension Strategy was used successfully. For the convenience of the user, both protocols are listed here.

In this paragraph we will comment briefly on primer design, which follows the same principles for both mutation approaches. The location of the desired exchange of a base pair triplet should always be in the center of the primer sequence. As a general rule, leaving 15 unchanged

nucleotides on both side of a mutation location always gave the desired results in our hands. The two primers cover the same stretch of cDNA (red letters indicate site of desired mutation):

(+)-Strand R58 ---GTGGAATGCTACTTT**TGG**AATACTGGGATCATCG--->

Sense W324D mutation primer GTGGAATGCTACTTT**GAT**AATACTGGGATCATCG--->

(-)-Strand R58 <---CACCTTACGATGAAA**ACC**TTATGACCCTAGTAGC---

(-)-Strand R58 (reversed) ---CGATGATCCCAGTATT**CCA**AAAGTAGCATTCCAC--->

Antisense W324D mutation primer CGATGATCCCAGTATT**ATC**AAAGTAGCATTCCAC--->

If subcloning of the PCR amplicon by restriction digestion is desired, the user should ensure that suitable restriction sites are present in the amplified PCR product. Useful information about primer design can be found at the following URL: http://www.genomics.agilent.com/primerDesignProgram.jsp.

*Note:* The pSBET vector (Schenk, Baumann, Mattes & Steinbiβ, 1995) was previously demonstrated to be an excellent choice for producing recombinant 4*S*-limonene synthase (R58) in *E. coli* (Williams, McGarvey, Katahira & Croteau, 1998). Mutant versions of the gene were therefore obtained using a plasmid that contained R58 in pSBET.

## 4.1 Quick Change PCR

### *4.1.1. Amplification of Mutated cDNA Sequence*

| | |
|---|---|
| DNA template (plasmid containing target gene) | 5-20 ng |
| 5 x HF Phusion polymerase buffer (New England Biolabs) | 10.0 µl |
| 5 µM Gene-specific primer (forward) | 2.5 µl |
| 5 µM Gene-specific primer (reverse) | 2.5 µl |
| Dimethyl sulfoxide (DMSO) | 1.0 µl |
| 10 mM Deoxynucleotide mix (dNTPs) | 1.0 µl |
| Phusion DNA polymerase (2.5 units) (New England Biolabs) | 1.0 µl |

Add deionized, sterile-filtered water to a final volume of 50 µl.

PCR conditions: initial denaturing at 98°C for 1.5 min; 20 cycles of 98°C for 15 s, 55°C for 30 s, 72°C for 5min; final extension at 72°C for 10 min; chill to 4°C for 15 min.

The original plasmid (containing non-mutated target sequence), which was maintained in a bacterial host strain and is therefore partially methylated, is then digested by adding Dpn I (10 units) to the above PCR mixture and incubating the mixture at 37°C for 1 h. Only amplicons with a mutated target sequence are thus retained and further processed. An aliquot (10 µl) of the reaction is evaluated by agarose gel electrophoresis (Fig. 2). Note that the mutation PCR generates an amplicon of the size of the vector plus insert (not just the target cDNA).

[Insert Fig. 2 here]

*4.1.2 Transformation of Vector Containing Mutated cDNA into Host Cells*

An aliquot (3 µl) of the DpnI-digested PCR solution was added to 50 µl ice-cold, highly competent XL-1 Blue cells ($10^9$/µg transformation efficiency or greater) and the mixture kept on ice for 30 min. To allow entry of the vector, the reaction tube was placed at 42°C for 45 s (heat shock) and returned to ice for 2 min. LB broth (350 µl) was added and the mixture incubated at 37°C for 1 h. An aliquot of the transformation reaction (50-200 µl depending on experience values for transformation efficiency) was spread evenly onto LB-agar plates containing 50 µg/ml kanamycin and maintained at 37ºC for 16 h. Single colonies were picked and grown overnight in culture tubes containing 5 ml liquid LB broth and 50 µg/ml kanamycin. Plasmids were isolated using a MiniPrep kit (these can be purchased from various suppliers) and the mutation verified by sequencing using commercial services.

*Notes:* Although the method above lists Phusion as a DNA polymerase with proofreading capacity that gives blunt ends, Pfu Turbo (Agilent Technologies) was also used successfully, but PCR conditions have to be adjusted. The Dpn I enzyme will perform satisfactorily in any $Mg^{2+}$-containing polymerase buffer.

## 4.2 Overlap Extension Mutagenesis

This method involves three rounds of PCR: in the first round, two partial cDNAs containing the desired mutation are generated; in the second round, the partial cDNA are self-annealed; and in the third round, a full-length, mutated cDNA is amplified that also contains restriction sites for convenient subcloning.

### *4.2.1 Introduction of Point Mutations*

This first round of PCRs requires two consecutive PCRs, where one reaction (A) will amplify the nucleotides from the vector (sense primer) to the point mutation (antisense primer) and the second reaction (B) will amplify from the point mutation (sense primer) into the vector (antisense primer):

| | |
|---|---|
| Template | 5-20 ng |
| 10 x Buffer | 2.5 µl |
| dNTPs (10 mM) | 0.2 µl |
| Primers (10 µM each) | 0.6 µl |
| Reaction A: vector sense primer and gene-specific antisense primer; | |
| Reaction B: gene-specific sense primer and vector antisense primer | |
| Pfu Turbo Polymerase | 0.4 µl |

Add deionized, sterile-filtered water to a final volume of 25 µl.

Thermocycling was carried out with an initial denaturation at 95ºC for 2 min, 40 cycles at 95ºC for 30 s, 53ºC for 40 s, 72ºC for 2 min, and a final extension at 72ºC for 10 min. The amplicons were gel-purified in a 1.3 % (w/v) low melting point agarose gel, bands were excised and DNA obtained using a gel extraction and purification kit (Qiagen) with 30 µl elution buffer.

### *4.2.2 Self-Annealing of Amplicons to Generate a Full-Length Mutated cDNA*

The purified amplicons from 4.2.1 were mixed and self-annealed to create the full length cDNA with vector anchors:

| | |
|---|---|
| Template | 5.0 µl each (reactions A and B) |
| 10 x Buffer | 2.5 µl |
| dNTPs (10 mM) | 0.2 µl |
| Pfu Turbo Polymerase | 0.5 µl |

Add deionized, sterile-filtered water to a final volume of 25 µl.

Themocycling was performed with an initial denaturation at 95ºC for 2 min, 25 cycles at 95ºC for 30 s, 60ºC for 5min, 72ºC for 5 min, and a final extension at 72ºC for 20 min. The reaction mixture was diluted 1 : 20 with deionized, sterile-filtered water and then used as template in the last round of PCR:

| | |
|---|---|
| Template (1 : 20 dilution) | 1.0 µl |
| 10 x Buffer | 2.5 µl |
| dNTPs (10 mM) | 0.2 µl |
| Vector Primer Sense (5 µM) | 1.5 µl |
| Vector Primer Antisense (5 µM) | 1.5 µl |
| Pfu Turbo Polymerase | 0.4 µl |

Add deionized, sterile-filtered water to a final volume of 25 µl.

Thermocycling conditions included and initial denaturation at 95ºC for 2min, 40 cycles at 95ºC for 30 s, 53ºC for 50 s, and 72ºC for 2 min, and a final extension at 72ºC for 10 min.

The resulting amplicon was gel-purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey and Nagel), double-digested with NdeI (20 U; New England BioLabs) and BamHI (20 U; New England BioLabs) at 37ºC for 90 min and then gel-purified again as above. The purified insert was ligated into the pSBET vector (50-100 ng; pre-digested with NdeI and BamHI as above) using T4 DNA ligase (20 U; New England BioLabs) at 20ºC for 12 h. The ligated product was transformed into *E-coli* XL-1 Blue competent cells, the cells were plated on LB-agar plates containing 50 µg/ml kanamycin, and the plates incubated at 37ºC for 16 h. Single colonies were picked and cells grown overnight in 5ml LB liquid broth containing 50 µg/ml kanamycin. Plasmids were isolated using a

commercial Miniprep kit according to the manufacturer's instructions and the mutation confirmed by sequencing using a commercial service provider.

*Note:* Plasmid yields in our hands were greatest when using XL-1 Blue competent cells, while lower yields were obtained with DH5**α,** Top10 or Omnimax competent cells.

# 5. STEP 2 – PRODUCTION OF PURIFIED, RECOMBINANT TARGET ENZYME

## 5.1 *E. coli* Cultivation and Induction of Target Enzyme Expression

Plasmids containing cDNAs of R58 and mutated versions were transformed into chemically competent *E. coli* BL21 (DE3) cells, which were then plated on LB-agar plates (containing 50 µg/ml kanamycin for selection) and incubated for 16 h at 37ºC. A single colony was picked from each plate, transferred into 150 ml liquid LB medium, and bacteria incubated at 37ºC with shaking at 250 rpm in an Incubator Shaker (Series 25, New Brunswick). Upon reaching an optical density of 0.8 at 600 nm, the expression of the transgene was induced by the addition of 75µl of 1M IPTG Solution, and the bacterial suspension was transferred to a different Incubator Shaker set to 16ºC and 250 rpm for another 24 h.

*Note:* The induction of transgene expression under rapid growth conditions (37ºC) results in the precipitation of recombinant enzyme in insoluble inclusion bodies. Incubation at reduced temperature (16ºC) dramatically improves the solubility and yield of recombinant enzyme.

## 5.2 Isolation of Target Enzyme and Assessment of Purity

### *5.2.1 Cell Disruption*

Bacterial cells were harvested by centrifugation (RC-5B, Sorvall) at 2,500 x *g* for 10 min. The supernatant was discarded and the remainder resuspended in 500 µl MOPSO Buffer. The mixture was transferred to a 2 ml microcentrifuge tube and samples were kept on ice whenever possible for all subsequent steps. Cells were broken using an Ultrasonic Cell Disruptor (Model 475,

Virsonic; 3 x 15 s bursts on ice with a 3.2 mm microprobe operated at 20 % output power). The resulting homogenate was precipitated using a Micro-Centrifuge (Z180M, Hermle) at 15,000 x *g* for 30 min. The supernatant was used for the purification of recombinant enzyme as described under 5.2.2. An aliquot (15 µl) was mixed with 5 µl Protein Gel Loading Dye and the sample transferred to the slot of a protein gel. Sodium Dodecyl Sulfate-Poly-Acrylaminde Gel Electrophoresis (SDS-PAGE) was performed as described under 5.2.3.

*Note:* Various methods for cell disruption were tested, including sonication, grinding with mortar and pestle, and the use of a glass homogenizer. The most consistent results and highest recombinant enzyme yields were obtained with the sonication protocol outlined above.

### *5.2.2 Batch Purification*

In a 2 ml microcentrifuge tube, 100 mg ceramic hydroxyapatite type II (CHT, Bio-Rad) was suspended in 500 µl MOPSO Buffer, the supernatant obtained under 5.2.1 added, and the suspension mixed gently on a Multi-Tube Rotator (Labquake, Barnstead/Thermolyne) for 1 h at 4°C. The tube was put on ice and the CHT support with bound recombinant enzyme allowed to settle for 10 min (do not centrifuge as this results in desorption of recombinant protein). The supernatant was removed carefully and 500 µl MOPSO Buffer were added. The contents were mixed gently on a Multi-Tube Rotator (Labquake, Barnstead/Thermolyne) for 10 min at 4°C (this wash step removes weakly adsorbed proteins). The tube was put on ice and the CHT support with bound recombinant enzyme allowed to settle for 10 min. The supernatant was removed carefully and 300µl of Phosphate Buffer were added. Contents were mixed and CHT support subsequently allowed to settle as described above. Aliquots of the supernatant were used to quantify total protein content using the Bradford Assay (see 5.2.3 for details) and assess target enzyme purity by SDS-PAGE (see 5.2.4 for details).

*Note:* The majority of studies with recombinant enzymes are performed using poly-histidine or other tags for expedient purification. However, the expression of R58 using commercial vectors with a $(histidine)_6$-tag led to the co-purification of *E. coli* chaperones and a compromised target protein activity (Williams, McGarvey, Katahira & Croteau, 1998). Therefore, we obtained

recombinant R58 and mutant enzymes by expressing the corresponding gene from the pSBET vector (Schenk, Baumann, Mattes & Steinbiβ, 1995) without tag and employing a purification protocol based on a conventional mixed-mode CHT support. The fastest and most efficient purification was achieved in batch mode rather than by gravity column chromatography.

### *5.2.3 Total Protein Quantitation*

The Bradford Assay was employed to quantify total protein content by comparison to a calibration curve. A 1 mg/ml stock solution of bovine serum albumin (BSA) in deionized water was prepared and different volumes added to the Bradford Assay Reagent (Bio-Rad). A calibration curve was obtained by plotting the absorbance measured at 595 nm (Microplate Reader Synergy H1, BioTek) against the concentration of BSA in the reagent solution (Table 1). The total protein content was calculated based on a linear equation ($y = m \bullet x + b$; where $y$ is the concentration, $m$ is the slope, $x$ is the absorbance value, and $b$ is the y-intercept).

**Table 1.** Pipetting scheme to determine a calibration curve for quantifying total protein content using the Bradford Assay.

| Standard (three replicates for each dilution) | BSA Stock Solution [µl] | Bradford Reagent [µl] | Deionized Water [µl] | Protein Concentration [mg/ml] |
|---|---|---|---|---|
| Dilution 1 | 2 | 40 | 158 | 0.01 |
| Dilution 2 | 4 | 40 | 156 | 0.02 |
| Dilution 3 | 8 | 40 | 152 | 0.04 |
| Dilution 4 | 12 | 40 | 148 | 0.06 |
| Dilution 5 | 16 | 40 | 144 | 0.08 |
| Dilution 6 | 20 | 40 | 140 | 0.1 |

### *5.2.4 Assessment of Recombinant Enzyme Purity*

The purity of the recombinant enzyme was monitored using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gels can be purchased in precast form commercial manufacturers (which is a convenient but costly option) or can be prepared onsite (which is more cost-effective but requires extra effort and time). All materials needed to cast your own gels are listed under 3.3., but for experimental details the reader is referred to an excellent review article (Brunelle & Green, 2014). For assessing the purification of target enzymes we generally use SDS-PAGE gels (102mm x 74mm x 0.75mm) with 5 % (w/v) stacking and 12 % (w/v) resolving components.

Samples were prepared as follows: 5 µg of total protein (based on Bradford Assay outlined under 5.2.3) were mixed with 5 µl of the 4 x Protein Gel Loading Dye and the solution was heated to 95ºC for 5 min. Samples and the protein ladder control (Precision Plus Protein All Blue Prestained Standards, Bio-Rad) were loaded side-by-side into the wells of the stacking gel. The gel was run at 110 V in Protein Gel Running Buffer until the dye front reached the other end of the gel. The gel was then carefully transferred to a dish containing 50 ml Protein Staining Dye solution, and the dish rocked on a for 2 h. The stained gel was transferred to another dish containing 100 ml gel destaining solution and rocked until the blue background color disappeared and individual protein bands became visible. The progress of purification was determined by comparing protein band intensities using a Gel Imager (MultiImage II, Alpha Innotech). Preparations with a purity of > 90 % target protein (band at ~50 kDa) (Fig. 3) were further evaluated in enzyme assays (details under 6.).

[Insert Fig. 3 here]

# 6. STEP 3 – FUNCTIONAL EVALUATION OF RECOMBINANT MONOTERPENE SYNTHASES

## 6.1 Enzymatic Reaction

In a 2 ml screw-cap glass vial, 200 µg of the purified enzyme (volume depends on enzyme concentration as determined under 5.2.3) and 20 µl Geranyl Diphosphate (GPP) Stock Solution (final concentration of substrate in the assay is 0.5 mM) were mixed and Enzyme Assay Buffer

was added to a final reaction volume of 400µl. Each sample was overlaid with 100 µl n-hexanes (OmniSolv, EMD Millipore), the reaction allowed to proceed to completion at 30ºC on a tube rotator for 16 h. The enzymatic reaction was stopped by vigorous mixing of the aqueous and organic phases on a Single Tube Shaker (Vortex Genie 2, IKA) operated at the higher setting. To facilitate phase separation, reaction vials were kept at -20ºC for 2 h. The supernatant containing the organic phase was transferred to 2 ml glass vial with conical insert and stored at -20ºC until further use (maximum storage duration was less than one week).

### 6.2 Product Quantitation

The products formed during the enzymatic reaction described under 6.1 were quantified by Gas Chromatography (GC) (model 7890, Agilent Technologies) with a Flame Ionization Detection. Analytes were separated on a Cyclodex-B chiral column (J&W Scientific; 30 m x 0.25 mm, 0.25 µm film thickness). The following GC settings were used: injector temperature 250ºC; injector split 1:20; sample volume 1µl; flow rate 2 ml/min with He as carrier gas; oven heating program with an initial ramp from 40ºC to 120ºC at 2ºC/min, followed by a second ramp to 200ºC at 50ºC/ min, and a final hold at 200ºC for 2 min; detector temperature 250ºC; detector gas flows of 30 ml/min for $H_2$, 400 ml/min for air, and 25 ml/min for He as make-up gas. Quantitation was achieved by comparing detector responses, using a linear equation, with those of a calibration curve, which was obtained by injecting varying quantities of authentic standards as described under 5.2.3 for protein quantitation. Representative chromatograms for enzyme assays are shown in Fig. 4.

*Note:* A chiral GC column was employed to enable the analysis of enantiomeric monoterpenes. The identities of monoterpenes formed in enzymatic reactions were verified by Gas Chromatography-Mass Spectrometry as described elsewhere (Adams, 2007).

[Insert Fig. 4 here]

### 6.3 Analysis of Kinetic Data

Kinetic assays were carried out to determine the maximum reaction rate ($V_{max}$), the turnover number ($K_{cat}$), the Michaelis constant $K_m$ (inverse measure of the enzyme's affinity for the substrate), and the catalytic efficiency (calculated as $k_{cat}/K_M$) of recombinant monoterpene synthases. In a continuous assay the enzyme concentration is kept constant and the substrate concentration is varied. Purified, recombinant monoterpene synthase (25 µM) was reacted with 2.5 to 50 µM substrate (GPP) by gentle mixing rotator at 30ºC for 20 min (these conditions ensured that < 20 % of the available substrate was consumed). A Michaelis-Menten saturation curve was obtained by plotting substrate concentration against reaction rate (formation of product over time) (Fig. 5). The Michaelis constant $K_m$ is defined as the substrate concentration at which the reaction rate is half of the maximal rate ($V_{max}$), but these constants were determined more accurately by nonlinear regression analysis using a hill type equation $y = V_{max} * \frac{x^n}{K_m^n + x^n}$ (Origin 8; OriginLab). The turnover number ($K_{cat}$) was then calculated by dividing $V_{max}$ by the total enzyme concentration.

[Insert Fig. 5 here]

**Conclusions**

During recent years, various synthetic biology platforms have been developed for the production of commercially relevant terpenoids (from C5 to C40) by introducing, among others, plant-derived terpene synthase genes into microbial hosts. Starting with (4*S*)-limonene synthase from spearmint as a template, we generated a large number of mutant enzymes that produce specific monoterpenes, including acyclic, monocyclic and bicyclic terpenoid hydrocarbons and alcohols. The methods described in this article encompass mutant generation by site-directed mutagenesis, the introduction of a mutant gene into a bacterial host cell, and the production, purification and functional characterization of the recombinant mutant enzyme. Based on the strategies outlined here, readers can develop designer mutant enzymes optimized for the production of specific target monoterpenes.

**Acknowledgements**

This work was supported by the Division of Chemical Sciences, Geosciences, and Biosciences, Office of Basic Energy Sciences, U.S. Department of Energy (grant no. DE–SC0001553 to BML).

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## Figure Captions

**Fig 1.** Formation of novel monoterpenes by mutants of (4*S*)-limonene synthase. The primary reaction pathway toward (-)-limonene, as catalyzed by the pseudomature form of the wild-type enzyme (designated as R58), is highlighted in dark gray, while primary products formed by mutant enzymes W324H and W324E are highlighted with brighter shades of gray.

**Fig 2.** Agarose gel showing amplicons of quick change PCRs using primers to amplify the wild-type enzyme (R58) (**lane 1**) and the mutations leading to the exchanges of W324H (**lane 2**) and W324E (**lane 3**). A DNA size standard ladder was loaded in lane 4. Note that PCR bands are often weak when using this method but, in our hands, that usually does not have negative effects on downstream manipulations.

**Fig 3.** Purification of recombinant R58 as indicated by SDS-PAGE. **Lane 1**, crude protein extract from *E. coli* cells expressing R58 (note the large band for the target protein); **lane 2**, purified protein fraction following hydroxyapatite fractionation (note that the target protein is > 95 % pure); **lane 3**, protein size standard ladder; and **lane 4**, crude protein extract from uninduced control *E. coli* cells (note that the target protein constitutes only one of many detectable bands).

**Fig 4.** GC-FID chromatograms of enzyme assay products. **A**, Wild-type (R58); **B**, W324E mutant; and **C**, W324H mutant.

**Fig 5.** Kinetic plot for the R58 enzyme assay. The dotted line indicates the curve fit obtained by nonlinear regression analysis.

Fig. 1

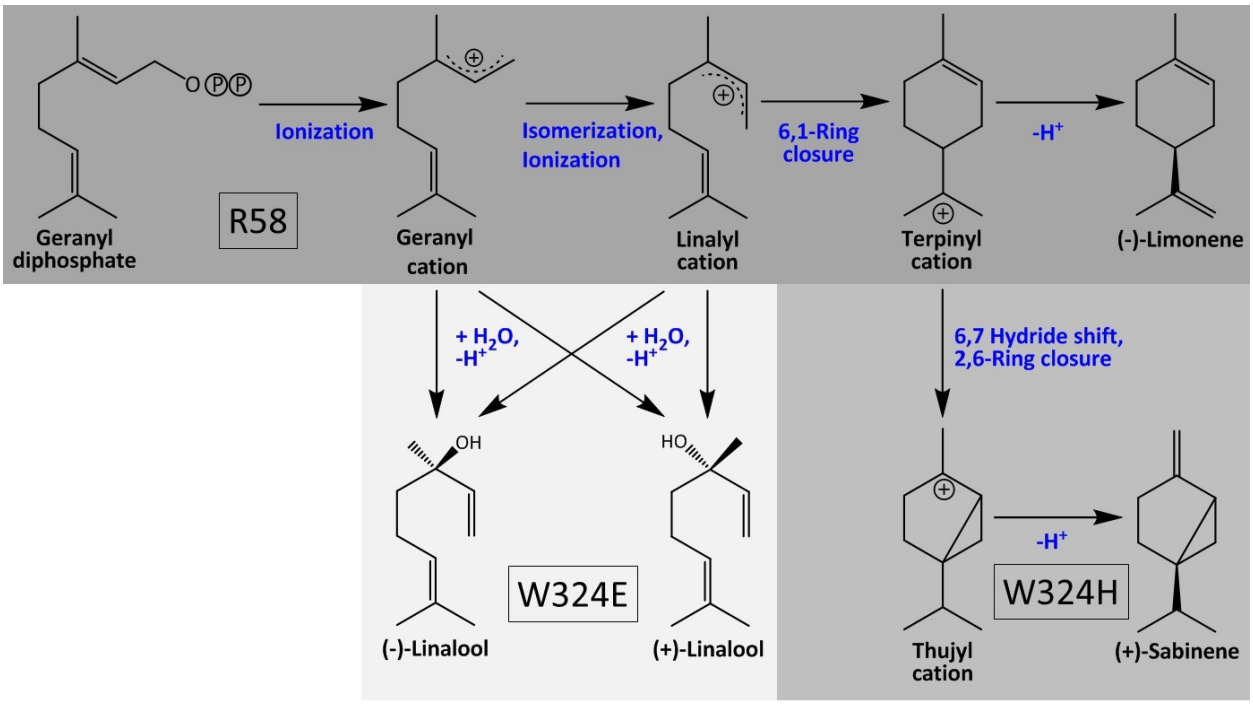

Geranyl diphosphate
R58
Ionization
Geranyl cation
Isomerization, Ionization
Linalyl cation
6,1-Ring closure
Terpinyl cation
-H+
(-)-Limonene
+ H2O, -H+
+ H2O, -H+
OH
HO
W324E
(-)-Linalool
(+)-Linalool
6,7 Hydride shift, 2,6-Ring closure
-H+
W324H
Thujyl cation
(+)-Sabinene

Fig. 2

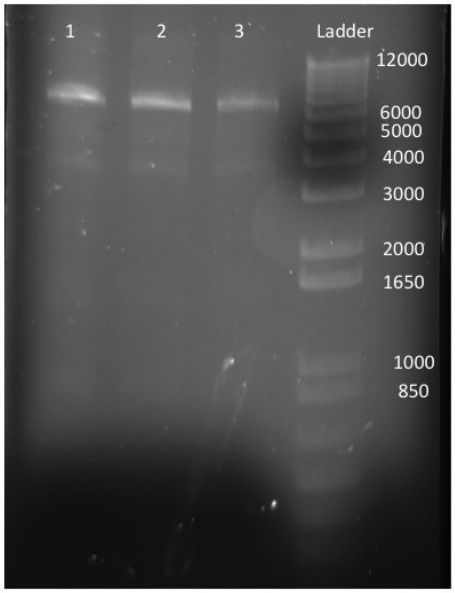

1
2
3
Ladder
12000
6000
5000
4000
3000
2000
1650
1000
850

Fig. 3

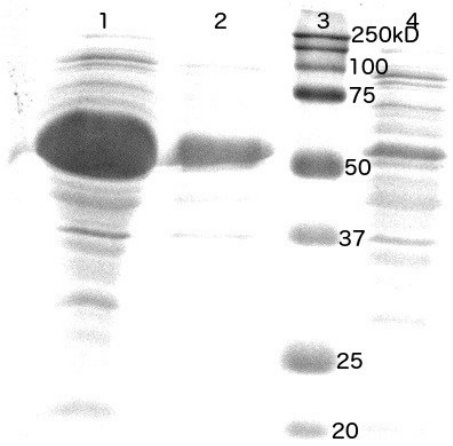

1
2
3
4
250kD
100
75
50
37
25
20

Fig. 4

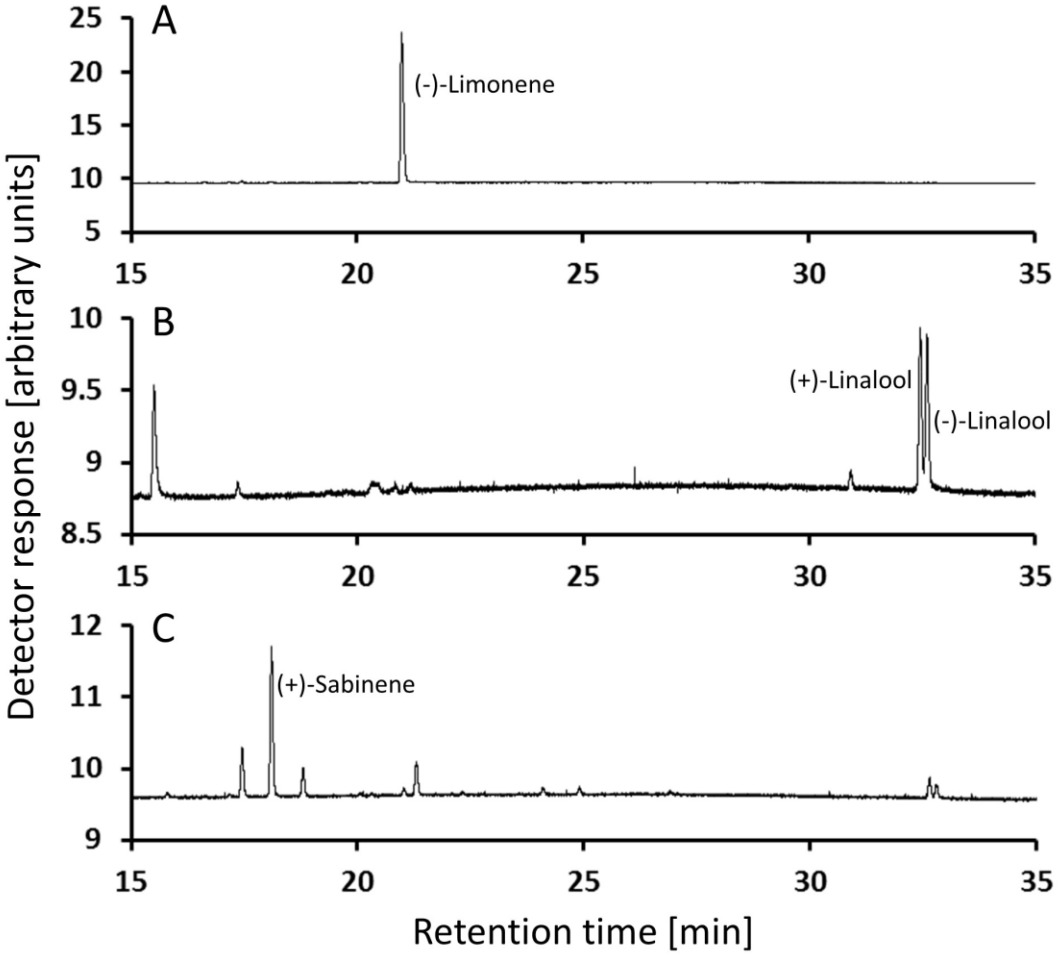
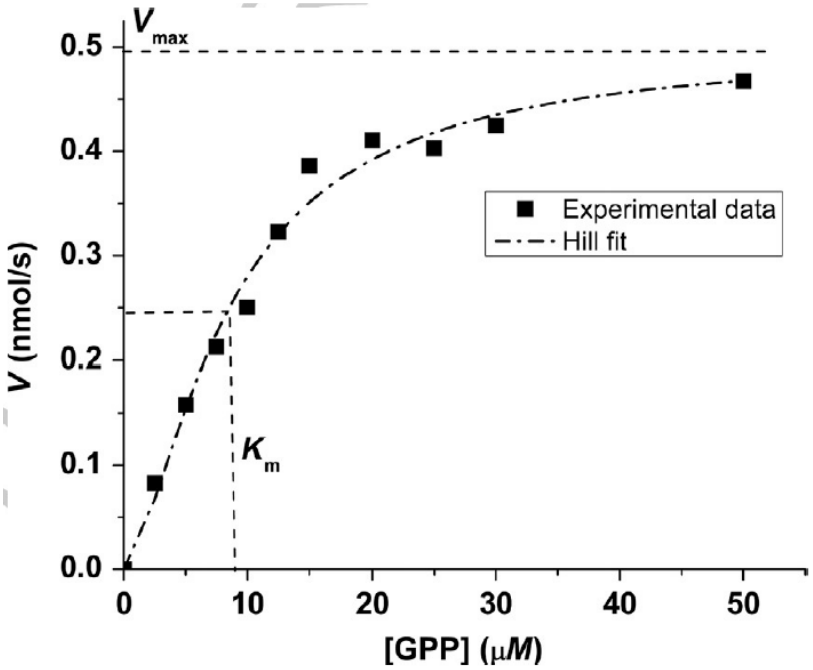

A
(-)-Limonene
B
(+)-Linalool
(-)-Linalool
C
(+)-Sabinene
Detector response [arbitrary units]
Retention time [min]

Fig. 5


V max
0.5
0.4
0.3
0.2
0.1
0.0
V (nmol/s)
Experimental data
Hill fit
K m
0
10
20
30
40
50
[GPP] (μM)