CHAPTER ELEVEN

Natural Product Biosynthesis in *Escherichia coli*: *Mentha* Monoterpenoids

H.S. Toogood*, S. Tait*, A. Jervis†, A. Ní Cheallaigh‡, L. Humphreys§, E. Takano*, J.M. Gardiner‡, N.S. Scrutton*,1

*Manchester Institute of Biotechnology, Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom
†Manchester Institute of Biotechnology, SYNBIOCHEM, University of Manchester, Manchester, United Kingdom
‡School of Chemistry, Manchester Institute of Biotechnology, University of Manchester, Manchester, United Kingdom
§GlaxoSmithKline, Medicines Research Centre, Stevenage, United Kingdom
1Corresponding author: e-mail address: Nigel.Scrutton@manchester.ac.uk

Contents

Abstract

The era of synthetic biology heralds in a new, more "green" approach to fine chemical and pharmaceutical drug production. It takes the knowledge of natural metabolic pathways and builds new routes to chemicals, enables nonnatural chemical production, and/or allows the rapid production of chemicals in alternative, highly performing organisms. This route is particularly useful in the production of monoterpenoids in microorganisms, which are naturally sourced from plant essential oils. Successful pathways are constructed by taking into consideration factors such as gene selection, regulatory

Methods in Enzymology, Volume 575
ISSN 0076-6879
http://dx.doi.org/10.1016/bs.mie.2016.02.020

elements, host selection and optimization, and metabolic considerations of the host organism. Seamless pathway construction techniques enable a "plug-and-play" switching of genes and regulatory parts to optimize the metabolic functioning in vivo. Ultimately, synthetic biology approaches to microbial monoterpenoid production may revolutionize "natural" compound formation.

1. INTRODUCTION

Natural products are organic compounds produced enzymatically by living organisms, often through pathways of secondary metabolism (Yonekura-Sakakibara & Saito, 2009). While secondary metabolites are not essential for survival, they often give the organism advantages for survival. For example, cytotoxic compounds produced by some plants can act as a form of chemical warfare against predators and competing organisms (Hunter, 2008). As natural products often have biological or pharmacological activity, they have traditionally been included, or are the active components of both traditional and modern medicines (Atanasov et al., 2015). Additional structural diversity is achievable through chemical, and more recently enzymatic biosynthesis, and synthetic analogues can be prepared with increased potency and purity. It is therefore not surprising that natural products have often been seen as the starting points in drug discovery screens (Hunter, 2008; Li & Vederas, 2009).

Many natural products have complex structures, often containing regiospecific functionalization and chiral centers. These features are largely responsible for their biological activity and potency. Traditionally, these compounds were obtained by extractions and extensive purifications from native plant species. This route is often problematic for several reasons: (i) plant natural products are often in low concentrations and require long growth periods; (ii) yields of compound(s) are subject to regional, seasonal, and climate variations; and (iii) purification procedures are often complex, costly, and challenging if pure enantiomeric compounds are needed. Consequently, there have been many studies focussed on standardizing and increasing the yields of primary and secondary natural products in both native plants and cell cultures (Hussain et al., 2012). Complete chemical synthesis of natural products provides an alternative route; however, the lengthy steps involved can result in an overall low yield of products, and the use of costly and/or toxic chemicals. For example, the total chemical synthesis of Taxol, a potent anticancer agent originally isolated from *Taxus brevifolia*

(Pacific yew tree), yielded only 0.02% of the final product (Nicolaou, Yang, Liu, Ueno, & Nantermet, 1994).

Limonene and derivatives are the most abundant monocyclic monoterpenoids in nature (Duetz, Bouwmeester, van Beilen, & Witholt, 2003). (*S*)-Limonene is naturally found in herbs such as *Mentha* species (peppermint and spearmint), while the more common (*R*)-enantiomer is a major oil constituent of orange and lemon peels. Limonene derivatives are often used in fragrances, perfumes, and flavors, such as menthol isomers and carvone giving the distinctive aroma of peppermint and spearmint, respectively (Fig. 1). Menthol isomers are commonly used as flavors in oral hygiene products, food, and beverages. Carveol is an additive of cosmetics, while pulegone is frequently found in perfumes and aromatherapy products. Within the pharmaceutical industry, carvone and carveol are known to have anticancer properties, while menthol has antibacterial properties against *Staphylococcus aureus* and *Escherichia coli* (Ajikumar et al., 2008).

There is a high demand for limonene and derivatives (eg, menthol oil ca. 31,000 t/$373–401 US million pa); however, the flavor and fragrance industries supply most of these compounds from natural sources (extract from *Mentha* spp.) to be compatible with use in food products. These sources are at the mercy of environmental conditions, volatile prices, and arable land competition for more profitable crops (eg, for biofuels production). Additionally, menthol oil requires expensive and low yielding steam distillation and filtration processes to produce a usable commodity (Lange et al., 2011; Lawrence, 2007). There are two major synthetic routes to menthol; the Haarmann–Reimer route from *m*-cresol and the Takasago synthesis starting with β-pinene (Sell, 2003). These processes generate annual yields of crude menthol to the value of around US$ 300 million (Croteau et al., 2005). However, given that the latter product is not from natural sources, alternative clean (bio)synthetic routes to these compounds are a commercially attractive prospect.

An alternative "natural" route to pure organic compounds is to utilize microorganisms as biological factories, using either existing or introduced de novo pathways (Chemler & Koffas, 2008; Mitchell, 2011). This synthetic biology approach generates fine chemicals via an enzymatic route utilizing rapidly growing, cost effective, and even food-compatible microorganisms grown on nonpetroleum-based renewable feedstock (Ajikumar et al., 2010; Friedman & Ellington, 2015; Winter & Tang, 2012). For example, *E. coli*, *Saccharomyces cerevisiae* (Baker's yeast), and cyanobacteria have been shown to produce plant terpenoids, such as limonene, by the inclusion of plant terpene

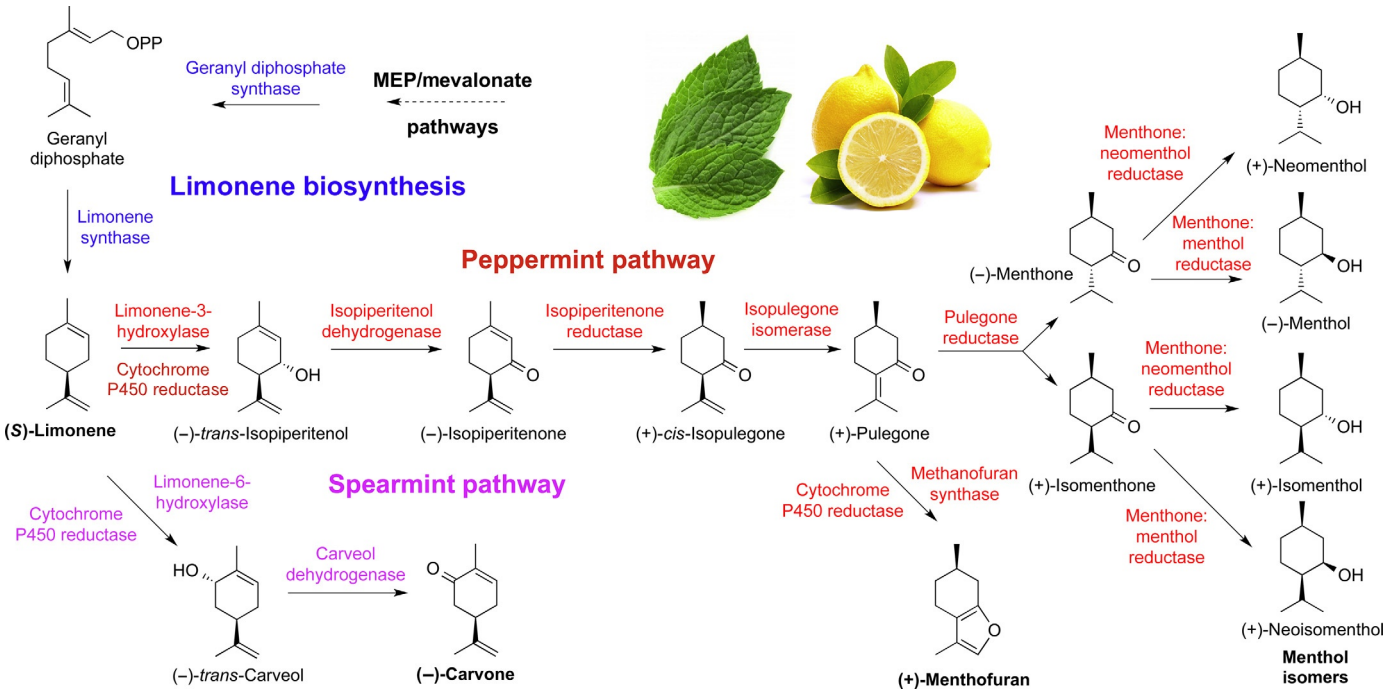

Fig. 1 Biosynthetic pathways of essential oil monoterpenoids by *Mentha piperita* (peppermint) and *M. spicata* (spearmint). The enzymes involved in each pathway are color coded for limonene (*blue*), peppermint (*red*), and spearmint (*magenta*) biosynthetic pathways (Croteau, Davis, Ringer, & Wildung, 2005; Toogood et al., 2015).

synthases, and the upregulation of enzymes involved in isoprene precursor production (Alonso-Gutierrez et al., 2013; Carter, Peters, & Croteau, 2003; Farmer & Liao, 2001; Jackson, Hart-Wells, & Matsuda, 2003; Kiyota, Okuda, Ito, Hirai, & Ikeuchi, 2014; Martin, Pitera, Withers, Newman, & Keasling, 2003). The semisynthetic industrial-scale production (ca. 35 t per annum) of the sesquiterpene lactone artemisinin by Sanofi, a major active ingredient in modern malarial treatments, has shown commercial success, in comparison to traditional extractions and purifications from natural sources (sweet wormwood) (Chang & Keasling, 2006). In the case of semisynthetic Taxol production, a multivariate-modular approach was successful, where the pathways were partitioned into (i) native upstream methylerythritol phosphate (MEP) pathway to form isopentenyl pyrophosphate and (ii) a heterologous downstream taxadiene (taxol precursor)-forming pathway (Ajikumar et al., 2010).

Recent work has shown progress in a synthetic biology approach to the formation of menthol isomers by *E. coli* using biosynthetic pathway precursors (Fig. 1) as the starting material (Toogood et al., 2015). In this approach, a de novo enzymatic route based on the *Mentha piperita* biosynthetic pathway was constructed in *E. coli*, and whole-cell extracts were successful in the synthesis of menthol from pulegone. Given the potential of synthetic biology techniques to revolutionize the production of "naturally sourced" organic compounds (Breitling & Takano, 2015), this paper will discuss general techniques applicable to this rapidly advancing technology, using the menthol production study protocols of "operon" construction and natural product synthesis/analysis (Toogood et al., 2015; Yadav, De Mey, Giaw Lim, Kumaran Ajikumar, & Stephanopoulos, 2012). The methodologies are suitable for generating constructs complete with reporter tags and multiple repeating sequences, the latter of which is often challenging to obtain.

2. OPERON CONSTRUCTION

2.1 Vector and Gene Selections

One advantage of constructing biosynthetic pathways in *E. coli*, as opposed to yeast, is the ability to create de novo synthetic polycistronic gene modules (operons) (Blount, Weenink, & Ellis, 2012). This reduces the DNA burden, as a single promoter/terminator pair can potentially control multiple genes. The eukaryotic nature of yeast makes it more challenging to predict gene regulation; however, the inclusion of synthetic regulatory networks in *S. cerevisiae* shows the potential of generating biofactories in yeast (Blount

et al., 2012). Given that the *E. coli* molecular biology "toolkit" is considerably more extensive than in yeast and other organisms, it is not surprising that new synthetic biology pathways are often constructed and tested in bacterial systems. Successful constructs can then be imported into more publically acceptable microorganisms (eg, Baker's yeast) and grown on renewable waste feedstock. To date, terpenoid metabolic engineering in microorganisms has been focused mostly on the production of carotenoids, such as lycopene and β-carotene, precursors for approved drugs, such as artemisinin and paclitaxel, and other terpenoids (Ajikumar et al., 2008).

There are a variety of commercially available vectors for the expression of one or multiple genes in *E. coli*. Perhaps one of the most commonly used collections within research facilities is the pET system (Merck Millipore, Billerica, Massachusetts; http://www.merckmillipore.com), where protein expression is controlled by the presence of an IPTG-inducible bacteriophage T7 RNA polymerase within the host cell. The arabinose-inducible pBAD system is also popular (Invitrogen, ThermoFisher, Waltham, Massachusetts; https://www.thermofisher.com), but it typically yields lower protein expression levels. However, it has the advantage of a tighter regulation of protein expression over pET vectors. Inclusive within these vectors are a variety of antibiotic (selection) genes and a variety of protein tags and cleavage sites. The addition of these N- or C-terminal tags to enzymes can be adventitious for several reasons: (i) increase soluble protein expression, (ii) rapid affinity chromatography of the target protein(s), and (iii) detection of recombinant protein by Western blotting.

Transcription of multiple genes within a bacterial operon is possible due to the presence of Shine–Dalgarno (SD) sequences; ribosomal-binding sites within messenger RNA upstream of the start codon of successive genes. This enables the ribosome to continue to initiate protein synthesis from the next gene without the need for a second full promoter sequence. The SD sequences can vary in length and nucleotide composition, which ultimately affects the expression levels of the downstream gene. There are a variety of algorithms' available for both predicting SD sequences in bacterial genomes (Starmer, Stomp, Vouk, & Bitzer, 2006), as well as designing novel sequences to maximize expression of a specific gene (eg, http://parts.igem.org/Ribosome_Binding_Sites/Design). Other factors to consider when designing synthetic operons are the choice of the promoter/terminator pair and even the order of the genes in the construct (Starmer et al., 2006). With very long constructs (greater than five genes), secondary promoter elements may be incorporated to boost downstream gene expression.

The selection of the gene homologues is an important consideration in successful biofactory design. For example, simply choosing the exact gene sequences encoding enzymes responsible for a native plant secondary metabolite pathway may not be successful in *E. coli*. This could be due to factors including insoluble protein expression, absence of plant-specific posttranslational modifications, incorrect protein folding and/or cofactor incorporation, and poor *E. coli*-specific codon usage in the plant genes. Therefore, gene sequences may need to be altered, such as codon optimization for the chosen host and the removal of any plant-specific targeting sequences. Alternatively, selection of gene homologues from bacteria may increase the likelihood of soluble, functional protein expression, as may selecting genes with a higher predicted protein solubility (Wilkinson & Harrison, 1991).

A recent study describing the generation of menthol isomers from pulegone in *E. coli* (Toogood et al., 2015) highlights the importance of some of the considerations above when designing operons in *E. coli*. In this approach, the vector pET21b was chosen due to the high expression levels of the individual proteins and the presence of hexa-histidine tags. The latter was important to enable the positive identification of recombinant protein expression via Western blotting and to enable successful gene expression in one case (menthone: neomenthol reductase (MNMR); Fig. 1). Gene selections were taken from both the native *M. piperita* pathway (Croteau et al., 2005) and a previously published bacterial homologue (Mansell et al., 2013). Each gene was synthesized, employing codon-optimization for improved *E. coli* soluble expression. The following sections will describe techniques employed in the construction of partial mint biosynthetic pathways and discuss the general applicability of these techniques in operon generation.

2.2 Assembly of *Mentha*-Like Biosynthetic Pathways in *E. coli*

The ideal cloning strategy involves a seamless stitching of genes and regulatory parts together, without the limitations of restriction digests and the associated cloning "scars." A number of techniques and commercial kits are available that fulfill these requirements, such as In-Fusion cloning (Zhu, Cai, Hall, & Freeman, 2007), Gibson assembly (Gibson et al., 2009), ligase cycling reaction (de Kok et al., 2014), and megaprimer PCR (Unger, Jacobovitch, Dantes, Bernheim, & Peleg, 2010). The chosen assembly method for the *Mentha* pathways in *E. coli* was the In-Fusion system (Toogood et al., 2015), where linearized vectors and gene insert(s) are fused

in a precise order and orientation by the presence of 15 bp overlaps at each end of the DNA fragments.

2.2.1 General Assembly Protocols

The success of seamless cloning DNA assembly is dependent on the presence of unique sequences at each end of the DNA parts, to enable DNA fragments to be joined in the correct order and orientation. Any intergenic regions or other DNA alterations (eg, SD sequences) are added to the parts by PCR amplification prior to construct assembly. The vector must also be linearized at the required insertion site.

The following are the general protocols utilized to enable the successful generation of the three-gene expression construct (Toogood et al., 2015). The vector (pET21b) already contained the first gene, namely, double bond reductase (NtDBR) from *Nicotiana tabacum*, containing a C-terminal His_6-tag. The two genes to be inserted were menthone: menthol reductase (MMR) and MNMR from *M. piperita* to form the DMN construct (Fig. 1). Both genes contained a C-terminal His_6-tag, however, MNMR also required an N-terminal His_6-tag to enable successful protein expression in *E. coli* (Toogood et al., 2015). Additionally, both MMR and MNMR required the addition of SD sequences at the 5′ end to enable coexpression of the three genes.

1. Inverse PCR of NtDBR-pET21b to generate a linearized single-gene construct. The forward and reverse primers anneal to the vector terminator region and 3′-end of NtDBR, respectively. This is followed by selective digestion of the template DNA by the restriction enzyme *Dpn*I.

   Note: *Dpn*I requires the presence of N6-methyladenine within the recognition sequence to cleave DNA. Therefore, the template DNA must have been produced by a Dam^+ *E. coli* strain for digestion to occur.
2. PCR amplification of the two additional genes, incorporating a SD sequence at the 5′ end of the genes. The 5′ and 3′ regions also contain a 15 bp overlap with the DNA sequences to be ligated to.
3. In-Fusion ligation reaction between the three DNA-linearized parts followed by transformation into Stella competent *E. coli* cells (Clontech, Mountain View, California; www.clontech.com). The transformation mixture is plated onto an antibiotic selection plate and incubated overnight at 37°C.

   Note: This strain of *E. coli* is specifically designed to survive the presence of toxic additives found in the In-Fusion reaction mixture. Using other competent cell strains may lead to no colony formation.

4. Colonies are screened for the presence of the two additional genes by colony pick PCR (Woodman, 2008). Confirmation of the correct sequence and gene orientations is performed by DNA sequencing.

2.2.2 In-Fusion Reactions with Repeating Sequences

Difficulties may arise when one or both termini of multiple pairs of DNA parts contain the same sequence with a high melting temperature. For example, with construct DMN, the junction between both NtDBR–MMR and MMR–MNMR contain identical C-terminal regions (CTCGAGCACCACC ACCACCACCACTGA) and 5′ SD sequence (GGAGGACAGCTAA), with a combined melting temperature of 73°C (Toogood et al., 2015). To avoid this, C-terminal His_6-tags can be eliminated, and/or nonidentical SD sequences may be used between different gene pairs. Where this is not optimal, primer design for gene amplification is critical to ensure there are no repeating units contained within the 15 bp overhangs.

One solution is to ensure that the repeating unit is wholly contained within either the 3′ end of one gene or 5′ end of the next gene, with no repeating sequence spanning both genes of the interface. For example, construct DMN could be constructed with nonidentical SD sequences (S1 and S2; Fig. 2A), while maintaining the C-terminal His_6-tags. In this case, NtDBR-pET21b could be linearized with the addition of the first SD sequence by PCR. The second gene MMR is amplified with both the first and second SD sequences at the 5′ and 3′ end, respectively. The final gene MNMR is amplified with the second SD sequence, and vector terminator region overhangs at the 5′ and 3′ ends, respectively. Therefore, the required 15 bp overlaps are contained wholly within either the nonidentical SD sequences or the terminator region of the vector (Fig. 2A). This would allow a one-step assembly reaction to be performed with all genes containing C-terminal His_6-tags.

In cases where the repeating sequence spans both genes within the fusion interface, a multistep approach can be employed (Fig. 2B). The published DMN construct method required both NtDBR-pET21b and MMR to be PCR amplified with identical SD sequences added as the 15 bp overhang at the 3′ and 5′ ends, respectively (Toogood et al., 2015). However, the 3′ end of MMR was amplified without its stop codon, and contained the 5′ end of the vector terminator region. This was followed by a two-gene construct In-Fusion ligation, then latter linearization by PCR at the junction between MMR and the terminator region. The final gene

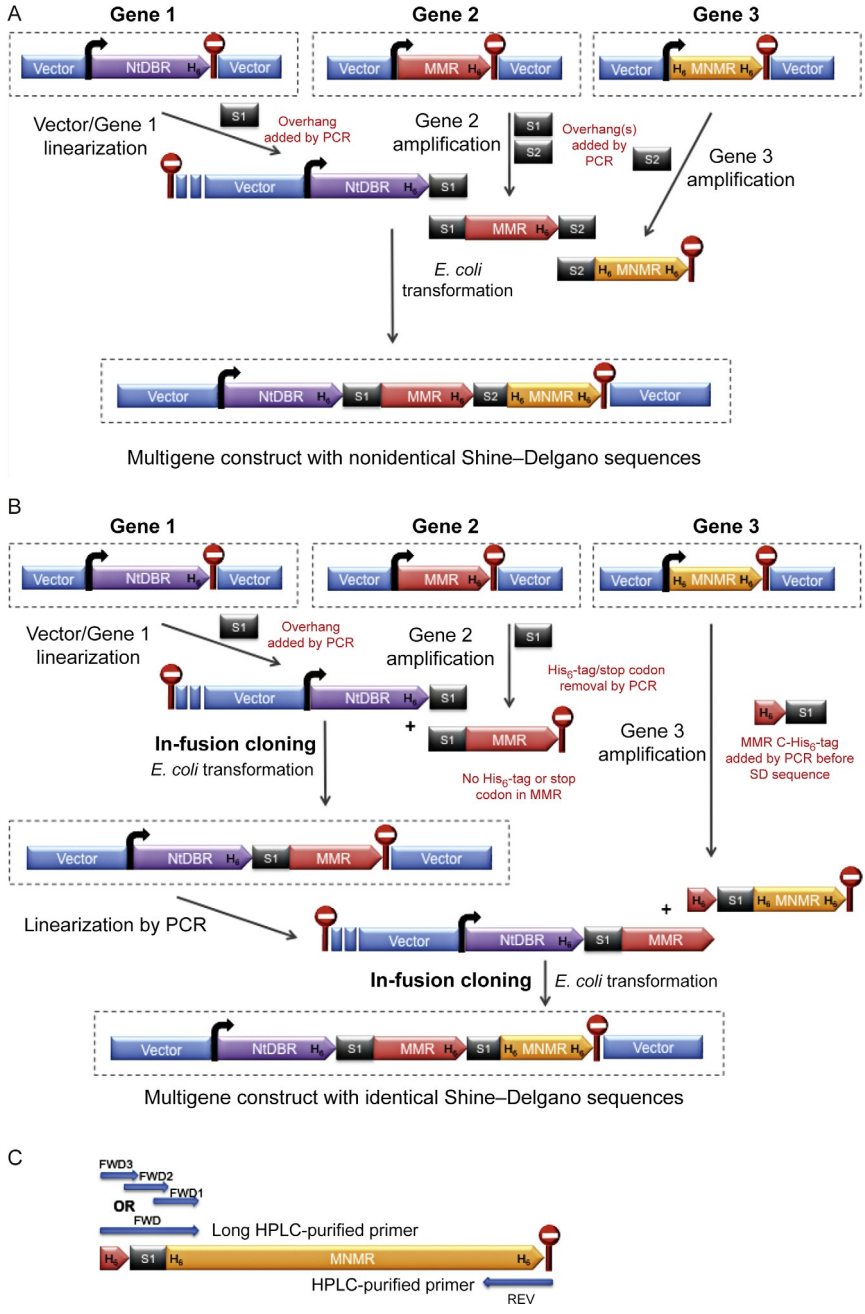

Fig. 2 PCR-based In-fusion cloning to create multigene expression constructs. (A) Single In-fusion cloning step protocol for constructs without significant repeating sequences. (B) Multiple In-fusion cloning steps protocol where repeating sequences (His_6-tag—Shine–Dalgarno sequence) occur at the cloning termini. (C) Construction of large sequence overhangs at the 5′ end of gene MNMR by PCR. *H6*, hexa-histidine tag; *S1/S2*, Shine–Dalgarno sequences.

MNMR was amplified with a very long 5′ overhang containing the His_6-tag/stop codon of MMR followed by the SD sequence (Fig. 2B). The 3′ end aligns to the vector terminator region, and the final three-gene In-Fusion assembly was conducted. Generation of lengthy overhangs (30–80 bp) can be readily achieved by PCR using either very long HPLC-purified primers, or a cascading series of smaller overlapping oligos (Fig. 2C). In the latter case, the ratio of the oligos is biased in favor of the outermost oligo (eg, oligos FWD1:FWD2:FWD3 with ratio 1:2:5) to maximize the amplification of full-length PCR product (Toogood et al., 2015). Therefore, the presence of repeating units at the junction between genes to be assembled does not necessarily limit the success of seamless cloning strategies.

3. MULTIGENE PROTEIN EXPRESSION AND VALIDATION

Successful production of monoterpenoids by microbial hosts is not solely dependent on sufficient expression levels of soluble, active recombinant enzymes. Other factors play important roles such as (i) impact on central metabolism, (ii) toxicity of the recombinant enzyme overexpression and monoterpenoid substrate(s)/product, (iii) colocalization of the enzymes with the substrate(s) of secondary metabolite production, (iv) competing pathways depleting monoterpenoid precursors or catalyzing side reactions, and (v) sufficient cofactor/coenzyme supply for both primary and secondary metabolism. These latter factors often require microbial strain engineering to generate robust, productive monoterpenoid biofactories. However, when designing and testing a new biosynthetic pathway in *E. coli*, it is important to demonstrate successful expression and catalytic functioning of the recombinant enzymes independent of other constraints of in vivo monoterpenoid production. Western blots are a useful technique to positively identify the presence of recombinant proteins separated by SDS-PAGE analysis.

1. Transformation of each operon constructs into *E. coli* expression strains. Grow small starter cultures in selective medium under noninducing conditions.

   Note: Constructs in pET vectors require *E. coli* (DE3) strains for expression of the genes. To minimize leaky expression in uninduced cultures, add 0.2% glucose to the medium (Novy & Morris, 2001).

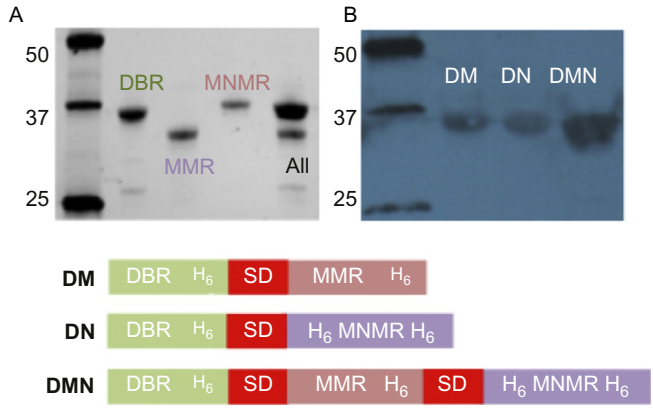

Fig. 3 (A) SDS-PAGE analysis of purified recombinant enzymes. (B) Western blot analysis of cell extracts of three operons of menthol biosynthesis.

2. Inoculate larger selective media with the starter cultures and express the recombinant proteins under conditions optimal for maximal soluble protein production.

   Note: Optimal conditions are determined by varying expression conditions such as inducer concentration, induction growth phase, postinduction temperature, and medium composition. In this case, constructs DMN, DM (NtDBR–MMR), and DN (NtDBR–MNMR) were successfully expressed in autoinduction medium (Fig. 3).
3. Generate cell-free extracts of expressed recombinant proteins. SDS-PAGE analysis will indicate the presence of overexpression bands (Fig. 3A).

   Note: Recombinant proteins containing affinity tags (eg, N- or C-His_6) can be partially purified prior to SDS-PAGE analysis to improve the detection of moderately expressed proteins.
4. Confirmation of expression is performed by a Western blot using anti-6X His tag antibodies (Fig. 3B).

   Note: Detection of recombinant proteins of similar migration distance by SDS-PAGE may be separated by native PAGE prior to Western blots.

4. BIOTRANSFORMATIONS

To avoid the complications of in vivo biotransformations (eg, cofactor supply and substrate transportation), initial monoterpenoid production tests

are performed with either cell-free extracts or permeabilized cells (Weyler & Heinzle, 2015), with an external cofactor (recycling) supply. This also allows the detection of by-products catalyzed by native *E. coli* enzymes, giving insight into possible strain engineering requirements to knock out/down competing pathways. The volatility of these monoterpenoids enables their easy detection by GC-based methods.

4.1 General Monoterpenoid Reaction and Detection

1. Biotransformations (1 mL) are composed of buffer (50 m*M* Tris, pH 7.0) containing substrate (1 m*M* pulegone), external hydride source ($NADP^+$/glucose dehydrogenase/glucose cofactor recycling system), and enzyme(s) source (cell-free extract or permeabilized cells). Reactions are incubated at 25–30°C for 2–24 h.

   Note: Extract quantities within the reactions are dependent on the state of the samples. For example, permeabilized cells and crude cell extracts may be viscous and/or precipitate during the reaction, making later solvent extractions difficult. Cofactor recycling systems can be replaced with NADPH at three to five times the concentration of the initial substrate. Shorter reaction times are particularly suitable for substrates and/or products that rapidly degrade under the reaction conditions. Monoterpenoid losses due to high volatility can be minimized by the addition of a solvent overlay in the reaction, such as 10% dodecane.
2. Substrate and product(s) are extracted into ethyl acetate (0.9 mL) containing an internal standard (0.5% *sec*-butylbenzene), dried with anhydrous magnesium sulfate, and quantified by GC.

   Note: Quantitative analysis was performed by a comparison of peak areas to authentic standards of known concentrations. GC–MS can also be used as the compound analyzer, enabling positive identification of any side products.
3. Reaction extracts (1 μL) are analyzed by gas chromatography on a DB-WAX column (30 m; 0.32 mm; 0.25-μm film thickness) with an FID detector. This column successfully separates the two isomers of menthone and four isomers of menthol (Fig. 4). Unknown products are identified by GC analysis in combination with mass spectrometry (GC–MS; inert XL-EI/CI MSD with triple axis detector) using a Zebron ZB-Semi Volatiles column (15 m × 0.25 mm × 0.25-μm film thickness).

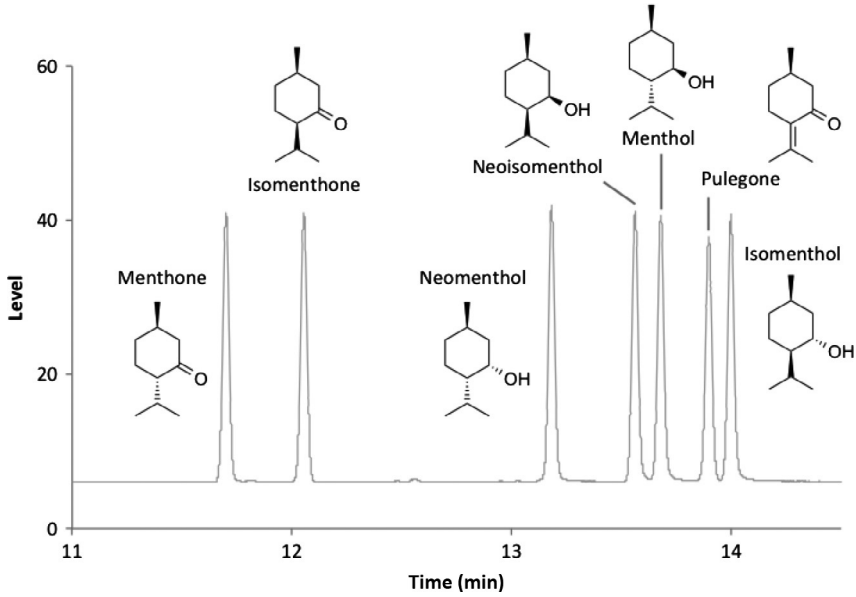

Fig. 4 Gas chromatography separation of seven monoterpenoids of the *Mentha* menthol biosynthetic pathway. Analysis was performed on a DB-WAX column (30 m; 0.32 mm; 0.25-μm film thickness; JW Scientific) on an Agilent Technologies 7890A GC system equipped with an FID detector. *Adapted from Toogood, H. S., Cheallaigh, A. N., Tait, S., Mansell, D. J., Jervis, A., Lygidakis, A., et al. (2015). "Enzymatic menthol production: One-pot approach using engineered* Escherichia coli*." ACS Synthetic Biology 4(10), 1112–1123.*

Note: GC method: injector temperature was 220°C (split ratio of 20:1) and helium flow rate of 1 mL/min (5.1 psi). The program started at 40°C, with a 1 min hold followed by a 10°C/min increase of temperature, and a final hold at 210°C for 1 min. The FID detector temperature was 250°C with a hydrogen flow of 30 mL/min. GC–MS method: injector temperature was 220°C (split ratio of 10:1) and helium flow rate of 1 mL/min (5.1 psi). The program started at 40°C, with a 3 min hold followed by a 10°C/min increase of temperature, and a final hold at 210°C for 3 min. The mass spectra fragmentation patterns were analyzed by the NIST/EPA/NIH 11 mass spectral library database for compound identification (Toogood et al., 2015).

4.2 Alternative Protocols

The inherent volatility of the menthol pathway components allows alternative analytical methods to be employed. Early work to determine the native

Mentha metabolic pathways described the use of in vivo detection of volatile monoterpenoid metabolites via simultaneous steam distillation and solvent extraction techniques combined with GC–MS analysis (Ringer, Davis, & Croteau, 2005; Ringer, McConkey, Davis, Rushing, & Croteau, 2003). This in situ method could be applied to the detection of enzyme activity during expression trials of constructs under different optimization conditions. It has the advantage of eliminating nonvolatile compound background, allowing cleaner samples to be analyzed. However, the extraction process is lengthy compared to standard solvent extraction, and the apparatus limits the minimal size of sample volumes to be analyzed.

A simpler approach to detecting monoterpenoid components is headspace sampling of biotransformation reactions coupled to GC or GC–MS analysis. This enables the detection of very light volatile organics without the need to perform extractions on complex mixtures (eg, cell extracts). Purge and trap technology (Lee, Lee, Hsiang, & Chen, 1997) can effectively concentrate the volatile compounds from the headspace prior to GC injection, allowing the analysis of trace levels of compounds. A downside of this technique is the lack of detection of some semi- and higher boiling point volatiles. However, for simple compound identification, rather than in-depth analysis of all monoterpenoid analytes, this may be a suitable method for routine compound quantification and identification.

5. FURTHER STUDIES

After the successful demonstration of an active biosynthetic pathway in *E. coli*, the next stage is to undergo a variety of optimization trials to improve the efficiency and yields of the desired product(s). These studies are critical to move from a proof-of-concept state to ultimately a commercially viable process. The following Section 5.1 discusses some microbial and other factors that may play a role in limiting the industrially relevance of a newly established synthetic biology approach to valuable compound production.

5.1 Expression Constructs

Expression of multiple recombinant genes controlled by the same promoter does not guarantee equivalent production levels of each protein. Protein levels are dependent on factors such as genomic sequence, number of genes in the operon, promoter and SD sequence strengths, vector copy number, and even the order of genes in the operon. The screening of homologous genes for optimal functioning in *E. coli* may improve biocatalytic ability,

as could a screen of variant libraries of existing biocatalysts. It is very important to look at the flux through the pathway to identify bottlenecks and specifically aims to modify the construct to overcome the problem. For example, The DM construct shows significantly higher NtDBR expression and activity than for MMR, as seen by incomplete menthol production, and an accumulation of the NtDBR reaction products menthone and isomenthone (Toogood et al., 2015).

An earlier study described the addition of four genes into *E. coli* encoding sequential steps of the biosynthesis of carvone from C5 isoprenoid precursors (Carter et al., 2003). Unfortunately, accumulation and cell export of the first enzymatic product limonene occurred due to a severe limitation in the availability of the precursors within central metabolism of the organism. An upregulation of the latter stage genes may improve this by reacting with the limonene before it is exported out of the cell. This may be accomplished by the addition of extra promoters within the construct to boost the levels of latter gene products. The synthesis of the Taxol precursor taxadiene by *E. coli* from glucose was achieved by partitioning the required genes into a native upstream MEP pathway and a downstream terpenoid-forming pathway (Ajikumar et al., 2010). The study described a systematic multivariate search was performed to identify conditions to balance the two pathways to maximize taxadiene production (Ajikumar et al., 2010).

5.2 Strain and Expression Optimization

The nature of the *E. coli* strain can have a dramatic impact on the expression levels of recombinant proteins. Different strains have been developed to overcome specific problems such as the expression of toxic proteins or the minimization of insoluble protein formation. The expression of the DMN construct was screened with 12 different strains of *E. coli* to determine the optimal host organism (Toogood et al., 2015). Dramatic, yet nonpredictive differences in menthol yields were obtained with the different strains (Fig. 5A). Additional improvements in protein expression can be achieved by varying the induction conditions (inducer concentration, induction time, and postinduction temperature) and growth medium (Fig. 5B).

Strain engineering is a crucial aspect in microbial monoterpenoid formation as these compounds are often cytotoxic, such as limonene (Chubukov et al., 2015; Chueca, Pagán, & García-Gonzalo, 2014). One approach to alleviate this toxicity is to incorporate terpenoid efflux pumps into *E. coli* to facilitate the excretion of the compounds into the culture medium.

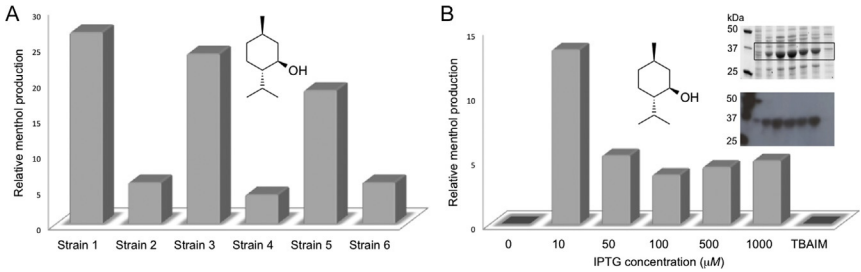

Fig. 5 Biotransformation data showing the effect of varying the (A) *E. coli* strain and (B) inducer concentration on menthol production. Inset in (B) shows the SDS-PAGE (*top*) and Western blot (*bottom*) analyses of the cultures. *TBAIM*, terrific broth auto-induction medium.

For example, a synthetic biology approach to microbial biofuel production included the screening of 43 known efflux pumps in bacteria for improved survivability of the cultures (Dunlop et al., 2011). This approach also has the added advantage of simplifying the product extraction and purification processes. Another bacterial efflux pump outer membrane protein AlkL from *Pseudomonas fluorescens* CHA0 in *E. coli* was shown to increase the dodecanoic acid methyl ester oxygenation activity 28-fold (Julsing et al., 2012). It was shown to be an efficient tool in boosting productivities of whole-cell biotransformations with hydrophobic and aliphatic substrates.

Microbial biocatalysis protocols can be complicated by the presence of competing pathways by constitutive *E. coli* enzymes, leading to unwanted by-products. For example, a synthetic biology approach to propane biosynthesis showed reduced titres due to a competition for the precursor molecule butyraldehyde by *E. coli* aldehyde dehydrogenases Ahr and YqhD (Menon et al., 2015). To overcome this, a double knockout strain Δ*ahr* and Δ*yqhD* was constructed, and propane production was increased significantly.

5.3 High-Throughput Optimization Studies

The successful redesign of in vivo biofactories to optimize monoterpenoid production is likely to require a combinatorial approach to overcome existing enzymatic and host bottlenecks and/or other constraints. In an ideal world, reliable prediction of the behavior of the organism during monoterpenoid pathway would enable a limited, rational approach to optimal biofactory redesign. In the real world, high-throughput optimization studies are often needed to overcome the often nonpredictive, competing factors involved in regulating secondary metabolite production. Advances in

new computational techniques and algorithms have enabled the development of "-omics" technologies, significantly impacting on our understanding of the complex nature of gene regulation and metabolite production (Cascante & Marin, 2008). Metabolomic analyses focus on the identification and quantification of all the metabolites within an organism. This enables an understanding of the physiological state of an organism under defined growth conditions. Complementary flux analyses (fluxomics) looks at the dynamic nature of the metabolome, and how it changes under environmental and genetic pressures (Cascante & Marin, 2008). Additional regulatory control can be discerned by investigating the transcriptome; the complete set of RNA transcripts generated under certain growth conditions. Therefore, a true synthetic biology approach to novel pathway design must include a more holistic paradigm approach, where cell metabolism regulation in addition to enzymatic considerations informs on key metabolite production.

The current menthol-producing *E. coli* strains utilize pulegone as the initial substrate (Toogood et al., 2015). A more industrially useful process would be linking menthol generation to *E. coli* central metabolism through the isoprenoid biosynthesis pathway (MEP/mevalonate pathways). This would require an upregulation of biosynthesis of the isoprenoid precursor GPP and incorporation of plant genes to generate pulegone from (*S*)-limonene (Fig. 1). Therefore, the addition of more genes and its concomitant effect on central metabolism must be investigated to enable successful menthol production. Similar approaches have been taken to produce artemisinin in *E. coli*. Initial studies showed production was hampered by limited isoprenoid precursor supply (Chang & Keasling, 2006). To overcome this, the *E. coli* nonmevalonate (DXP) pathway for precursor production was bypassed, and the heterologous mevalonate pathway from *S. cerevisiae* was incorporated in two synthetic operons. Growth inhibition, due to an accumulation of toxic isoprenoid precursors, was overcome by the coexpression of the latter stage biocatalyst amorpha-4,11-diene (amorphadiene) synthase (Chang & Keasling, 2006).

Recently, the Centre for Synthetic Biology of Fine and Speciality Chemicals (SYNBIOCHEM; http://synbiochem.co.uk) was set up to develop new faster, more predictable, and reliable "greener" routes to sustainable fine and speciality chemicals production. It aims to accelerate the delivery of bespoke chemicals syntheses by adopting a modular "plug-and-play" platform approaches, using a "design–build–test–deploy" life cycle (Fig. 6). At the Design stage, in silico whole-cell metabolic modelling

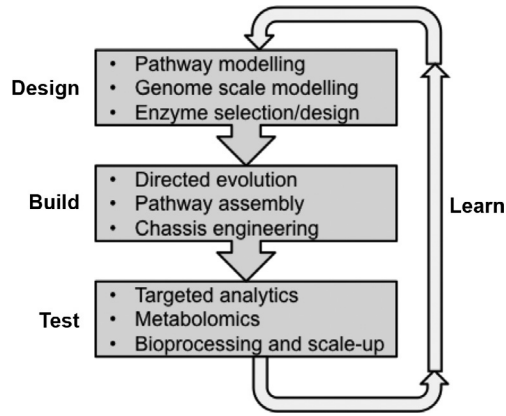

Fig. 6 The Design–Build–Test cycle employed in SYNBIOCHEM at Manchester University for the design of microbial biofactories for speciality biochemical production. Each part of the cycle is divided into three scales, parts, devices, and systems to represent the considerations in developing industrially relevant microbial production chassis.

and pathway flux modelling can produce strategies for engineering of the chassis to optimize flux through the pathway of interest, reduce competitive side reactions, and increase the supply of cofactors and precursors (Becker et al., 2007; Breitling, Achcar, & Takano, 2013; Sridhar, Suthers, & Maranas, 2010). To address poorly performing, or missing steps in a pathway, automated prediction and selection of enzyme alternatives using tools such as RetroPath (Carbonell, Parutto, Herisson, Pandit, & Jaulon, 2014) or PathPred (Moriya et al., 2010) can potentially identify enzymes displaying improved activity in vivo. Alternatively, directed evolution of biocatalysts can be employed to improve fitness toward the desired activity (Currin, Swainston, Day, & Kell, 2014; Currin, Swainston, Day, & Kell, 2015; Swainston, Currin, Day, & Kell, 2014).

These design tools can give rise to high numbers of test parameters including testing of regulatory features (eg, promoters, RBS, vector, gene order), alternative biocatalytic parts, addition of accessory pathways and genome modifications. Automated experimental procedures are therefore required to allow HTP acceleration of assembly and screening of production chassis. Robotic liquid handling platforms are ideally suited for DNA manipulations such as one-step pathway assembly but also for rapid screening of the resultant production chassis. Automated colony picking and culturing in microtiter plates or microfluidics (Pico droplet) followed by sample processing allow a flexible Build pipeline which can directly feed Test

platforms. Typical Test systems include mass spectrometry for targeted analysis (GC–MS for monoterpenoids) (Alonso-Gutierrez et al., 2013; Toogood et al., 2015) or metabolomics (Chokkathukalam, Kim, Barrett, Breitling, & Creek, 2014; Muhamadali et al., 2015; Nguyen et al., 2012), proteomics, and transcriptomics. The output-of-Test data can then feedback into the Design platforms to improve accuracy and predictability of the in silico models and facilitates predictable downstream scale-up. This iterative cycle implements a multidisciplinary approach for the iterative development of industrially relevant production chassis.

6. CONCLUSIONS

The synthetic biology approach to secondary metabolite production within microorganisms heralds a new era in natural and "green," sustainable fine chemicals production. It goes beyond the classical biocatalysis technology of optimizing enzyme expression/functionality to include considerations for enabling compound production from cheap, renewable waste feed stocks via linking biosynthesis to central metabolism. Recent studies in the production of monoterpenoids in *E. coli* have demonstrated the commercial potential of such technologies, bringing fine chemical production in line with the increasing world-wide economic and political pressures for environmentally friendly and nonfossil fuel-derived sustainable production processes.

The method of construction of successful biosynthetic constructs is important to enable seamless and scar-less "stitching" of both genes and suitable regulatory elements. Adaptations of existing ligation existing cloning methodologies enable the successful incorporation of DNA parts containing repeating sequences with high annealing capability, compared to lengthy and possibly costly whole operon gene synthesis. Initial constructs can then undergo functional screening, followed by plug-and-play construct optimization to improve its biosynthetic ability. New and emerging technologies may further advance how we will regulate secondary metabolite production in vivo. For example, there is current interest in the use of both riboswitches (Morra et al., 2015) and light-activated promoter elements for novel regulatory control (Hardman, Hauck, Clark, Heyes, & Scrutton, 2014; Tabor, Levskaya, & Voigt, 2011).

Whole organism metabolic constraints must also be taken into consideration in construct design. The use of fluxomics/metabolomics and transcriptomics enables dynamic metabolic pathway modelling, and the impact

of compound production on the overall performance of the microbial biofactory. These modelling technologies, combined with pathway design and in vitro biotransformation performance studies, enable the tuning of the host microorganism by upregulating/downregulating key genes to eliminate competing pathway, optimize flux balance and potentially overcome toxicity issues.

The future outlook for the synthetic biology approach could be to revolutionize the manufacture of fine chemicals and pharmaceuticals. We are in an exciting new era of not only reading and understanding DNA, but also now writing our own constructs and building nonnative metabolic pathways to existing or novel chemicals. This transition from understanding biology to informed editing and rewriting metabolic processes has exciting prospects for the future.

ACKNOWLEDGMENTS

This work was funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC BB/J015512/1; BB/M017702/1) and GlaxoSmithKline. SYNBIOCHEM is funded by the BBSRC (BB/M017702/1). N.S.S. is an Engineering and Physical Sciences Research Council (EPSRC; EP/J020192/1) Established Career Fellow.

REFERENCES

Ajikumar, P. K., Tyo, K., Carlsen, S., Mucha, O., Phon, T. H., & Stephanopoulos, G. (2008). Terpenoids: Opportunities for biosynthesis of natural product drugs using engineered microorganisms. *Molecular Pharmaceutics*, *5*(2), 167–190.

Ajikumar, P. K., Xiao, W. H., Tyo, K. E. J., Wang, Y., Simeon, F., Leonard, E., et al. (2010). Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science*, *330*(6000), 70–74.

Alonso-Gutierrez, J., Chan, R., Batth, T. S., Adams, P. D., Keasling, J. D., Petzold, C. J., et al. (2013). Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production. *Metabolic Engineering*, *19*, 33–41.

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P., et al. (2015). Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances*, *33*(8), 1582–1614.

Becker, S. A., Feist, A. M., Mo, M. L., Hannum, G., Palsson, B. O., & Herrgard, M. J. (2007). Quantitative prediction of cellular metabolism with constraint-based models: The COBRA toolbox. *Nature Protocols*, *2*(3), 727–738.

Blount, B. A., Weenink, T., & Ellis, T. (2012). Construction of synthetic regulatory networks in yeast. *FEBS Letters*, *586*(15), 2112–2121.

Breitling, R., Achcar, F., & Takano, E. (2013). Modeling challenges in the synthetic biology of secondary metabolism. *ACS Synthetic Biology*, *2*(7), 373–378.

Breitling, R., & Takano, E. (2015). Synthetic biology advances for pharmaceutical production. *Current Opinion in Biotechnology*, *2*, 46–51.

Carbonell, P., Parutto, P., Herisson, J., Pandit, S. B., & Jaulon, J.-L. (2014). XTMS: Pathway design in an eXTended metabolic space. *Nucleic Acids Research*, *42*, W389–W394.

Carter, O. A., Peters, R. J., & Croteau, R. (2003). Monoterpene biosynthesis pathway construction in *Escherichia coli*. *Phytochemistry*, *64*(2), 425–433.
Cascante, M., & Marin, S. (2008). Metabolomics and fluxomics approaches. *Essays in Biochemistry*, *45*, 67–81.
Chang, M. C. Y., & Keasling, J. D. (2006). Production of isoprenoid pharmaceuticals by engineered microbes. *Nature Chemical Biology*, *2*(12), 674–681.
Chemler, J. A., & Koffas, M. A. (2008). Metabolic engineering for plant natural product biosynthesis in microbes. *Current Opinion in Biotechnology*, *19*(6), 597–605.
Chokkathukalam, A., Kim, D. H., Barrett, M. P., Breitling, R., & Creek, D. J. (2014). Stable isotope-labeling studies in metabolomics: New insights into structure and dynamics of metabolic networks. *Bioanalysis*, *6*(4), 511–524.
Chubukov, V., Mingardon, F., Schackwitz, W., Baidoo, E. E. K., Alonso-Gutierrez, J., Hu, Q., et al. (2015). Acute limonene toxicity in Escherichia coli is caused by limonene-hydroperoxide and alleviated by a point mutation in alkyl hydroperoxidase (AhpC). *Applied and Environmental Microbiology*, *81*(14), 4690–4696.
Chueca, B., Pagán, R., & García-Gonzalo, D. (2014). Oxygenated monoterpenes citral and carvacrol cause oxidative damage in Escherichia coli without the involvement of tricarboxylic acid cycle and Fenton reaction. *International Journal of Food Microbiology*, *189*, 126–131.
Croteau, R. B., Davis, E. M., Ringer, K. L., & Wildung, M. R. (2005). (−)-Menthol biosynthesis and molecular genetics. *Naturwissenschaften*, *92*(12), 562–577.
Currin, A., Swainston, N., Day, P. J., & Kell, D. B. (2014). SpeedyGenes: An improved gene synthesis method for the efficient production of error-corrected, synthetic protein libraries for directed evolution. *Protein Engineering, Design & Selection*, *27*, 273–280.
Currin, A., Swainston, N., Day, P. J., & Kell, D. B. (2015). Synthetic biology for the directed evolution of protein biocatalysts: Navigating sequence space intelligently. *Chemical Society Reviews*, *44*(5), 1172–1239.
de Kok, S., Stanton, L. H., Slaby, T., Durot, M., Holmes, V. F., Patel, K. G., et al. (2014). Rapid and reliable DNA assembly via ligase cycling reaction. *ACS Synthetic Biology*, *3*(2), 97–106.
Duetz, W. A., Bouwmeester, H., van Beilen, J. B., & Witholt, B. (2003). Biotransformation of limonene by bacteria, fungi, yeasts, and plants. *Applied Microbiology and Biotechnology*, *61*(4), 269–277.
Dunlop, M. J., Dossani, Z. Y., Szmidt, H. L., Chu, H. C., Lee, T. S., Keasling, J. D., et al. (2011). Engineering microbial biofuel tolerance and export using efflux pumps. *Molecular Systems Biology*, *7*, 1–7.
Farmer, W. R., & Liao, J. C. (2001). Precursor balancing for metabolic engineering of lycopene production in *Escherichia coli*. *Biotechnology Progress*, *17*(1), 57–61.
Friedman, D. C., & Ellington, A. D. (2015). Industrialization of biology. *ACS Synthetic Biology*, *4*(10), 1053–1055.
Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A. r., & Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods*, *6*(5), 343–345.
Hardman, S. J., Hauck, A. F., Clark, I. P., Heyes, D. J., & Scrutton, N. S. (2014). Comprehensive analysis of the green-to-blue photoconversion of full-length Cyanobacteriochrome Tlr0924. *Biophysical Journal*, *107*(9), 2195–2203.
Hunter, P. (2008). Harnessing Nature's wisdom. *EMBO Reports*, *9*(9), 838–840.
Hussain, M. S., Fareed, S., Ansari, S., Rahman, M. A., Ahmad, I. Z., & Saeed, M. (2012). Current approaches toward production of secondary plant metabolites. *Journal of Pharmacy & Bioallied Sciences*, *4*(1), 10–20.
Jackson, B. E., Hart-Wells, E. A., & Matsuda, S. P. T. (2003). Metabolic engineering to produce sesquiterpenes in yeast. *Organic Letters*, *5*(10), 1629–1632.

Julsing, M. K., Schrewe, M., Cornelissen, S., Hermann, I., Schmid, A., & Buhler, B. (2012). Outer membrane protein AlkL boosts biocatalytic oxyfunctionalization of hydrophobic substrates in Escherichia coli. *Applied and Environmental Microbiology*, *78*(16), 5724–5733.

Kiyota, H., Okuda, Y., Ito, M., Hirai, M. Y., & Ikeuchi, M. (2014). Engineering of cyanobacteria for the photosynthetic production of limonene from CO_2. *Journal of Biotechnology*, *185*, 1–7.

Lange, B. M., Mahmoud, S. S., Wildung, M. R., Turner, G. W., Davis, E. M., Lange, I., et al. (2011). Improving peppermint essential oil yield and composition by metabolic engineering. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(41), 16944–16949.

Lawrence, B. M. (Ed.), (2007). In *Medicinal and aromatic plants—Industrial profiles*: *Vol. 44. Mint: The genus Mentha* (pp. 156–158). Florida: CRC Press.

Lee, M.-R., Lee, J.-S., Hsiang, W.-S., & Chen, C.-M. (1997). Purge-and-trap gas chromatography–mass spectrometry in the analysis of volatile organochlorine compounds in water. *Journal of Chromatography A*, *775*(1–2), 267–274.

Li, J. W. H., & Vederas, J. C. (2009). Drug discovery and natural products: End of an era or an endless frontier? *Science*, *325*(5937), 161–165.

Mansell, D. J., Toogood, H. S., Waller, J., Hughes, J. M. X., Levy, C. W., Gardiner, J. M., et al. (2013). Biocatalytic asymmetric alkene reduction: Crystal structure and characterization of a double bond reductase from *Nicotiana tabacum*. *ACS Catalysis*, *3*, 370–379.

Martin, V. J. J., Pitera, D. J., Withers, S. T., Newman, J. D., & Keasling, J. D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nature Biotechnology*, *21*(7), 796–802.

Menon, N., Pásztor, A., Menon, B. R., Kallio, P., Fisher, K., Akhtar, M. K., et al. (2015). A microbial platform for renewable propane synthesis based on a fermentative butanol pathway. *Biotechnology for Biofuels*, *8*(1), 61.

Mitchell, W. (2011). Natural products from synthetic biology. *Current Opinion in Chemical Biology*, *15*(4), 505–515.

Moriya, Y., Shigemizu, D., Hattori, M., Tokimatsu, T., Kotera, M., Goto, S., et al. (2010). PathPred: An enzyme-catalyzed metabolic pathway prediction server. *Nucleic Acids Research*, *38*, W138–W143.

Morra, R., Shankar, J., Robinson, C. J., Halliwell, S., Butler, L., Upton, M., et al. (2015). Dual transcriptional-translational cascade permits cellular level tuneable expression control. *Nucleic Acids Research*, *4*(3), e21.

Muhamadali, H., Xu, Y., Ellis, D. I., Allwood, J. W., Rattray, N. J., Correa, E., et al. (2015). Metabolic profiling of *Geobacter sulfurreducens* during industrial bioprocess scale-up. *Applied and Environmental Microbiology*, *81*(10), 3288–3298.

Nguyen, Q. T., Merlo, M. E., Medema, M. H. M., Jankevics, A., Breitling, R., & Takano, E. (2012). Metabolomics methods for the synthetic biology of secondary metabolism. *FEBS Letters*, *586*, 2177–2183.

Nicolaou, K. C., Yang, Z., Liu, J. J., Ueno, H., & Nantermet, P. G. (1994). Total synthesis of taxol. *Nature*, *367*(6464), 630–634.

Novy, R., & Morris, B. (2001). Use of glucose to control basal expression in the pET System. *Innovations Biotechniques*, *12*, 1–3.

Ringer, K. L., Davis, E. M., & Croteau, R. (2005). Monoterpene metabolism. Cloning, expression, and characterization of (–)-isopiperitenol/(–)-carveol dehydrogenase of peppermint and spearmint. *Plant Physiology*, *137*(3), 863–872.

Ringer, K. L., McConkey, M. E., Davis, E. M., Rushing, G. W., & Croteau, R. (2003). Monoterpene double-bond reductases of the (−)-menthol biosynthetic pathway: Isolation and characterization of cDNAs encoding (−)-isopiperitenone reductase and (+)-pulegone reductase of peppermint. *Archives of Biochemistry and Biophysics*, *418*(1), 80–92.

Sell, C. (2003). *A fragrant introduction to terpenoid chemistry*. Cambridge, UK: Royal Society of Chemistry.
Sridhar, R., Suthers, P. F., & Maranas, C. D. (2010). OptForce: A optimization procedure for identifying all genetic manipulations leading to targeted overproductions. *PLoS Computational Biology*, *6*(4), e1000744.
Starmer, J., Stomp, A., Vouk, M., & Bitzer, D. (2006). Predicting Shine–Dalgarno sequence locations exposes genome annotation error. *PLoS Computational Biology*, *2*(5), e57.
Swainston, N., Currin, A., Day, P. J., & Kell, D. B. (2014). GeneGenie: Optimized oligomer design for directed evolution. *Nucleic Acids Research*, *12*, W395–W400.
Tabor, J. J., Levskaya, A., & Voigt, C. A. (2011). Multichromatic control of gene expression in *Escherichia coli*. *Journal of Molecular Biology*, *405*(2), 315–324.
Toogood, H. S., Cheallaigh, A. N., Tait, S., Mansell, D. J., Jervis, A., Lygidakis, A., et al. (2015). Enzymatic menthol production: One-pot approach using engineered *Escherichia coli*. *ACS Synthetic Biology*, *4*(10), 1112–1123.
Unger, T., Jacobovitch, Y., Dantes, A., Bernheim, R., & Peleg, Y. (2010). Applications of the restriction free (RF) cloning procedure for molecular manipulations and protein expression. *Journal of Structural Biology*, *172*(1), 34–44.
Weyler, C., & Heinzle, E. (2015). Multistep synthesis of UDP-glucose using tailored, permeabilized cells of E. coli. *Applied Biochemistry and Biotechnology*, *175*(8), 729–736.
Wilkinson, D. L., & Harrison, R. G. (1991). Predicting the solubility of recombinant proteins in *Escherichia coli*. *Bio/Technology*, *9*, 443–448.
Winter, J. M., & Tang, Y. (2012). Synthetic biological approaches to natural product biosynthesis. *Current Opinion in Biotechnology*, *23*(5), 736–743.
Woodman, M. E. (2008). Appendix 3D direct PCR of intact bacteria (colony PCR). *Protocols in Microbiology*, *9*(A.3D), 1–6.
Yadav, V. G., De Mey, M., Giaw Lim, C., Kumaran Ajikumar, P., & Stephanopoulos, G. (2012). The future of metabolic engineering and synthetic biology: Towards a systematic practice. *Metabolic Engineering*, *14*(3), 233–241.
Yonekura-Sakakibara, K., & Saito, K. (2009). Functional genomics for plant natural product biosynthesis. *Natural Product Reports*, *26*(11), 1466–1487.
Zhu, B., Cai, G., Hall, E., & Freeman, G. (2007). In-Fusion™ assembly: Seamless engineering of multidomain fusion proteins, modular vectors, and mutations. *Biotechniques*, *43*(3), 354–359.