catalysts

MDPI

Review

Advances in the Metabolic Engineering of *Escherichia coli* for the Manufacture of Monoterpenes

Si-si Xie †, Lingyun Zhu †, Xin-yuan Qiu, Chu-shu Zhu and Lv-yun Zhu *

Department of Biology and Chemistry, College of Liberal Arts and Sciences, National University of Defense Technology, Changsha 410073, Hunan, China; xiesisi_bio@126.com (S.-s.X.); lingyunzhu@nudt.edu.cn (L.Z.); qiuxinyuan12@nudt.edu.cn (X.-y.Q.); zhuchushu13@163.com (C.-s.Z.)

* Correspondence: zhulvyun@nudt.edu.cn; Tel.: +86-731-84574281

† These authors contributed equally to this work.

Received: 1 April 2019; Accepted: 6 May 2019; Published: 9 May 2019

Abstract: Monoterpenes are commonly applied as pharmaceuticals and valuable chemicals in various areas. The bioproduction of valuable monoterpenes in prokaryotic microbial hosts, such as *E. coli*, has progressed considerably thanks to the development of different outstanding approaches. However, the large-scale production of monoterpenes still presents considerable limitations. Thus, process development warrants further investigations. This review discusses the endogenous methylerythritol-4-phosphate-dependent pathway engineering and the exogenous mevalonate-dependent isoprenoid pathway introduction, as well as the accompanied optimization of rate-limiting enzymes, metabolic flux, and product toxicity tolerance. We suggest further studies to focus on the development of systematical, integrational, and synthetic biological strategies in light of the inter disciplines at the cutting edge. Our review provides insights into the current advances of monoterpene bioengineering and serves as a reference for future studies to promote the industrial production of valuable monoterpenes.

Keywords: monoterpene; prokaryotic microbial factory; metabolic engineering; MEP pathway; MEV pathway

1. Introduction

Terpenoids are widely distributed natural compounds that are extracted from plants, algae, mosses, and even insects and microbes. Terpenoids are composed of isoprene five-carbon units (C5) as the basic skeleton in accordance with the biogenetic isoprene rule and then further classified as monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenoids (C30), tetraterpenes (C40), and polyterpenes. These numerous compounds are normally used as medicines, insecticides, and fragrances [1]. For example, artemisinin is an endoperoxide sesquiterpene lactone isolated from *Artemisia annua Linn* and used as an anti-malarial drug [2]; paclitaxel is a cyclic diterpene hydrocarbon derived from the pacific yew and broadly applied in clinic as an anticancer drug [3,4]; squalene is the precursor of triterpenoids and is used as a pharmaceutical intermediate and bactericide [5,6]. All isoprenoids are synthesized from co-precursor isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), and this process is catalyzed by a series of corresponding isoprenoid synthases. IPP and DMAPP are subsequently transformed to geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), or geranylgeranyl pyrophosphate (GGPP), which are the precursors of monoterpenes, sesquiterpenes, and diterpenes, respectively [7]. Two major pathways are involved in the natural synthesis of isoprenoid precursors IPP and DMAPP: the methylerythritol-4-phosphate-dependent pathway (MEP pathway), also termed as deoxyxylulose phosphate pathway (DXP pathway) and the mevalonate-dependent isoprenoid pathway (MEV pathway) [8,9]. Almost all eukaryotes and archaea use the MEV pathway,

whereas most prokaryotes take advantage of the MEP pathway. Plants can utilize both biosynthetic pathways [10–12] (Figure 1).

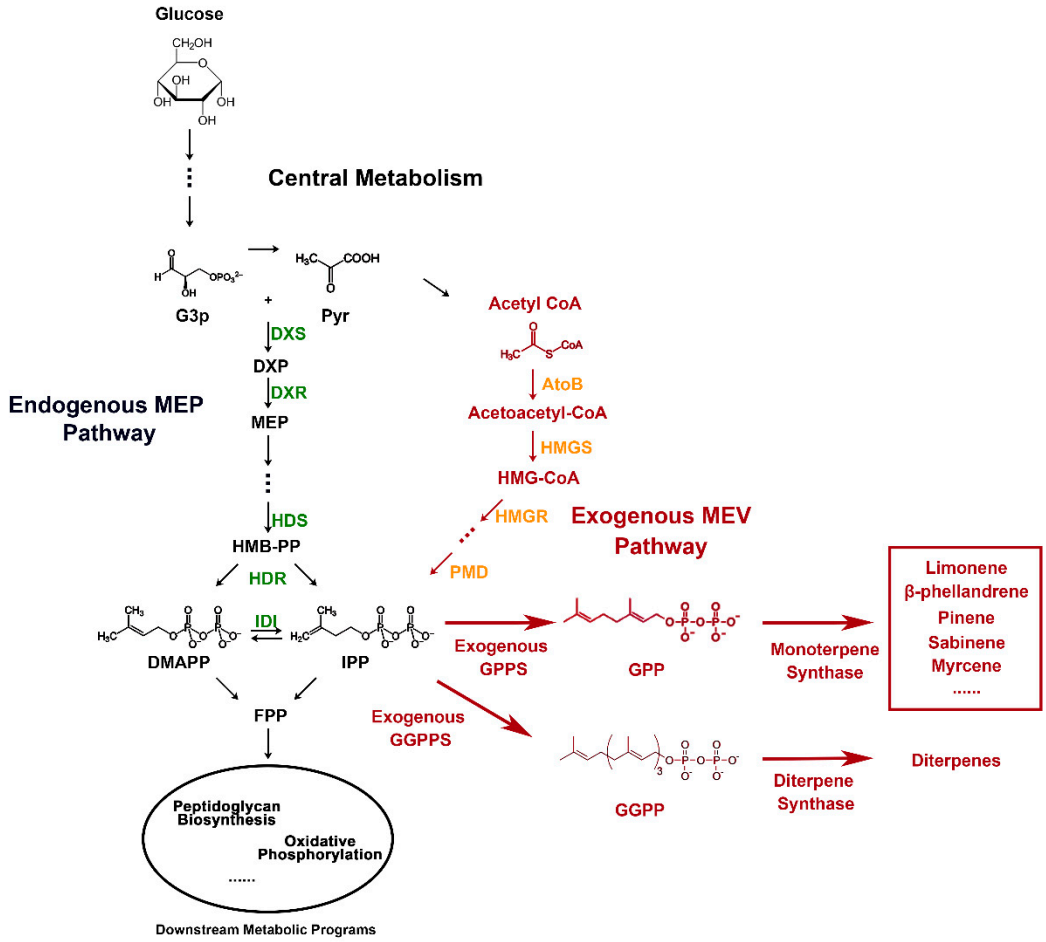

Figure 1. Monoterpene synthesis pathway in engineered prokaryotic host. GA3P, D-glyceraldehyde 3-phosphate; CoA, coenzyme A; MEP, methylerythritol 4-phosphate; DXS, 1-deoxyD-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5phosphate reductoisomerase; AtoB, acetoacetyl-CoA synthase; HMGS, 3-hydroxy3-methylglutaryl-CoA synthase; HMGR, 3-hydroxy-3methylglutaryl-CoA reductase; IDI, isopentenyl diphosphate isomerase; DMAPP, dimethylallyl diphosphate; IPP, isopentenyl diphosphate.

Monoterpenes, the members with the smallest molecular weight in terpenoids, are gaining significant attention because of their various applications in medicines, biofuels, and agriculture in addition to the traditional use of essential oils and flavor production. Monoterpenes have been gradually recognized as essential medicines and prophylactic formulations because of their characteristics that can be easily absorbed by the body and transferred into blood and their ability to treat severe chronic diseases, including cancer [13]. Limonene, which is used to produce fragrance, flavor, and medicinal products, is a compound generally recognized as safe because of its earth-friendly cleaning performance [14,15]. Geraniol is often used in the production of perfumes and cosmetics, and can also be used as a clinical anticancer drug against pancreatic, colon, and other cancers [16–18]. Meanwhile, some monoterpenes and their derivatives, such as α-pinene, camphene, and limonene, have high calorific value of combustion and low freezing point; these advantages make them a favorable choice for next-generation clean jet-biofuels to replace the traditional jet fuels such as JP-10 and RJ-5 [19,20]. Moreover, some monoterpenes, such as carvacrol, p-cymene, and gamma-terpinene, are toxic to microbes and insects, and are thus often used as antibiotics and insecticides [21,22].

In the past, monoterpenes were mainly obtained from plant biomass, but this traditional production method restricts their wide applications because of its low yields, high costs, long reaction cycles and difficult purification [23]. Similar problems also exist in the subsequently developed chemical synthesis strategy with the complex reaction process and the environment pollution risks, even though the productivity could be increased. Moreover, such modes of production could hardly synthesize compounds with complex molecular structure and specific affinity and specificity [24]. To address these problems and achieve broad commercial applications, scholars have developed a series of biological manufacturing methods with the advantages of self-assembly, proliferation, mild reaction condition requirements, and environment-friendly features to produce valuable monoterpenes, especially in this rapid development era of synthetic biology and bioengineering [25,26]. Several microorganisms, such as *Escherichia coli* and *Saccharomyces cerevisiae*, are considered as perfect chassis and have been designed as microbial cell factories for the industrialized production of significant monoterpenoids [27,28]. Both MEV and MEP pathways and their downstream enzyme systems are available to be incorporated and engineered in these chassis with different strategies to produce various valuable monoprenoids with high yield. This review summarizes the advances of different strategies for the establishment and optimization of heterologous monoterpene synthesis in the prokaryotic cell factory (Table 1).

Table 1. Strategies used for monoterpene production.

Pathway	Strains of the *E. coli* Chassis	Origin of the Integrated Enzymes for the Monoterpene Production	Engineering Design	Monoterpene Product	Maximal Monoterpene Yield, Culture and Recovery Methods	Reference
MEP	BLR (DE3) Codon Plus-RIL cells	1. tGPPS from *Abies grandis* 2. tLS from *Mentha spicata*	1. Absence of enhanced MEP or MVA pathway 2. Adjusting promoter strength	Limonene	~5 mg/L, Steam distillation;	[29]
	BL21 (DE3)	1. tGPPS from *Abies grandis* 2. tLS from *Mentha spicata* 3. DXS and IDI from *E. coli* K12 MG1655	1. Codon optimization 2. Plasmid vector and enzyme arrangement selection 3. Integration of *gpps* and *ls* in one plasmid; integration of *dxs* and *idi* in another plasmid	Limonene	35.8 mg/L, two-phase culture of *n*-hexadecane organic layer	[30]
MEV	DH1 ΔacrAB	1. AACT and IDI from *E. coli* 2. HMGS and tHMGR from *Staphylococcus Aureus* 3. MVK, PMK, and PMD from *Saccharomyces cerevisiae* 4. tGPPS from *Abies grandis* 5. tLS from *Mentha spicata* 6. efflux pump from *Alcanivorax borkumensis*	1. Codon optimization 2. Exogenous pathway introduction 3. Replication origin and promoter strength selection 4. Integration of seven MEV pathway genes in one plasmid; integration of *gpps* and *ls* in another plasmid; efflux pump genes integrated in the last plasmid alone.	Limonene	~60 mg/L, two-phase culture of dodecane organic layer	[31]
	DH1	1. AtoB and IDI from *E. coli* 2. HMGS and tHMGR from *Staphylococcus Aureus* 3. MK, PMK, and PMD from *Saccharomyces cerevisiae* 4. tGPPS from *Abies grandis* 5. tLS from *Mentha spicata* 6. Cytochrome P450 from *Mycobacterium*	1. Codon optimization 2. Exogenous pathway introduction 3. Stronger promoter replacement 4. Integration of seven MEV pathway genes in one plasmid; integration of *gpps* and *ls* in one plasmid; integration of limonene-producing genes in one plasmid; integration of P450 system genes in one plasmid	Limonene, Perillyl alcohol	~435 mg/L, two-phase culture of dodecane organic layer; ~34 mg/L, *in situ* product recovery strategy based on Amberlite IRA 410 Cl (A)	[32]
	Rosetta	1. AtoB from *E. coli* 2.HMGS and HMGR from *Enterococcus faecalis* 3. FNI, MK, PMK and PMD from *Streptococcus pneumoniae R6* 4. tGPPS from *Picea abies* 5. PHLS from *Lavandula angustifolia*	1. Codon optimization 2. Exogenous pathway introduction 3. Integration of seven MEV pathway genes in one plasmid; integration of *gpps* and *phls* in one plasmid	β-phellandrene	25 mg/g_{dcw}, two-phase culture of hexane organic layer	[33]

Table 1. *Cont.*

Pathway	Strains of the *E. coli* Chassis	Origin of the Integrated Enzymes for the Monoterpene Production	Engineering Design	Monoterpene Product	Maximal Monoterpene Yield, Culture and Recovery Methods	Reference
	MG1655	1. AtoB and IDI from *E. coli* 2. HMGS and tHMGR from *Saccharomyces cerevisiae* 3. MK, PMK, and PMD from *Saccharomyces cerevisiae* 4. tGPPS and tPS from *Abies grandis*	1. Codon optimization 2. Exogenous pathway introduction 3. Fusion protein 4. Integration of seven MEV pathway genes in one plasmid; integration of *gpps* and *ps* in another plasmid	Pinene	32.4 mg/L, two-phase culture of dodecane organic layer	[34]
	BL21 (DE3)	1. AtoB and IDI from *E. coli* 2. HMGS and tHMGR from *Saccharomyces cerevisiae* 3. MK, PMK, and PMD from *Saccharomyces cerevisiae* 4. tGPPS from *Abies grandis* 5. tLS from *Mentha spicata*	1. Codon optimization 2. Exogenous pathway introduction 3. Integration of seven MEV pathway genes in one plasmid; integration of *gpps* and *ls* in another plasmid	Limonene	2.7 g/L, two-phase culture of diisonoylphtalate organic layer	[35]
	BL21(DE3)	1. MvaE and MvaS from *Enterococcus faecalis* 2. MK, PMK, PMD and IDI from *Saccharomyces cerevisiae* 3. GPPS from *Abies grandis* 4. SabS from *Salvia pomifera*	1. Codon optimization 2. Exogenous pathway introduction 3. Integration of three upper MEV pathway genes, *gpps* and *sabs* in one plasmid; integration of four lower MEV pathway genes in another plasmid	Sabinene	2.65 g/L	[36]
	MG1655	1. MvaE and MvaS from *Enterococcus faecalis* 2. MvaK1, MvaK2, MvaD from *Streptococcus pneumoniae* 3. IDI from *E. coli* 4. GPPS from site-directed mutation of FPPS 5. tGES from *Ocimum basilicum*	1. Codon optimization 2. Exogenous pathway introduction 3. Deletion of *E. coli* gene *yjgB* 4. Integration of seven MEV pathway genes in one plasmid; integration of *gpps* and *ges* in one plasmid	Geraniol	182.5 mg/L; two-phase culture of decane organic layer	[37]
	DH1	1. AtoB and IDI from *E. coli* 2. HMGS and tHMGR from *Saccharomyces cerevisiae* 3. MK, PMK, and PMD from *Saccharomyces cerevisiae* 4. tGPPS from *Abies grandis* 5. tMS from *Quercus ilex* L.	1. Codon optimization 2. Exogenous pathway introduction 3. Integration of seven MEV pathway genes and *gpps* in one plasmid; integration of *ms* alone in one plasmid	Myrcene	58.19 mg/L two-phase culture of dodecane organic layer	[38]
	BL21 (DE3)	1. MvaE and MvaS from *Enterococcus faecalis* 2. MK, PMK, PMD and IDI from *Saccharomyces cerevisiae* 3. tGPPS from *Abies grandis* 4. tGES from *Ocimum basilicum*	1. Codon optimization 2. Exogenous pathway introduction 3. Identification of the role of acetylesterase for converting geranyl acetate to geraniol 4. Integration of three upper MEV pathway genes, *gpps* and *ges* in one plasmid; integration of four lower MEV pathway genes in another plasmid	Geraniol	~ 2.0 g/L; two-phase culture of isopropyl myristate organic layer	[39]
	XL1-Blue	1. AtoB and IDI from *E. coli* 2. HMGS and tHMGR from *Staphylococcus Aureus* 3. MK, PMK, and PMD from *Saccharomyces cerevisiae* 4. tGPPS from *Abies grandis* 5. PS from *Pinus taeda*	1. Codon optimization 2. Exogenous pathway introduction 3. Directed evolution of PS 4. Integration of seven MEV pathway genes and *gpps* in one plasmid; integration of mutant *ps* alone in another plasmid	Pinene	140 mg/L; two-phase culture of dodecane organic layer	[40]

Abbreviations: AtoB, acetoacetyl-CoA synthase; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, mevalonate pyrophosphate decarboxylase; MvaE, bifunctional acetoacetyl-CoA thiolase and HMG-CoA reductase; MvaS, HMG-CoA synthase; MvaK1, mevalonate kinase; MvaK2, phosphomevalonate kinase; MvaD, mevalonate diphosphate decarboxylase; IDI, isopentenyl diphosphate isomerase; GPPS, geranyl diphosphate synthase; FPPS, farnesyl diphosphate synthase; LS, limonene synthase; PS, pinene synthase; SabS, sabinene synthase; GES, geraniol synthase; MS, myrcene synthase; AES, acetylesterase.

2. Engineering Endogenous MEP Pathway in Prokaryotic Chassis

As a pivotal microbial chassis for bioengineering, prokaryotic *E. coli* mainly use MEP pathway for isoprenoid biosynthesis, which usually unable to produce sufficient quantities of monoterpenes for industrial production. Although several groups indicated that the engineered MEP pathway could increase the level of isoprenoid production in *E. coli*, the common precursors IPP and DMAPP are

primarily synthesized to FPP and higher polyprenyl diphosphates rather than to the intermediate of GPP and its downstream monoterpenoids [41,42]. Thus, introducing exogenous catalytic enzyme genes that encode GPP synthase (GPPS) and other monoterpene synthases to improve GPP production could be an appropriate solution. Carter et al. tested the function of a short exogenous metabolic pathway for the biosynthesis of the simple monoterpene carvone [29]. They incorporated four enzymes, namely, GPPS, limonene synthase (LS), cytochrome P450 limonene hydroxylase (L6H), and carveol dehydrogenase (CdH), into *E. coli*, which could theoretically catalyze IPP and DMAPP to carvone. However, the production of the intermediate limonene increased to nearly 5 mg/L, whereas the target product of carvone could hardly be detected. Intriguingly, feeding with exogenous limonene could push forward the carbon flux to the synthesis of carvone probably through increasing the supply of substrates. This strategy could improve the production of carvone into 0.25 mg/L. These results suggest that the endogenous supplies of the crucial precursors of IPP and DMAPP are at a relative low flux to the monoterpene synthesis. The requirement of the industrial production of monoterpenes may hardly be satisfied by simply incorporating several downstream enzymes to the MEP pathway.

Since the efficiency of the endogenous MEP pathway became a limiting factor, several groups attempted to engineer the MEP pathway to acquire higher titers of monoterpene. Enzymes 1-deoxy-d-xylulose-5-phosphate synthase (DXS), DXP reductoisomerase (DXR), and isopentenyl diphosphate isomerase (IDI) were demonstrated as the rate-limiting factors in the MEP pathway [43,44]. DXS catalyzes the formation of DXP, DXR reduces DXP to 2-C-methyl-D-erythritol-4-phosphate, and IDI catalyzes the conversion of the relatively unreactive IPP to the more-reactive electrophile DMAPP. The strategies of introducing exogenous rate-limiting enzymes with high expression by codon-optimization and/or increasing the expression levels of these endogenous enzymes through integrating with strong promoters could successfully control flux from the target precursors to the subsequent desired compounds. Du et al. embedded two exogenous genes encoding GPPS and LS in *E. coli* so that the production of limonene reaches 4.87 mg/L. Subsequently, they overexpressed DXS and IDI through plasmid transient transformation, which could enhance the production of limonene ultimately to 17.4 mg/L at 48 h. After a series of modifications to optimize the two-phase culture medium, the titer of limonene continuously elevated up to 35.8 mg/L, approximately 7-fold greater than the initial yield [30]. Despite the significant improvement in monoterpene biosynthesis, the wide applications of MEP pathway engineering are limited because of the presence of inherent regulation mechanisms and the unknown physiological control elements in the host cell, which cause the bottleneck of monoterpene production efficiency [45].

3. Introduction of Heterologous MEV Pathway

Bypassing the inherently metabolic synthesis pathway through replacing it with a heterologous mevalonate-dependent pathway provides a pioneering strategy for the production of valuable terpenoids [46]. For example, Martin's group introduced a heterologous MEV pathway into *E. coli* to increase amorphadiene titer greater than 100 mg/L [47]. This mechanism might be the overproduction of universal precursors IPP and DMAPP by the heterologous expression of MEV pathway enzymes with terpene synthases, which could enhance the conversion efficiency of IPP and DMAPP to relevant terpenoids. Similar strategies were inspired to increase the titers and yields of various monoterpenes. For instance, Gutierrez et al. engineered *E. coli* by introducing all seven enzyme-encoding genes involved in the MEV pathway and downstream limonene and perillyl alcohol (POH) synthesis-dependent genes encoding GPPS, LS, and cytochrome P450 [32]. Considering that the dispersion of the seven genes of the MEV pathway into multiple plasmids could aggravate the metabolic burden and hinder the target product synthesis in the host cell due to the raw materials and energy consumption [48,49], the authors integrated the seven genes into one plasmid and generated various versions of plasmid constructs for further study. The appropriately modified metabolic route increased the yield of limonene to 435 mg/L during 72 h of incubation with supplement of 1% glucose in the culture medium. Thus, the high titer of limonene could overcome its uptake and trafficking restrictions, which might promote the

conversion efficiency of limonene to the target monoterpene such as carvone. Accordingly, the strain harboring the reasonably combinatory plasmids was induced to produce its derivative POH to about 34 mg/L. For α-pinene production, Yang et al. embedded the heterologous hybrid MEV pathway and the co-expressed GPPS and α-pinene synthase (PS).

The final biosynthesis production accumulated up to 5.44 mg/L and 0.97 g/L under the culture conditions of flask and fed-batch fermentation, respectively [50]. Another intriguing work indicated that the incorporation of GPPS and β-phellandrene synthase (PHLS) in *E. coli* could not result in the measurable yield of β-phellandrene. However, after introducing the MEV pathway in collaboration with the GPPS and the PHLS, the output of β-phellandrene reached 11 mg/g_{dcw} after over 20 h of incubation. This titer could be further improved to 25 mg/g_{dcw} by optimizing LB broth with 1% glucose supplement and then extending the incubation time to over 72 h [33]. Notably, when the endogenous MEP pathway and the exogenous MEV pathway were used individually, the production of sabinene generated from integrated GPPS and sabinene synthase in the MEV pathway was 20-fold higher than that in the MEP pathway. The final production via a series optimization for culture condition and inducer concentration achieved a maximum titer of 82.18 mg/L under shake-flask culture and 2.65 g/L under fed-batch culture [36]. However, the method of heterologous MEV pathway introduction is not always available for different monoterpene biosyntheses to reach an industrial grade, which is probably due to restrictions of the rate-determining step and the imbalance of the metabolic flux. These bottlenecks may result in the accumulation of some toxic intermediates and the limitation of the downstream essential products in the intracellular space.

4. Optimization of the Expression and Function of the Rate-Limiting Enzymes

A common method to break the restrictions of the rate-determining step is to increase the expression level of rate-limiting enzymes in prokaryotic hosts. Three strategies are available: (1) insertion of stronger promoters into the operons directing the enzyme gene expression; (2) codon optimization of enzyme-coding regions; and (3) screening of enzymes from different species for a higher compatible and efficient homolog [51,52]. For the integration of MEV pathway and monoterpene synthases, a series of strong promoters, including T7, lacUV5, and trc, was utilized [32,33]. In another way, the codon optimization scheme is also essential for the heterologous metabolic system due to relatively diminished expression efficiency by biased codon usage [53]. Moreover, for the enzyme homolog screening, GPPS is considered a crucial candidate, which could be classified as homomeric and heteromeric isoforms [54,55]. The heteromeric GPPS extracted from *Mentha piperita* is composed of two subunits with different sizes, which shows no single catalytic activity. This heterodimer could transform IPP and DMAPP to C_{10} GPP and C_{20} GGPP [56]. The homomeric GPPS from *Arabidopsis thaliana* is a polyprenyl pyrophosphate synthase that synthesizes multiple terpenes ranging from C_{25} to C_{45}, while another type from conifers, such as *Picea abies* and *Abies grandis*, yields only GPP [41,57,58]. Because the GPP specific-producing manner can avoid or suppress the byproduct generation and effectively control the metabolic flux into the monoterpenes, the fully characterized GPPSs from *P. abies* and *A. grandis* were confirmed the better synthetases for the monoterpene production in host microbes, which need further codon optimization and truncation of tail sequences encoding plastid signal peptide for prokaryotic cell integration.

Another striking method for the functional improvement of the enzymes is directed evolution, which relies on random mutagenesis and high-throughput screening [59]. It confers the enzymes with an unnatural powerful catalytic efficiency and effectively overcomes the metabolic flux nodes [60,61]. Notably, the monoterpene synthase-mediated reactions are widely considered as the rate-limiting step in production. The improved activity and stability of enzymes could benefit to compete for the essential intermediate GPP from FPPS, which will lead the carbon flow to the final monoterpene. For instance, Tashiro's group isolated a PS variant that outperforms the wild-type through the directed enzyme evolution. After co-expression of this variant with IDI, GPPS, and the MEV pathway, pinene productivity could reach 140 mg/L in flask culture [40]. Technically, the visualization of substrate

consumption and the diversity of alternative variants could facilitate the establishment of the screening system for the directed evolution of enzymes [62,63]. Thus, this approach could rapidly select perfect mutants for advanced cellular performance, which is suggested to be suitable for many other enzymes in the metabolic route [59].

5. Controlling the Flux Distribution of Essential Intermediates

5.1. Fusion of Key Enzymes

Biofunctional fusion enzyme works as a multifunctional protein derived from a single nucleotide sequence that may contain different enzyme genes. This construction renders the active site of one enzyme face to another, which channels the intermediates directly though successive catalytic bioreactions. The spatial distance between enzymes lowers the substrate transmission loss and accelerates the reaction rate [64]. Meanwhile, the direction of internal enzyme components as well as the length and the amino acid composition of the linkers involved in the fusion enzymes are considered the optimizable parameters for the improvement of their catalytic efficiency [65,66]. Sarria et al. programed an engineered *E. coli* strain for pinene synthesis by introducing the exogenous MEV pathway and a series of GPPS-PS fusion enzymes with the components derived from three species. The highest-flux synthase combination elevated the concentration of pinene in host cells to about 28 mg/L, whereas the subsequent expression of assembled GPPS-PS fusion protein achieved a higher production of 32 mg/L, approximately 6-fold than that previously reported [34]. The high local accumulation and exchange of substances though the fusion enzyme system could theoretically overcome the metabolic burden of the essential intermediate leakage, such as GPP, and relieve or even eliminate its feedback inhibition effect and toxicity to the host. However, the critical drawback of this strategy is its high dependence to the accurate architecture of different enzyme components, which are restricted for expanding to higher-order enzyme fusion.

5.2. Spatial Organization of Heterologous Enzymes

A series of enzymes driving the engineered metabolic pathway could be spatially colocalized in a specific area of host cells by a programmable scaffold manner. This intriguing strategy has a significant scalability for enzyme population and can balance the overall pathway fluxes and alleviate metabolic burdens to optimize yields of target products [67]. The reason is that highly ordered enzymes reduce intermediate transmission time, protect them from diffusing or competing the bypass route, and circumvent undesirable equilibria and kinetics resulting from bulk-phase metabolite concentrations [68–70]. The synthetic scaffold strategy was initially inspired from the natural synthase complex exhibiting substrate channeling and the programmable nucleotide–protein interaction. Dueber et al. constructed synthetic protein scaffolds that spatially recruit three MEV biosynthetic enzymes to improve the mevalonate titers to 77-fold (~5 mM) with relatively low enzyme expression [71]. Another work arranged individual enzymes of the metabolic pathway at the proper stoichiometry via fusing enzymes to zinc-finger domains that specifically bind to corresponding DNA sequences. The titers of metabolites resveratrol, 1,2-propanediol, and mevalonate increased up to about 5-, 4.5-, and 2.5-fold compared with controls, respectively [72]. Our group demonstrated that fusing the acetoacetyl-CoA synthase (AtoB), 3-hydroxy3-methylglutaryl-CoA synthase (HMGS), and 3-hydroxy-3methylglutaryl-CoA reductase (HMGR) genes with rationally designed transcription activator-like effectors (TALEs) increases the mevalonate production by 3.7-fold [73]. RNA aptamers were also introduced to the scaffold systems for the designable colocalization of sequential metabolic enzymes [74,75]. The modularity and programmability of all these scaffold systems enable them to organize pathway enzymes in specific orientation and optimal stoichiometry. These assembled synthetic complexes could be conductive to the formation of a concentrated metabolic pool and benefit the manufacture of various multi-enzyme metabolic pathways.

5.3. Decrease the Flux of Essential Intermediates into Irrelevant Endogenous Pathways

In metabolic engineering, the irrelevant endogenous pathways utilizing the building blocks of introduced heterologous pathway in host could be the essential factor hindering the target monoterpene production. The loss could be avoided through blocking or rerouting irrelevant flux [76]. Zhou et al. introduced recombinant GPPS and the bottom portion of the MEV pathway into *E. coli* to yield geraniol up to 13.3 mg/L. With the combination of geraniol synthase (GES) heterologous expression, the geraniol production reached 105.2 mg/L. However, endogenous dehydrogenization and isomerization of geraniol into other geranoids restrained the production. After engineering the strain via deleting the microbial gene that is highly homologous to plant geraniol dehydrogenase, the conversion of geraniol significantly reduced and its productivity reached 129.7 mg/L. This titer could be further increased to 182.5 mg/L by the whole MEV pathway integration [37]. Liu's group explored an inverse conversion approach to acquire an approximate industrial productivity of geraniol. They identified that the acetylesterase (AES) from *E. coli* could transform geranyl acetate into geraniol; simultaneously, *E. coli* could reuse acetate as carbon source in the absence of glucose. By stopping glucose supply for the engineered strain after 48 h incubation, the geraniol production under fed-batch fermentation increased up to 2.0 g/L [39]. Thus, accompanied by the engineering of the MEV pathway, identification and deletion of the endogenous metabolic routes interfering the target product synthesis pathway will be a broadly applicable approach for controlling the metabolic flux and improving the production of various terpenoids.

6. Improvement of the Toxicity Tolerance for the Host Strain

Almost all monoterpenes exert critical toxicity to the host cell. Thus, continuously elevated production of monoterpenes may exacerbate the exhaustion of the cell factory. Their toxicity could inhibit the growth of producing hosts, lower the biotransformation activity of enzymes, and impede the availability of the metabolic route. In the end, the total production of monoterpenes by microbial strains is eventually reduced [21,77,78]. Commonly used approaches to improve toxicity tolerance include modification of membrane proteins, expression of efflux pumps, and activation of the stress response system, such as expression and regulation of heat shock proteins [79–81]. Dunlop et al. employed a cellular export system to alleviate biofuel toxicity and enhance the host's tolerance. They screened bacterial genomes and read out all the efflux pumps, a class of membrane transport proteins driven by the proton motive force [82–84]. Afterward, 43 of the pumps were selected and cloned into a plasmid library, which were subsequently transformed into *E. coli* for heterologous expression. This export mechanism shows significant protective effects of host survival for five chosen biofuels, including geranyl acetate, geraniol, α-pinene, limonene, and farnesyl hexanoate [31]. The elevated tolerance to the product toxicity and the significant improvement of product yields might be due to the efficient transportation of toxins out of the host and the maintenance of biomass accumulation by the efflux pumps. However, the combination of more than one type of efflux pumps could inhibit cell growth, implying that antagonistic mechanism is involved in the multi efflux pump effects [85]. Nevertheless, this approach has a profound effect on the toxicity tolerance of the prokaryotic hosts and has a great potential to be applied in monoterpene production. Alternatively, another intelligent strategy called in situ product recovery (ISPR) based on anion exchange resin was developed [86,87]. This method uses a column containing a fluidized bed of resin combined with a bioreactor to specifically trap toxic products and remove them from the culture media. This manner could relieve the effect of toxic inhibition on bioconversion and finally improve the production of the target monoterpene. Alonso-Gutierrez et al. tested four commercially available resins and then found the Amberlite IRA 410 Cl, a most suitable resin for POH recovery. Amberlite resin traps the POH, which can maintain a low final product concentration in the media and maintain a high rate of metabolic flux toward it. Their results showed 1.5-fold higher POH production than that in the resin-absent group. Moreover, combination of this method with overexpression of the cytochrome P450 system could increase the ultimate concentration of POH with 2.5-fold in total and 3.5-fold in specific production [32].

Meanwhile, a method using an aqueous-organic two-phase system for host cell culture produces similar protective effects. Rational utilization of in situ separation and extraction for two-phase culture medium not only alleviates the product toxicity to cells but also prevents the target monoterpene from volatilizing [88,89]. By taking advantage of the in situ two-phase extraction, the bacteria harboring the heterologous MEV pathway, GPPS and MS could produce myrcene to a maximum of 58.19 ± 12.13 mg/L [38]. However, these progresses for toxic tolerance improvement are mainly focused on the effective transfer of products. Other ways of optimizing the metabolic systems or engineering the host cells in a more robust condition should also be considered in future studies.

7. Conclusions

The metabolic engineering of monoterpene production has already achieved substantial progress in recent years. In early studies, the native MEP pathway as a primary regulatory objective acquired considerable attention. Most studies focused on the overexpression of key enzymes in this pathway, which limit productivity. Unfortunately, the implementations of such strategy could not achieve the expected effect for the industrial production of monoterpenes possibly because of the intrinsic barrier of the MEP pathway for supplying IPP and DMAPP, two essential universal monoterpene-building components. The introduction of a stronger heterologous MEV pathway partially compensated for this shortage. This strategy was subsequently designed to combine with a series of optimization approaches, such as functional improvement of the rate-limiting enzymes, control of the metabolic flux, and increase of the host's tolerance to the product toxicity as discussed above, for further productivity improvement. In general, most monoterpene biosyntheses remain far from the industrialization by now. To address this problem, systematic analysis of the complicated metabolic system should be considered in future studies for monoterpene manufacturing. One notable study designed a computational tool named principal component analysis of proteomics (PCAP), for the multi-dimensional engineering of global metabolic pathways. This rational mathematical tool interrogated the data of the targeted proteomics and products based on principal component analysis, which could help researchers to modify the expression of some specific enzymes, balance the metabolic pathways, and predict product yields. Thus, the overall strategies for engineering the higher-efficient monoterpene-producing chassis could be rationally designed [90]. Future studies should also consider the systematical integration of multiple approaches demonstrated to be effective in the prokaryotic chassis. An intriguing research took advantage of a multi-level analytical method to define and assess four engineering strategies for fermentative limonene production in *E. coli*: (1) Construction of a metabolic route by transformation of recombinant plasmids harboring heterologous MEV pathway enzymes and the subsequent synthases into the host strains; (2) Determination of the enzyme activity for choosing an appropriate GPPS-exploited cell-free system with the functional proteins extracted from the induced host and then adding extra substrates to the system; (3) Selection of the most suitable host by analyzing the physiological properties of different *E. coli* strains after introduction of the MEV pathway and the downstream steps; (4) Utilization of two liquid-phase carbon source fed-batch fermentation with glucose and glycerol, as well as adding a non-toxic organic solvent for the in situ extraction of monoterpenes [35]. This attempt of the integration scheme from genetic modification to process optimization surprisingly increased target productivity. Further studies are necessary to find other complementary advantages of various metabolic engineering approaches and systematically elongate the pipelines of engineering for monoterpene biomanufacturing.

Other interesting opportunities for future studies of monoterpene biomanufacturing will rely on the rapid development of the synthetic biology, which could interrogate the organizational principles of metabolic systems and easily rewire the prokaryotic cells into highly efficient cell factories. The growing strategies generated from this field, such as artificial life creation, gene circuit reconstruction, and metabolism redirection, would open new opportunities not only for the synthesis of monoterpenes but also for many other valuable important chemicals. Their applications in the construction and optimization of microbial cell factories will inevitably shorten the distance from

laboratory trials into industrial production. Along with the continuous breakthrough of the bottlenecks and restrictive factors, we can expect monoterpenes to play valuable roles in health and environment applications over the coming years.

Author Contributions: S.-s.X. and L.-y.Z. wrote the manuscript, X.-y.Q. and L.-y.Z. design and visualize the figure, L.Z. and C.-s.Z. provided important advises.

Funding: This review was funded by the National Natural Science Foundation of China (grant numbers 31500686, 31870855), the Hunan Provincial Natural Science Foundation of China (grant number 2017JJ3358), and the National University of Defense Technology project (grant numbers ZK17-03-58).

Acknowledgments: The authors thank Shine Write for its linguistic assistance during the preparation of this manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gershenzon, J.; Dudareva, N. The function of terpene natural products in the natural world. *Nat. Chem. Biol.* **2007**, *3*, 408–414. [CrossRef]
2. Fidock, D.A.; Rosenthal, P.J.; Croft, S.L.; Brun, R.; Nwaka, S. Antimalarial drug discovery: Efficacy models for compound screening. *Nat. Rev. Drug Discov.* **2004**, *3*, 509–520. [CrossRef] [PubMed]
3. Jennewein, S.; Croteau, R. Taxol: Biosynthesis, molecular genetics, and biotechnological applications. *Appl. Microbiol. Biotechnol.* **2001**, *57*, 13–19. [PubMed]
4. Croteau, R.; Ketchum, R.E.; Long, R.M.; Kaspera, R.; Wildung, M.R. Taxol biosynthesis and molecular genetics. *Phytochem. Rev.* **2006**, *5*, 75–97. [CrossRef]
5. Spanggord, R.J.; Wu, B.; Sun, M.; Lim, P.; Ellis, W.Y. Development and application of an analytical method for the determination of squalene in formulations of anthrax vaccine adsorbed. *J. Pharm. Biomed. Anal.* **2002**, *29*, 183–193. [CrossRef]
6. Arias, J.L.; Reddy, L.H.; Othman, M.; Gillet, B.; Desmaele, D.; Zouhiri, F.; Dosio, F.; Gref, R.; Couvreur, P. Squalene based nanocomposites: A new platform for the design of multifunctional pharmaceutical theragnostics. *ACS Nano* **2011**, *5*, 1513–1521. [CrossRef] [PubMed]
7. Tholl, D. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Curr. Opin. Plant Biol.* **2006**, *9*, 297–304. [CrossRef]
8. Lange, B.M.; Rujan, T.; Martin, W.; Croteau, R. Isoprenoid biosynthesis: The evolution of two ancient and distinct pathways across genomes. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 13172–13177. [CrossRef]
9. Kuzuyama, T. Mevalonate and nonmevalonate pathways for the biosynthesis of isoprene units. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 1619–1627. [CrossRef]
10. Rohmer, M. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. *Nat. Prod. Rep.* **1999**, *16*, 565–574. [CrossRef] [PubMed]
11. Smit, A.; Mushegian, A. Biosynthesis of isoprenoids via mevalonate in Archaea: The lost pathway. *Genome Res.* **2000**, *10*, 1468–1484. [CrossRef]
12. Vranová, E.; Coman, D.; Gruissem, W. Network analysis of the MVA and MEP pathways for isoprenoid synthesis. *Annu. Rev. Plant Biol.* **2013**, *64*, 665–700. [CrossRef] [PubMed]
13. Murthy, K.N.C.; Jayaprakasha, G.K.; Mantur, S.M.; Patil, B.S. Citrus Monoterpenes: Potential Source of Phytochemicals for Cancer Prevention. In *Emerging Trends in Dietary Components for Preventing and Combating Disease*; American Chemical Society: Washington, DC, USA, 2012; pp. 545–558.
14. Sun, J. D-Limonene: Safety and clinical applications. *Altern. Med. Rev.* **2007**, *12*, 259–264.
15. Ciriminna, R.; Lomeli-Rodriguez, M.; Demma Cara, P.; Lopez-Sanchez, J.A.; Pagliaro, M. Limonene: A versatile chemical of the bioeconomy. *Chem. Commun.* **2014**, *50*, 15288–15296. [CrossRef]
16. Chen, W.; Viljoen, A. Geraniol—A review of a commercially important fragrance material. *S. Afr. J. Bot.* **2010**, *76*, 643–651. [CrossRef]
17. Kim, S.H.; Bae, H.C.; Park, E.J.; Lee, C.R.; Kim, B.J.; Lee, S.; Park, H.H.; Kim, S.J.; So, I.; Kim, T.W.; et al. Geraniol inhibits prostate cancer growth by targeting cell cycle and apoptosis pathways. *Biochem. Biophys. Res. Commun.* **2011**, *407*, 129–134. [CrossRef]

18. Sun, H.; Wang, Z.; Yakisich, J.S. Natural products targeting autophagy via the PI3K/Akt/mTOR pathway as anticancer agents. *Anticancer Agents Med. Chem.* **2013**, *13*, 1048–1056. [CrossRef]
19. George, K.W.; Alonso-Gutierrez, J.; Keasling, J.D.; Lee, T.S. Isoprenoid Drugs, Biofuels, and Chemicals-Artemisinin, Farnesene, and Beyond. *Adv. Biochem. Eng. Biotechnol.* **2015**, *148*, 355–389. [PubMed]
20. Gupta, P.; Phulara, S.C. Metabolic engineering for isoprenoid-based biofuel production. *J. Appl. Microbiol.* **2015**, *119*, 605–619. [CrossRef]
21. Trombetta, D.; Castelli, F.; Sarpietro, M.G.; Venuti, V.; Cristani, M.; Daniele, C.; Saija, A.; Mazzanti, G.; Bisignano, G. Mechanisms of antibacterial action of three monoterpenes. *Antimicrob. Agents Chemother.* **2005**, *49*, 2474–2478. [CrossRef] [PubMed]
22. Cristani, M.; D'Arrigo, M.; Mandalari, G.; Castelli, F.; Sarpietro, M.G.; Micieli, D.; Venuti, V.; Bisignano, G.; Saija, A.; Trombetta, D. Interaction of four monoterpenes contained in essential oils with model membranes: Implications for their antibacterial activity. *J. Agric. Food Chem.* **2007**, *55*, 6300–6308. [CrossRef] [PubMed]
23. Geron, C.; Rasmussen, R.; Arnts, R.R.; Guenther, A. A review and synthesis of monoterpene speciation from forests in the United States. *Atmos. Environ.* **2000**, *34*, 1761–1781. [CrossRef]
24. Koeller, K.M.; Wong, C.H. Enzymes for chemical synthesis. *Nature* **2001**, *409*, 232–240. [CrossRef]
25. Brenner, K.; You, L.; Arnold, F.H. Engineering microbial consortia: A new frontier in synthetic biology. *Trends Biotechnol.* **2008**, *26*, 483–489. [CrossRef] [PubMed]
26. Keasling, J.D. Synthetic biology for synthetic chemistry. *ACS Chem. Biol.* **2008**, *3*, 64–76. [CrossRef]
27. Keasling, J.D. Synthetic biology and the development of tools for metabolic engineering. *Metab. Eng.* **2012**, *14*, 189–195. [CrossRef]
28. Kim, J.; Salvador, M.; Saunders, E.; Gonzalez, J.; Avignone-Rossa, C.; Jimenez, J.I. Properties of alternative microbial hosts used in synthetic biology: Towards the design of a modular chassis. *Essays Biochem.* **2016**, *60*, 303–313. [CrossRef] [PubMed]
29. Carter, O.A.; Peters, R.J.; Croteau, R. Monoterpene biosynthesis pathway construction in Escherichia coli. *Phytochemistry* **2003**, *64*, 425–433. [CrossRef]
30. Du, F.-L.; Yu, H.-L.; Xu, J.-H.; Li, C.-X. Enhanced limonene production by optimizing the expression of limonene biosynthesis and MEP pathway genes in E. coli. *Bioresour. Bioprocess.* **2014**, *1*, 10. [CrossRef]
31. Dunlop, M.J.; Dossani, Z.Y.; Szmidt, H.L.; Chu, H.C.; Lee, T.S.; Keasling, J.D.; Hadi, M.Z.; Mukhopadhyay, A. Engineering microbial biofuel tolerance and export using efflux pumps. *Mol. Syst. Biol.* **2011**, *7*, 487. [CrossRef]
32. Alonso-Gutierrez, J.; Chan, R.; Batth, T.S.; Adams, P.D.; Keasling, J.D.; Petzold, C.J.; Lee, T.S. Metabolic engineering of Escherichia coli for limonene and perillyl alcohol production. *Metab. Eng.* **2013**, *19*, 33–41. [CrossRef] [PubMed]
33. Formighieri, C.; Melis, A. Carbon partitioning to the terpenoid biosynthetic pathway enables heterologous beta-phellandrene production in Escherichia coli cultures. *Arch. Microbiol.* **2014**, *196*, 853–861. [CrossRef] [PubMed]
34. Sarria, S.; Wong, B.; Garcia Martin, H.; Keasling, J.D.; Peralta-Yahya, P. Microbial synthesis of pinene. *ACS Synth. Biol.* **2014**, *3*, 466–475. [CrossRef]
35. Willrodt, C.; David, C.; Cornelissen, S.; Buhler, B.; Julsing, M.K.; Schmid, A. Engineering the productivity of recombinant Escherichia coli for limonene formation from glycerol in minimal media. *Biotechnol. J.* **2014**, *9*, 1000–1012. [CrossRef] [PubMed]
36. Zhang, H.; Liu, Q.; Cao, Y.; Feng, X.; Zheng, Y.; Zou, H.; Liu, H.; Yang, J.; Xian, M. Microbial production of sabinene—A new terpene-based precursor of advanced biofuel. *Microb. Cell Fact.* **2014**, *13*, 20. [CrossRef]
37. Zhou, J.; Wang, C.; Yoon, S.H.; Jang, H.J.; Choi, E.S.; Kim, S.W. Engineering Escherichia coli for selective geraniol production with minimized endogenous dehydrogenation. *J. Biotechnol.* **2014**, *169*, 42–50. [CrossRef] [PubMed]
38. Kim, E.M.; Eom, J.H.; Um, Y.; Kim, Y.; Woo, H.M. Microbial Synthesis of Myrcene by Metabolically Engineered Escherichia coli. *J. Agric. Food Chem.* **2015**, *63*, 4606–4612. [CrossRef]
39. Liu, W.; Xu, X.; Zhang, R.; Cheng, T.; Cao, Y.; Li, X.; Guo, J.; Liu, H.; Xian, M. Engineering Escherichia coli for high-yield geraniol production with biotransformation of geranyl acetate to geraniol under fed-batch culture. *Biotechnol. Biofuels* **2016**, *9*, 58. [CrossRef]

40. Tashiro, M.; Kiyota, H.; Kawai-Noma, S.; Saito, K.; Ikeuchi, M.; Iijima, Y.; Umeno, D. Bacterial Production of Pinene by a Laboratory-Evolved Pinene-Synthase. *ACS Synth. Biol.* **2016**, *5*, 1011–1020. [CrossRef] [PubMed]
41. Burke, C.; Croteau, R. Geranyl diphosphate synthase from Abies grandis: cDNA isolation, functional expression, and characterization. *Arch. Biochem. Biophys.* **2002**, *405*, 130–136. [CrossRef]
42. Burke, C.; Croteau, R. Interaction with the small subunit of geranyl diphosphate synthase modifies the chain length specificity of geranylgeranyl diphosphate synthase to produce geranyl diphosphate. *J. Biol. Chem.* **2002**, *277*, 3141–3149. [CrossRef]
43. Eisenreich, W.; Rohdich, F.; Bacher, A. Deoxyxylulose phosphate pathway to terpenoids. *Trends Plant Sci.* **2001**, *6*, 78–84. [CrossRef]
44. Eisenreich, W.; Bacher, A.; Arigoni, D.; Rohdich, F. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell. Mol. Life Sci.* **2004**, *61*, 1401–1426. [CrossRef]
45. Banerjee, A.; Wu, Y.; Banerjee, R.; Li, Y.; Yan, H.; Sharkey, T.D. Feedback inhibition of deoxy-D-xylulose-5-phosphate synthase regulates the methylerythritol 4-phosphate pathway. *J. Biol. Chem.* **2013**, *288*, 16926–16936. [CrossRef] [PubMed]
46. Pitera, D.J.; Paddon, C.J.; Newman, J.D.; Keasling, J.D. Balancing a heterologous mevalonate pathway for improved isoprenoid production in Escherichia coli. *Metab. Eng.* **2007**, *9*, 193–207. [CrossRef] [PubMed]
47. Martin, V.J.; Pitera, D.J.; Withers, S.T.; Newman, J.D.; Keasling, J.D. Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. *Nat. Biotechnol.* **2003**, *21*, 796–802. [CrossRef]
48. Bhattacharya, S.K.; Dubey, A.K. Metabolic burden as reflected by maintenance coefficient of recombinant Escherichia coli overexpressing target gene. *Biotechnol. Lett.* **1995**, *17*, 1155–1160. [CrossRef]
49. Rozkov, A.; Avignone-Rossa, C.A.; Ertl, P.F.; Jones, P.; O'Kennedy, R.D.; Smith, J.J.; Dale, J.W.; Bushell, M.E. Characterization of the metabolic burden on Escherichia coli DH1 cells imposed by the presence of a plasmid containing a gene therapy sequence. *Biotechnol. Bioeng.* **2004**, *88*, 909–915. [CrossRef]
50. Yang, J.; Nie, Q.; Ren, M.; Feng, H.; Jiang, X.; Zheng, Y.; Liu, M.; Zhang, H.; Xian, M. Metabolic engineering of Escherichia coli for the biosynthesis of alpha-pinene. *Biotechnol. Biofuels* **2013**, *6*, 60. [CrossRef]
51. Fell, D.A. Increasing the flux in metabolic pathways: A metabolic control analysis perspective. *Biotechnol. Bioeng.* **1998**, *58*, 121–124. [CrossRef]
52. Kholodenko, B.N.; Lyubarev, A.E.; Kurganov, B.I. Control of the metabolic flux in a system with high enzyme concentrations and moiety-conserved cycles. The sum of the flux control coefficients can drop significantly below unity. *Eur. J. Biochem.* **2010**, *210*, 147–153. [CrossRef]
53. Gustafsson, C.; Govindarajan, S.; Minshull, J. Codon bias and heterologous protein expression. *Trends Biotechnol.* **2004**, *22*, 346–353. [CrossRef]
54. Burke, C.C.; Wildung, M.R.; Croteau, R. Geranyl diphosphate synthase: Cloning, expression, and characterization of this prenyltransferase as a heterodimer. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 13062–13067. [CrossRef]
55. Schmidt, A.; Gershenzon, J. Cloning and characterization of two different types of geranyl diphosphate synthases from Norway spruce (Picea abies). *Phytochemistry* **2008**, *69*, 49–57. [CrossRef] [PubMed]
56. Chang, T.H.; Hsieh, F.L.; Ko, T.P.; Teng, K.H.; Liang, P.H.; Wang, A.H. Structure of a heterotetrameric geranyl pyrophosphate synthase from mint (Mentha piperita) reveals intersubunit regulation. *Plant Cell* **2010**, *22*, 454–467. [CrossRef]
57. Aubourg, S.; Lecharny, A.; Bohlmann, J. Genomic analysis of the terpenoid synthase (AtTPS) gene family of Arabidopsis thaliana. *Mol. Genet. Genom.* **2002**, *267*, 730–745. [CrossRef]
58. Schmidt, A.; Wächtler, B.; Temp, U.; Krekling, T.; Séguin, A.; Gershenzon, J. A bifunctional geranyl and geranylgeranyl diphosphate synthase is involved in terpene oleoresin formation in Picea abies. *Plant Physiol.* **2010**, *152*, 639–655. [CrossRef] [PubMed]
59. Furubayashi, M.; Ikezumi, M.; Kajiwara, J.; Iwasaki, M.; Fujii, A.; Li, L.; Saito, K.; Umeno, D. A high-throughput colorimetric screening assay for terpene synthase activity based on substrate consumption. *PLoS ONE* **2014**, *9*, e93317. [CrossRef]
60. Jürgens, C.; Strom, A.; Wegener, D.; Hettwer, S.; Wilmanns, M.; Sterner, R. Directed evolution of a ($\beta\alpha$) 8-barrel enzyme to catalyze related reactions in two different metabolic pathways. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9925–9930. [CrossRef] [PubMed]
61. Keasling, J.D. Manufacturing molecules through metabolic engineering. *Science* **2010**, *330*, 1355–1358. [CrossRef]

62. Lopez-Gallego, F.; Wawrzyn, G.T.; Schmidt-Dannert, C. Selectivity of fungal sesquiterpene synthases: Role of the active site's H-1 alpha loop in catalysis. *Appl. Environ. Microbiol.* **2010**, *76*, 7723–7733. [CrossRef] [PubMed]
63. Vardakou, M.; Salmon, M.; Faraldos, J.A.; O'Maille, P.E. Comparative analysis and validation of the malachite green assay for the high throughput biochemical characterization of terpene synthases. *MethodsX* **2014**, *1*, 187–196. [CrossRef] [PubMed]
64. Dale, G.E.; Oefner, C.; D'Arcy, A. The protein as a variable in protein crystallization. *J. Struct. Biol.* **2003**, *142*, 88–97. [CrossRef]
65. Chen, X.; Zaro, J.L.; Shen, W.C. Fusion protein linkers: Property, design and functionality. *Adv. Drug Deliv. Rev.* **2013**, *65*, 1357–1369. [CrossRef]
66. Reddy Chichili, V.P.; Kumar, V.; Sivaraman, J. Linkers in the structural biology of protein-protein interactions. *Protein Sci.* **2013**, *22*, 153–167. [CrossRef]
67. Lee, H.; DeLoache, W.C.; Dueber, J.E. Spatial organization of enzymes for metabolic engineering. *Metab. Eng.* **2012**, *14*, 242–251. [CrossRef] [PubMed]
68. Miles, E.W.; Rhee, S.; Davies, D.R. The molecular basis of substrate channeling. *J. Biol. Chem.* **1999**, *274*, 12193–12196. [CrossRef]
69. Zhang, Y.H. Substrate channeling and enzyme complexes for biotechnological applications. *Biotechnol. Adv.* **2011**, *29*, 715–725. [CrossRef]
70. Siu, K.H.; Chen, R.P.; Sun, Q.; Chen, L.; Tsai, S.L.; Chen, W. Synthetic scaffolds for pathway enhancement. *Curr. Opin. Biotechnol.* **2015**, *36*, 98–106. [CrossRef]
71. Dueber, J.E.; Wu, G.C.; Malmirchegini, G.R.; Moon, T.S.; Petzold, C.J.; Ullal, A.V.; Prather, K.L.; Keasling, J.D. Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* **2009**, *27*, 753–759. [CrossRef]
72. Conrado, R.J.; Wu, G.C.; Boock, J.T.; Xu, H.; Chen, S.Y.; Lebar, T.; Turnsek, J.; Tomsic, N.; Avbelj, M.; Gaber, R.; et al. DNA-guided assembly of biosynthetic pathways promotes improved catalytic efficiency. *Nucleic Acids Res.* **2012**, *40*, 1879–1889. [CrossRef]
73. Xie, S.S.; Qiu, X.Y.; Zhu, L.Y.; Zhu, C.S.; Liu, C.Y.; Wu, X.M.; Zhu, L.; Zhang, D.Y. Assembly of TALE-based DNA scaffold for the enhancement of exogenous multi-enzymatic pathway. *J. Biotechnol.* **2019**, *296*, 69–74. [CrossRef] [PubMed]
74. Delebecque, C.J.; Lindner, A.B.; Silver, P.A.; Aldaye, F.A. Organization of intracellular reactions with rationally designed RNA assemblies. *Science* **2011**, *333*, 470–474. [CrossRef] [PubMed]
75. Sachdeva, G.; Garg, A.; Godding, D.; Way, J.C.; Silver, P.A. In vivo co-localization of enzymes on RNA scaffolds increases metabolic production in a geometrically dependent manner. *Nucleic Acids Res.* **2014**, *42*, 9493–9503. [CrossRef] [PubMed]
76. Zhao, J.; Li, C.; Zhang, Y.; Shen, Y.; Hou, J.; Bao, X. Dynamic control of ERG20 expression combined with minimized endogenous downstream metabolism contributes to the improvement of geraniol production in Saccharomyces cerevisiae. *Microb. Cell Fact.* **2017**, *16*, 17. [CrossRef] [PubMed]
77. Cowan, M.M. Plant products as antimicrobial agents. *Clin. Microbiol. Rev.* **1999**, *12*, 564–582. [CrossRef] [PubMed]
78. Cox, S.D.; Mann, C.M.; Markham, J.L.; Bell, H.C.; Gustafson, J.E.; Warmington, J.R.; Wyllie, S.G. The mode of antimicrobial action of the essential oil of Melaleuca alternifolia (tea tree oil). *J. Appl. Microbiol.* **2010**, *88*, 170–175. [CrossRef]
79. Shah, A.A.; Wang, C.; Chung, Y.R.; Kim, J.Y.; Choi, E.S.; Kim, S.W. Enhancement of geraniol resistance of Escherichia coli by MarA overexpression. *J. Biosci. Bioeng.* **2013**, *115*, 253–258. [CrossRef] [PubMed]
80. Wang, J.-F.; Xiong, Z.-Q.; Li, S.-Y.; Wang, Y. Enhancing isoprenoid production through systematically assembling and modulating efflux pumps in Escherichia coli. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 8057–8067. [CrossRef]
81. Jongedijk, E.; Cankar, K.; Buchhaupt, M.; Schrader, J.; Bouwmeester, H.; Beekwilder, J. Biotechnological production of limonene in microorganisms. *Appl. Microbiol. Biotechnol.* **2016**, *100*, 2927–2938. [CrossRef]
82. Putman, M.; van Veen, H.W.; Konings, W.N. Molecular properties of bacterial multidrug transporters. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 672–693. [CrossRef]

83. Ramos, J.L.; Duque, E.; Gallegos, M.T.; Godoy, P.; Ramos-Gonzalez, M.I.; Rojas, A.; Teran, W.; Segura, A. Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.* **2002**, *56*, 743–768. [CrossRef]
84. Nikaido, H.; Takatsuka, Y. Mechanisms of RND multidrug efflux pumps. *Biochim. Biophys. Acta* **2009**, *1794*, 769–781. [CrossRef] [PubMed]
85. Turner, W.J.; Dunlop, M.J. Trade-Offs in Improving Biofuel Tolerance Using Combinations of Efflux Pumps. *ACS Synth. Biol.* **2015**, *4*, 1056–1063. [CrossRef]
86. Newman, J.D.; Marshall, J.; Chang, M.; Nowroozi, F.; Paradise, E.; Pitera, D.; Newman, K.L.; Keasling, J.D. High-level production of amorpha-4, 11-diene in a two-phase partitioning bioreactor of metabolically engineered Escherichia coli. *Biotechnol. Bioeng.* **2006**, *95*, 684–691. [CrossRef] [PubMed]
87. Dafoe, J.T.; Daugulis, A.J. In situ product removal in fermentation systems: Improved process performance and rational extractant selection. *Biotechnol. Lett.* **2014**, *36*, 443–460. [CrossRef] [PubMed]
88. Schewe, H.; Holtmann, D.; Schrader, J. P450(BM-3)-catalyzed whole-cell biotransformation of alpha-pinene with recombinant Escherichia coli in an aqueous-organic two-phase system. *Appl. Microbiol. Biotechnol.* **2009**, *83*, 849–857. [CrossRef] [PubMed]
89. Brennan, T.C.; Turner, C.D.; Krömer, J.O.; Nielsen, L.K. Alleviating monoterpene toxicity using a two-phase extractive fermentation for the bioproduction of jet fuel mixtures in Saccharomyces cerevisiae. *Biotechnol. Bioeng.* **2012**, *109*, 2513–2522. [CrossRef] [PubMed]
90. Alonso-Gutierrez, J.; Kim, E.M.; Batth, T.S.; Cho, N.; Hu, Q.; Chan, L.J.G.; Petzold, C.J.; Hillson, N.J.; Adams, P.D.; Keasling, J.D.; et al. Principal component analysis of proteomics (PCAP) as a tool to direct metabolic engineering. *Metab. Eng.* **2015**, *28*, 123–133. [CrossRef]