Chapter 9
Synthetic Biology Enables Photosynthetic Production of Limonene from CO_2 and H_2O

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What You Will You Learn from This Chapter:

- The physical and chemical properties of limonene, a C_{10} isoprenoid with applications in green solvents, pharmaceuticals, perfumes, and food flavorings
- An overview of efforts to genetically engineer cyanobacteria to synthesize limonene
- Perspectives on developing integrated systems to produce limonene at the industrial-scale

9.1 Introduction

Photosynthesis is the ultimate life-sustaining biological process on the planet and is the main driver behind the worldwide production of food, fiber, and renewable fuels. With the human population projected to increase by ~2 billion by 2050 (Cohen 2003), innovations in agriculture that will meet the demand for these resources are critically important. More solar energy reaches the Earth's surface every hour (4.3×10^{14} MJ) than is consumed annually (4.1×10^{14} MJ), making solar radiation a plentiful energy resource (Zhu et al. 2008). However, the energy losses of incident solar radiation from cell interception to the formation of chemical energy create a limit on biomass yields and productivity. This is especially true for most crop plants, which exhibit relatively low photosynthetic efficiencies (2–4%) (Zhu et al. 2008). Furthermore, the extensive energy needed for planting, pesticide and herbicide application, harvesting, and year-round land management means that modern

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agriculture is a major energy user and contributes to deforestation, soil erosion, and pollution (Foley et al. 2005; Montgomery 2007). To ensure sustainable agriculture for the future, innovations that can more efficiently fix and sequester carbon without negative effects to the environment are key areas for scientific investigation and economic investment.

Microalgae and cyanobacteria are photosynthetic microorganisms that have been researched extensively as models for carbon fixation (Parmar et al. 2011; Chisti 2007). They exhibit higher photosynthetic efficiency and biomass productivity than traditional oil-seed crops, can be grown on nonarable land, and can utilize a wide variety of water sources (e.g., wastewater). These characteristics, along with their abundant composition of lipids and sugars, give them an advantage over terrestrial crops as a carbon feedstock (Quintana et al. 2011). Over the last few decades, advancements in genomics, recombinant DNA technology, and synthetic biology have provided researchers with a sophisticated toolset for genetically engineering cyanobacteria to overproduce high-value compounds originally harvested from crops. These include naturally synthesized carbon feedstocks, such as fatty acids and triacylglycerides (TAGs), and fermentable sugars (sucrose, glycogen) that can be used for biodiesel and bioethanol, respectively (Quintana et al. 2011). Furthermore, genetic engineering efforts have created cyanobacteria able to directly synthesize and secrete tailor-made compounds from their cells, eliminating the need for harvesting, processing, or fermentation (Fig. 9.1). These new products include fuels, as well as a diverse portfolio of medicines, polymers, food flavorings, fragrances, and industrial solvents (Ducat et al. 2011).

One group of compounds that have gained recent attention as a commercial product from engineered cyanobacteria is the isoprenoids (also referred to as terpenoids), a large class of organic molecules that are naturally synthesized by plants, animals, bacteria, and archaea (Vranova et al. 2013). Their energetic composition make them ideal precursors for drop-in diesel and jet fuels (Rude and Schirmer 2009). Given their structural diversity, they also have industrial applications as solvents, nutraceuticals, natural pesticides, and drugs (Harrewijn 2001). During the last 15 years, a number of genetic engineering projects have made great strides in reprogramming different microorganisms to overproduce and secrete isoprenoids from their cells (Wang et al. 2015). In time, these achievements may become the foundation of commercial systems that can utilize CO_2, H_2O, and solar radiation to produce isoprenoids and similar molecules for human use.

Although great achievements have been made in this field, the cost of making isoprenoids from cyanobacteria and microalgae still outweigh the risk for most commercial investors. This is primarily due to low productivities from currently engineered strains, technicalities of isoprenoid collection, and high start-up costs (Parmar et al. 2011). To make large-scale isoprenoid production from single-celled photosynthetic microorganisms a reality, future research and ingenuity will be needed to make the photons-to-bioproduct strategy economically viable. Increasing our knowledge of cyanobacterial genetics, metabolism, and energy distribution and defining the regulatory networks involved in isoprenoid biosynthesis will expand our ability to predict the outcomes of genetic manipulation. This information,

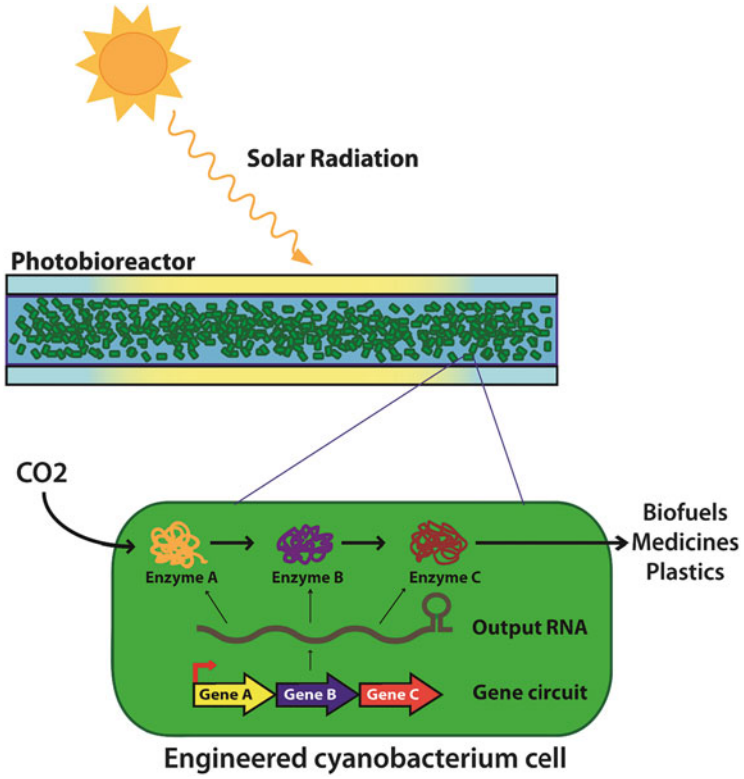

Fig. 9.1 Engineered cyanobacteria with designed gene circuits in a photobioreactor (PBR) have demonstrated advanced biofuel production technology that can utilize solar radiation to transform CO_2 directly to renewable fuels, medicines, and biomaterials

coupled with advancements in gene editing and programming, will enable us to design better engineering strategies that increase productivity in large-scale photosynthetic systems.

In this chapter, we discuss using engineered cyanobacteria for the photosynthetic production of limonene, a C_{10} cyclic isoprenoid that has applications not only as an alternative fuel but also in green solvents, pharmaceuticals, perfumes, and food flavorings.

Table 9.1 Chemical and physical properties of limonene

Parameter	Value	References
Melting point	−74.3 °C	Lide (1991)
Boiling point	175.5–176 °C	Budavari (1989)
Density	0.8411 g ml^{-1} at 20 °C	Larrañaga et al. (2016)
Solubility	Insoluble in water. Soluble in benzene, carbon tetrachloride, diethyl ether, ethanol, and petroleum ether. Slightly soluble in glycerine	Lide (1991), STN International (1992)
Stability	Oxidizes to film in air. Store in dark at −18 °C	Larrañaga et al. (2016) Ranganna et al. (1983)
Refractive index	1.4730	Lide (1991)
Optical rotation	125.6°	Lide (1991)
Flash point	50 °C	Lide (1991)
Energy density		
Volatility (vapor pressure)	133 Pa at 14 °C; 665 Pa at 40.4 °C	National Toxicology Program (1991)

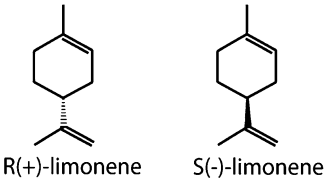

Fig. 9.2 Limonene structure, showing both *R*- and *S*- enantiomers

9.2 Limonene: A Cyclic Chemical with Important Societal Benefits

9.2.1 Physical and Chemical Properties

Limonene ($C_{10}H_{16}$, molecular weight 136.24 g/mol) is classified as a cyclic terpene, with the properties listed in Table 9.1. It is also named 1-methyl-4-(1-methylethenyl) cyclohexene under the National Institute of Standards and Technology. Limonene is a chiral molecule, occurring in two enantiomers (Fig. 9.2). The most common is *R* (+)-limonene (also called *d*-limonene), which is mainly found in citrus fruits, and gives the characteristic scent of orange or lemon. The other enantiomer is *S*(-)-limonene (also called *l*-limonene), which has an earthy, mint aroma or the scent of pine. The racemic mixture of both isoforms is also known as dipentene (Simonsen 1947). Limonene is found mainly in the oils of citrus fruit rinds but is also found in other fruits and vegetables. It has been reported that limonene is present in the essential oils of more than 300 species (Flavor and Extract Manufacturers'

Association 1991). The *d*-form comprises the majority (98–100%) of limonene in citrus oils, while the *l*-form is most common in citronella and lemongrass oils (Burdock and Fenaroli 2010; Clayton et al. 1991; Larrañaga et al. 2016).

9.2.2 Safety Issues

d-Limonene is generally recognized as safe for human consumption by the US Food and Drug Association as a flavoring substance (US Food and Drug Administration 1991). However, limonene and its oxidized products can be skin and respiratory irritants during long-term industrial exposure (IARC 1993). High doses of limonene have resulted in renal cancer in male rats (National Toxicology Program 1990), but the IARC classifies *d*-limonene as a Group 3 carcinogen: *not classifiable as to its carcinogenicity to humans* (IARC 1993). In fact, limonene is being evaluated for chemopreventative or anticancer effects (Crowell 1999; Tsuda et al. 2004).

9.2.3 Sources and Production

The first reported extraction and purification of limonene was in Florida in the early 1940s from evaporator condensate of citrus molasses (Schulz 1972). Thereafter production expanded, with the primary feedstocks being orange, grapefruit, and lemons (Verghese 1968). Several methods can be used to recover limonene. Citrus peels are mechanically processed to rupture the oil sacs and release the oil into an aqueous emulsion from which the peel oil is recovered by centrifugation (Ranganna et al. 1983). The resulting peel oil can contain up to 95% *d*-limonene. Alternatively citrus peels and pulp can be cold pressed, and the resulting press liquor is evaporated to produce citrus molasses. The condensate from this process is referred to as stripper oil, and it may contain >95% *d*-limonene (Ranganna et al. 1983). *d*-Limonene may also be recovered during other aspects of processing, including deterpenation of citrus oils (Burdock and Fenaroli 2010). Distillation may be used to further concentrate *d*-limonene, due to its relatively high thermal stability.

9.2.4 Production Levels and Use

On a global basis, ~50,000 tons of limonene are extracted annually, primarily from the residue of harvested citrus fruits (Brennan et al. 2012). Production has only grown slightly over the past 30–40 years and largely comes from the southern USA, Mexico, the Caribbean basin and Southern Hemisphere countries. Since its discovery, limonene has been used primarily as a flavor or fragrance additive in cosmetics, beverages, and foods (Duetz et al. 2003). Another major use has been as a chemical

intermediate in resin and adhesive production, as a solvent and cleaner, and in some paints and air freshener products (Schulz 1972; Burdock and Fenaroli 2010; Larrañaga et al. 2016). Due to its low toxicity to mammals and acute effects on many insects, limonene has also been used as alternative to synthetic insecticides (Karr and Coats 1988; Hooser et al. 1986; Hooser 1990). Another relatively minor volume use has been as an alternative to treat gastroesophageal reflux disease and heartburn (Sun 2007).

Future demand for green solvents is expected to rise due to consumer preference for environmentally safe products with minimal health hazards, and younger generations are willing to pay more for sustainable, eco-friendly products up from 55% in 2014 to 72% in 2015 (The Nielsen Company 2015). Green solvents are included in cleaners, paints, coatings, printing inks, and pharmaceutical and personal care products such as cosmetics and toiletries. Major companies in the green solvents space include Bio Amber Inc. (USA), Cargill Inc. (USA), Florida Chemicals Inc. (USA), Dow Chemicals (USA), DuPont (USA), and Vertec Biosolvents (USA), BASF (Germany), and CSM (the Netherlands).

9.2.5 Use as a Fuel

A new use for limonene is as a third-generation biofuel (Brennan et al. 2012), especially in jet fuel and diesel applications due to its immiscibility in water, combustibility, high energy density, and low freezing point (Hellier et al. 2013). Limonene also may be further functionalized through cyclopropanation to increase its energetic content (Langlois and Lebel 2010). The key factor limiting limonene's use in biofuels is that global limonene production is far from sufficient to meet potential demands. As a result, many groups have sought to engineer microbes to produce limonene. This has included heterotrophic bacteria to synthesize limonene from sugars (Alonso-Gutierrez et al. 2013), as well as cyanobacteria to produce limonene from CO_2 and H_2O (Halfmann et al. 2014b; Davies et al. 2014; Kiyota et al. 2014), which will be in later sections.

Limonene is viewed as an attractive target compound for large-scale production due to its potential applications in many different industrial sectors. Furthermore, its chemical similarities to diesel and jet fuels make it a prime candidate as a third-generation biofuel. Photosynthetic limonene production from engineered cyanobacteria represents a viable alternative biofuel platform and could possibly be a future innovation in agriculture. Understanding the biosynthetic pathways that produce limonene and similar compounds will be critically important in designing engineering strategies that can create biofuels and bioproducts via photosynthesis with high productivity and yield. As discussed below, limonene belongs to a large family of functional compounds that are naturally synthesized in many organisms.

9.3 Isoprenoids: Functions in Biological Systems

Limonene is a member of the isoprenoid family, a structurally diverse group of compounds that function as primary and secondary metabolites (Chappell 1995; Rohmer 1999). The roles of isoprenoids in living organisms have been researched extensively, mostly in plants, but also in animals, yeasts, bacteria, and green algae. There are many excellent reviews which highlight the diverse roles of isoprenoids in cellular function (Vranova et al. 2013; Lichtenthaler 1999). As secondary metabolites, they play a central role in membrane fluidity, respiration, photosynthesis, protein prenylation, and the regulation of growth and development through hormones synthesis. A major proportion of isoprenoids synthesized in plants are used to construct photosynthetic pigments that participate in light harvesting, photoquenching, and transferring resonance energy in photosynthetic reaction centers (PRCs). Chlorophylls, which consist of a magnesium-bound tetrapyrrole ring attached to an isoprenoid-derived phytol chain, are essential molecules in the PRC for photon absorption and energy transfer. Accessory pigments, such as carotene and xanthophylls, are also derived from isoprenoids and are important for quenching excess excitation energy and protecting the light-harvesting complexes from photodamage. Plant sterols are steroid-like molecules derived from isoprenoid precursors and, like cholesterols in vertebrates, function to regulate the rigidity and integrity of cell membranes. Monoterpenes emitted from plants have a pleasant aroma and are utilized to attract insect pollinators and seed-dispersing animals. Isoprenoids also participate in plant-pathogen interactions and can aid in protection against herbivory.

9.3.1 Metabolic Routes for Isoprenoid Synthesis

All isoprenoids are formed through the universal precursor isopentenyl pyrophosphate (IPP) and its isomer, dimethylallyl pyrophosphate (DMAPP) (Vranova et al. 2013). The 1:1 condensation of IPP (C_5) and DMAPP (C_5) catalyzed by geranyl pyrophosphate synthase (GPPS; EC 2.5.5.1) creates geranyl pyrophosphate (GPP; C_{10}), the precursor for all C_{10} isoprenoids (monoterpenes), including limonene. The condensation of IPP to GPP creates farnesyl pyrophosphate (FPP), the precursor for all sesquiterpenes (C_{15}), the major constituents of hops in beer. The addition of IPP to FPP creates geranylgeranyl pyrophosphate (GGPP; C_{20}), the precursor for photosynthetic pigments (chlorophylls and carotenoids) and quinones involved in the light-dependent reactions (plastoquinones and ubiquinones). Subsequent additions of IPP subunits to longer prenylated pyrophosphates create the precursors needed to create triterpenes (C_{30}), tetraterpenes, (C_{40}), etc. IPP and DMAPP are synthesized from two separate pathways: the mevalonate (MVA) pathway and the more recently discovered 2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway (Vranova et al. 2013; Lichtenthaler 1999). Animals, archaea,

some gram-positive cocci bacteria, and yeast contain the MVA pathway, while most gram-negative bacteria, cyanobacteria, and green algae use the MEP pathway exclusively.

While most organisms have either one or the other terpenoid biosynthetic pathway, higher plants and some marine alga (e.g., the red alga *Cyanidium caldarium*) contain both the MVA and MEP pathway. Genomic analyses have revealed higher plants compartmentalize these pathways, with the MVA pathway operating in the endoplasmic reticulum and cytosol and the MEP pathway functioning in the chloroplast. Why plants have retained both pathways is currently unknown but brings an interesting discussion on evolutionary aspects regarding isoprenoid synthesis. By compartmentalizing the pathways in different organelles, plants may overcome the disadvantages of immobility by having a more stringent control over isoprenoid synthesis (e.g., localizing chlorophyll synthesis in the plastid; phytosterol synthesis in the cytoplasm). This would allow accurate and faster responses to environmental cues that affect growth, development, and metabolism (Vranova et al. 2013).

9.3.2 Isoprenoid Synthesis Through the MVA Pathway

Isoprenoids synthesized in the cytosol and mitochondria are synthesized through the MVA pathway (Fig. 9.3b) (Vranova et al. 2013; Lichtenthaler 1999). The pathway starts with the Claisen condensation of two molecules of acetyl-coenzyme A (Ac-CoA) to create acetoacetyl-CoA (AcAc-CoA) in a reversible reaction, catalyzed by the enzyme AcAc-CoA thiolase (AACT; EC 2.3.1.9). Thiolases are divided into two classes: class I, which are characterized as degradative thiolases and are involved in fatty acid oxidation, and class II, which are biosynthetic thiolases and can catalyze the first reaction of the MVA pathway. Following models of pathway regulation, AACT activity strictly adheres to substrate/product ratios and was found to be extremely sensitive to free CoA. In an effort to increase *n*-butanol production in *Clostridium acetobutylicum*, the AACT enzyme ThlA was engineered to be less sensitive to feedback inhibition through free CoA by substituting three amino acids in the protein. This lead to an increase of ethanol and butanol titers by 46% and 18%, respectively (Mann and Lutke-Eversloh 2013).

AcAc-CoA is further condensed to 3-hydroxy-3-metylglutaryl-CoA (HMG-CoA) by HMG synthase (HMGS; EC 2.3.3.10). In yeast and most plants, these enzymes are found in paralogs (a set of genes created from a gene duplication event) within the chromosome. The exception is found in *Arabidopsis thaliana*, where HMGS is encoded by a single gene found to complement *erg11* and *erg13* yeast mutants lacking HMGS (Vranova et al. 2013).

Next, HMG-CoA is converted to mevalonic acid (MVA) in a two-step reduction process, requiring NADPH as a reducing equivalent. This reaction is catalyzed by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR; EC 1.1.1.34), a major rate-limiting enzyme in the MVA pathway. *Saccharomyces cerevisiae* harbors two paralogs of HMGR (*hmg1p* and *hmg2p*), each consisting of two major domains:

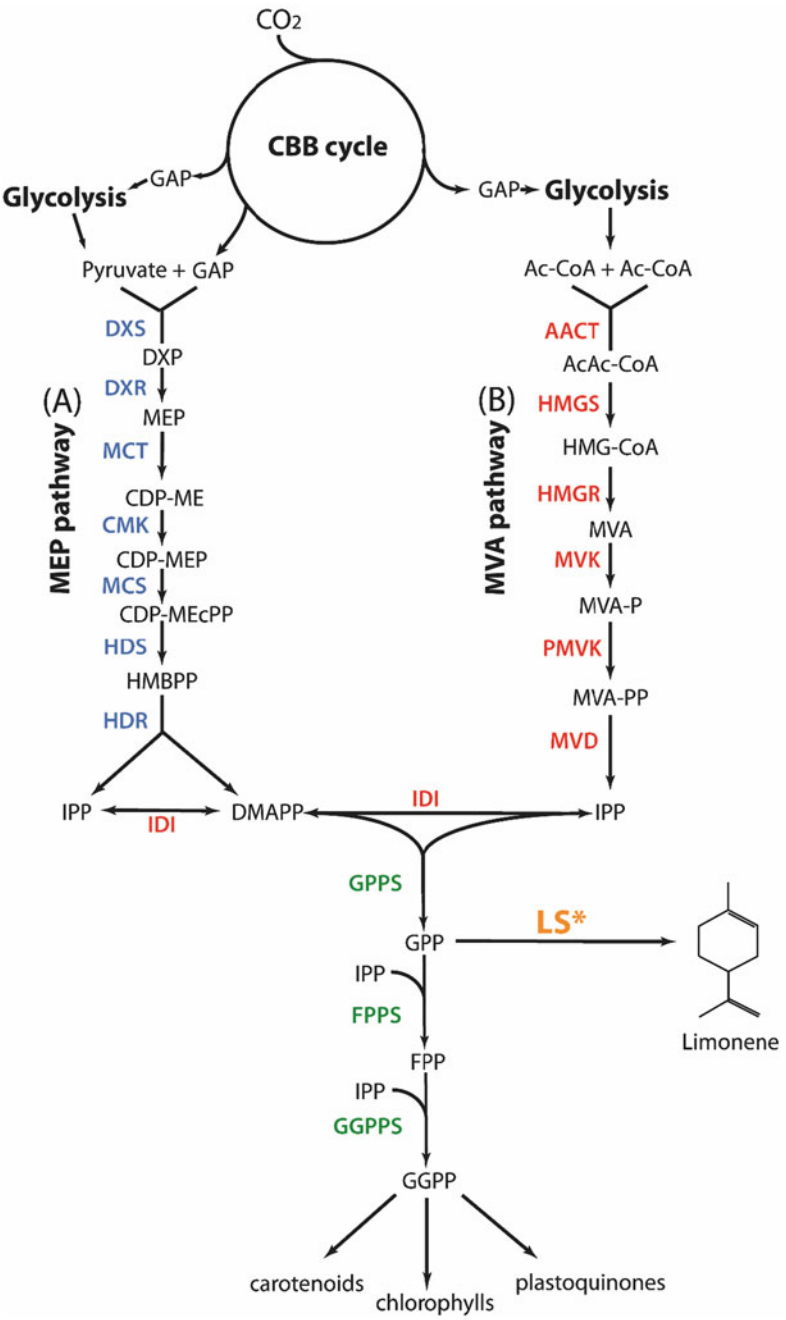

Fig. 9.3 Diagram of the two biosynthetic isoprenoid pathways. (**a**) The MEP pathway is found in most gram-negative bacteria, cyanobacteria, green algae, and plant plastids. (**b**) The MVA pathway is found in animals, archaea, fungi, plant cytosol, and some gram-positive cocci. In photosynthetic

an anchoring transmembrane domain associated with the endoplasmic reticulum (ER) and a catalytic domain facing the cytosol. Research has shown removal of the N-terminal anchoring domain and overexpression of the catalytic domain increased HMGR activity and the accumulation of squalene in yeast (Donald et al. 1997).

In two successive phosphorylations, mevalonate kinase (MVK; EC 2.7.1.36) phosphorylates MVA to create mevalonate-5-phosphate (MVA-P), which is then phosphorylated by phospho-MVA kinase (PMVK; EC2.7.4) to produce MVA-5-pyrophosphate (MVA-PP). These two enzymatic steps were identified as major bottleneck points in the MVA pathway, and overexpression of both genes in *E. coli* leads to improved productivity of desired bioproducts (Redding-Johanson et al. 2011).

The last steps of the pathway involve the decarboxylation of MVA-5-pyrophosphate to IPP, followed by the reversible isomerization of IPP to DMAPP. MVA-5-pyrophosphate to IPP is catalyzed by the enzyme MVA-pyrophosphate decarboxylase (MVD; EC 4.1.1.33). This step relies on the input of ATP and results in the decarboxylation of MVA-5-pyrophosphate to IPP, along with the release of CO_2. IPP isomerase (IDI; EC 5.3.3.2) then acts to convert IPP to its isomer DMAPP by transposing the position of a C-C double bond on the IPP molecule. This isomerization reaction is a noted rate-limiting step in the pathway, with equilibrium favored toward DMAPP production (Sun et al. 1998).

9.3.3 Isoprenoid Synthesis Through the MEP Pathway

The MEP pathway, also referred to as the 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, is an alternative route to the formation of IPP and DMAPP and is found in bacteria, various apicomplexan parasites, and plant chloroplasts (Lichtenthaler 1999) (Fig. 9.3a). The pathway starts with the condensation of pyruvate and glyceraldehyde-3-phosphate (GAP) to produce 1-deoxy-D-xylulose 5-phosphate (DXP). This reaction is catalyzed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS; EC 2.2.1.7), which requires a divalent cation (Mg^{2+} or Mn^{2+}) and thiamine pyrophosphate (TPP) as a cofactor. DXS plays an important regulatory role in the MEP pathway, since excess IPP and DMAPP can inhibit DXS activity by competing with TPP for binding with the enzyme (Banerjee and Sharkey 2014). In this way, excess IPP and DMAPP (created downstream in the pathway) can effectively slow or shut down MEP flux through an inhibitory feedback loop.

Fig. 9.3 (continued) organisms, substrates for both pathways are created during photosynthesis through the Calvin-Benson-Bassham (CBB) cycle. The isomerization of GPP through LS creates limonene, a cyclic isoprenoid with industrial applications. Enzymes in the MEP pathway are labeled in blue; enzymes in the MVA pathway are labeled in red. Enzymes downstream of both pathways are labeled in green. LS is labeled in orange and asterisked. Details on enzymes and pathway intermediates are described in the text

Next, DXP is converted to methylerythritol 4-phosphate (MEP) via DXP reductoisomerase (DXR, also known as IspC; EC 1.1.1.267). DXR requires reducing power in the form of NADPH, gained from the photosynthetic electron transport chain in photosynthetic organisms. Enzyme activity is controlled by phosphorylation of a particular serine residue in bacteria (Ser177 in *Francisella tularensis*; Ser186 in *Escherichia coli*) and acts as an important residue for binding of substrate (Banerjee and Sharkey 2014). The serine residue is conserved within plant DXRs; however, there is no information at present whether this same mechanism plays any role in regulating the MEP pathway.

The enzyme methylerythritol 4-phosphate cytidylyltransferase (MCT, also known as IspD; EC 2.7.7.60) converts MEP to diphosphocytidyl methylerythritol (CDP-ME) in a CTP-dependent reaction. Like DXR, this enzyme is thought to be dependent on a phosphorylation site on a threonine residue (Thr141 in *F. tularensis*; *Thr140 in E. coli*), which could also play a role in substrate binding (Banerjee and Sharkey 2014). CDP-ME is then phosphorylated from ATP to produce diphosphocytidyl methylerythritol 2-phosphate (CDP-MEP), catalyzed by CDP-ME kinase (CMK; EC 2.7.1.148). These two steps in the MEP pathway both require CTP and ATP, respectively.

The cyclic conversion of CDP-MEP to CDP-ME-cyclo-pyrophosphate (CDP-MEcPP) is performed by MEcPP synthase (MCS, also known as IspF; EC 4.6.1.12) and represents a major regulatory checkpoint in the MEP pathway (Banerjee and Sharkey 2014). Crystal structural analysis of the MCS from *E. coli* has shown a hydrophobic cavity present in the enzyme that may bind to different isoprenoids containing pyrophosphate moieties, such as IPP/DMAPP, GPP, and FPP. Furthermore, sequence alignments show strong conservation of this motif among MCSs from various organisms, suggesting a selective pressure for this specific structure (Banerjee and Sharkey 2014). These results suggest the motif in MCS might play a specific role in feedback regulation in the MEP pathway.

The next two steps in the MEP pathway consist of successive redox reactions. The first enzyme, hydroxy-methylbutenyl diphosphate synthase (HDS, also known as IspG or GcpE; EC 1.17.7.1), converts CDP-MEcPP to hydroxyl-methylbutenyl pyrophosphate (HMBPP), which is then reduced to IPP and DMAPP by HMBPP reductase (HDR, also known as IspH or LytB; EC 1.17.1.2). Both enzymes utilize [4Fe-4S]-clusters and involve double one-electron transfers in their reaction mechanism. It was demonstrated in *A. thaliana* and the thermophilic cyanobacterium *Thermosynechococcus elongatus BP-1* that HDS reduces MEcPP through reduced ferredoxin, generated by the photosynthetic electron transport chain (Okada and Hase 2005; Seemann et al. 2006). In contrast, *E. coli* HDS requires the flavodoxin/flavodoxin reductase/NADPH system to reduce MEcDP (Xiao et al. 2009). The highest reported HDS activity in *E. coli* is remarkably low at 74–99 nmol min^{-1} mg^{-1}, which is roughly 300-fold lower than HDR (30.4 μmol min^{-1} mg^{-1}) and 100- to 5000-fold lower than all of the other MEP enzymes (10–500 μmol min^{-1} mg^{-1}). HDS and HDR both require a very negative reducing power to catalyze their reactions, which are naturally supplied by ferredoxin (plants and cyanobacteria) or the NADPH/flavodoxin system (bacteria). Reducing power is

often a limiting factor for HDS and HDR activity. Exchanging NADPH/flavodoxin for an artificial redox partner with a lower reducing potential (methyl viologen; −446 mV) increased the activity of the *E. coli* HDS to 550 nmol min^{-1} (Xiao et al. 2009).

9.3.4 Convergence of MEP and MVA Pathway Through IPP/DMAPP Formation

In contrast to the MVA pathway, which creates IPP and isomerizes it to DMAPP through IDI, the enzyme HDR catalyzes the simultaneous formation of both IPP and DMAPP in an approximate 5:1 proportion (Adam et al. 2002). This formation of IPP and DMAPP is the one step linking both the MVA and MEP pathway, although the pathways are separated by different organisms, or in the case of plants, different organelles. Two classes of IDI enzymes have been discovered which show no sequence similarities, display different reaction mechanisms, and require different cofactors for proper catalysis (Perez-Gil and Rodriguez-Concepcion 2013). The type I enzyme (IDI-I) is found in many bacteria (including *E. coli*) and is similar to IDIs found in fungi, plants, and animals. The type II enzyme (IDI-II) is found in archaea and some bacteria (*Streptomyces*) but is absent from plants and animals. Interestingly, genome sequencing has revealed some bacteria contain either IDI-I or II enzymes, while others contain both or neither. Since IDI activity is essential to produce DMAPP in organisms only containing the MVA pathway, it may not be surprising to find its absence in organisms harboring the MEP pathway, since they can form IPP and DMAPP through HDR. Some species of cyanobacteria, including *Synechocystis*, *Synechococcus*, and *Cyanothece*, contain orthologs of IDI-II, yet these enzymes are deemed nonessential for isoprenoid synthesis (Perez-Gil and Rodriguez-Concepcion 2013).

As stated earlier, the head-to-tail linking of IPP and DMAPP creates the prenyl pyrophosphate GPP, the substrate for all monoterpene synthases. Next, we focus on the monoterpene synthase that catalyzes the conversion of GPP to limonene.

9.3.5 Limonene Synthases in Nature

Limonene synthase (LS) is a monoterpene cyclase that catalyzes the cyclization of GPP to limonene. To date, there are two major classes of LS found in nature, each producing the separate *R*- and *S*-enantiomers of limonene. The most common is (4*R*)-LS (EC 4.2.3.20), which produces *R*(+)-limonene (*d*-limonene). The other major LS is (4*S*)-LS (EC 4.2.3.16), which produces S(-)-limonene (*l*-limonene). The first LSs to be characterized were the (4S)-LSs from the oil glands of mint plants (Alonso et al. 1992; Colby et al. 1993; Rajaonarivony et al. 1992). This opened the

door to the isolation and functional expression of LS cDNA from many members of the mint, herb, and citrus families. Across plant species, most LSs have similar features: they are localized to the chloroplast, have a molecular weight of ~65 kD, require divalent metal ions (Mg^{+2} or Mn^{+2}) for substrate binding and catalysis, and have a pI of ~5 and optimal pH of ~7 and a $k_{cat} < 1\ s^{-1}$ (Hyatt et al. 2007).

9.3.6 Reaction Mechanism and Conserved Sequences

Monoterpene synthases, such as LS, utilize GPP as a natural substrate. Since GPP cannot be directly cyclized due to the location of double bond between the 2 and 3 carbons (C2 and C3), the reaction mechanism involves isomerization and cyclization steps (Bohlmann et al. 1998). First, GPP is ionized with the help of a divalent metal ion (Mg^{+2} or Mn^{+2}) bound to the LS active site. The interaction between the resulting C2 carbocation and the migration of the pyrophosphate anion to C3 yields the enzyme-bound intermediate (3R)- or (3S)-linalyl pyrophosphate (LPP). Whether limonene takes either 4R- or 4S-form depends on the initial folding of the GPP substrate. After rotation from a trans-to-cis formation, LPP ionizes again and cyclizes to form the corresponding 4R- or 4S-α-terpinyl cation. From here, the cation intermediate is deprotonated, creating the final limonene product. Minor products, including α-pinene, β-pinene, and myrcene, have been reported from preparative LS assays, although their concentrations are significantly lower (Colby et al. 1993; Martin et al. 2004). The ability to create multiple products is an unusual enzymatic attribute of LS, yet common among characterized monoterpene synthases.

Comparative genomics have revealed sequence similarities between LSs and other isoprenoid synthases, giving us insight into important structural and catalytic elements of these enzymes (Bohlmann et al. 1998). In general, LS is composed of two distinct structural domains: an N-terminal transit domain and a C-terminal active site domain (Fig. 9.4). Transit peptides located in the N-terminus target the newly synthesized LS enzyme to the chloroplast. Posttranslational modifications then remove the N-terminal residues upstream of a conserved, tandem arginine repeat (RRx_8W), creating a mature protein. The active site of the LS is composed of an aspartate-rich DDxxD motif, which coordinates two Mg^{2+} ions for aligning the GPP substrate to the catalytic pocket of the enzyme. Mutagenesis of any of the three aspartates of the DDxxD motif reduces catalytic activity by 1000-fold, proving the absolute necessity of this element for LS activity (Bohlmann et al. 1998).

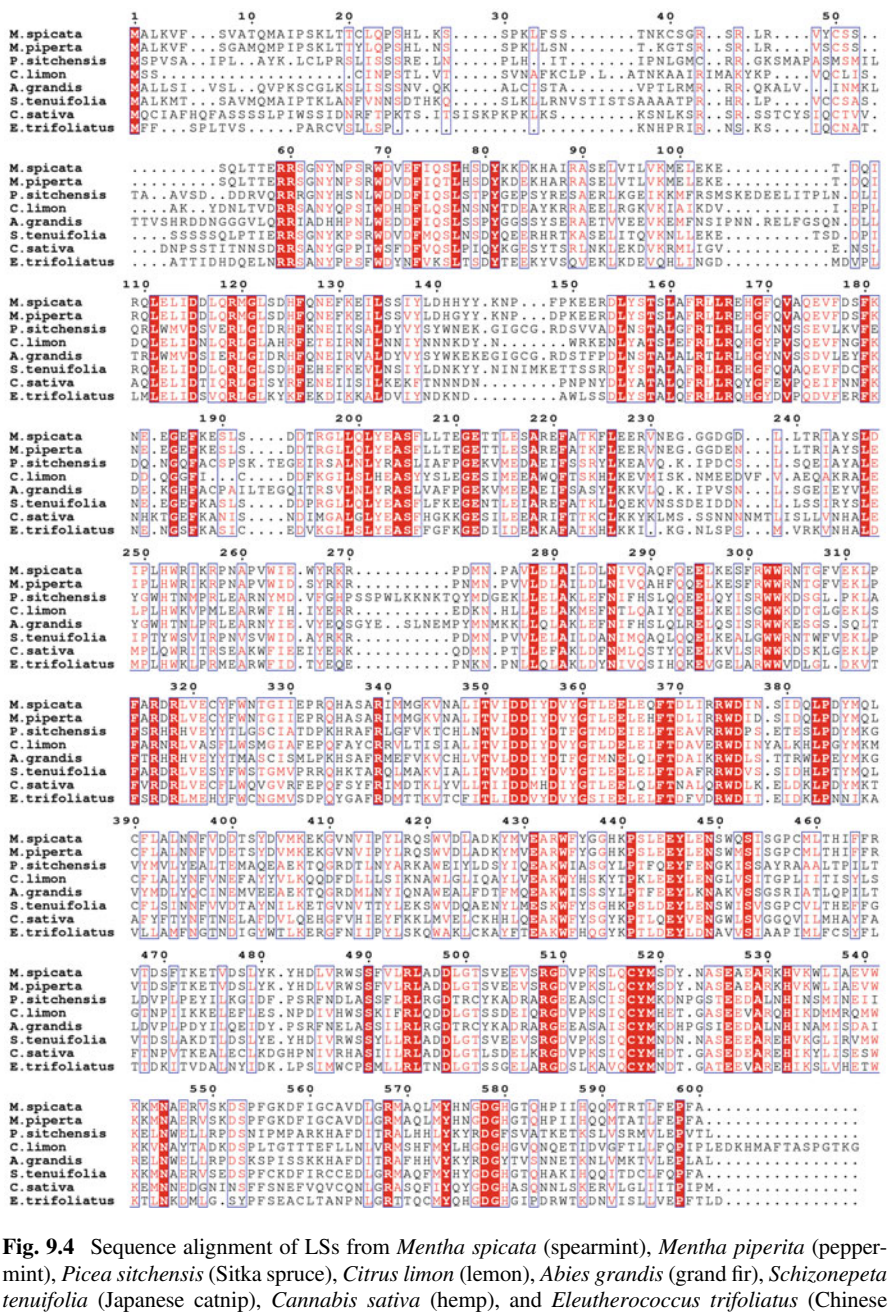

Fig. 9.4 Sequence alignment of LSs from *Mentha spicata* (spearmint), *Mentha piperita* (peppermint), *Picea sitchensis* (Sitka spruce), *Citrus limon* (lemon), *Abies grandis* (grand fir), *Schizonepeta tenuifolia* (Japanese catnip), *Cannabis sativa* (hemp), and *Eleutherococcus trifoliatus* (Chinese herb). Conserved amino acids are labeled in red, including the RRx_8W and DDxxD motif

9.4 Designing Cyanobacteria as Cellular Factories for Limonene Production

The last 20 years have seen a revolution in genome sequencing, unveiling the complete chromosome and plasmid sequences of many different cyanobacteria species (http://genome.microbedb.jp/cyanobase/). These efforts have allowed researchers to characterize the genes that create metabolic networks and to understand the extraordinary processes that convert CO_2 into sugars, amino acids, nucleotides, lipids, pigments, and other cellular components. Advancements in DNA-editing technology provide new opportunities to not only improve production of metabolites naturally produced by an organism but to add new genetic components to allow an organism to generate new metabolites. In the case of limonene, this would require insertion of new genes into the cyanobacterium to redirect intermediate metabolites into limonene. The expression and regulation of genes involved in biosynthetic pathways can boost carbon flux toward limonene synthesis, resulting in the higher limonene productivities needed for commercialization.

Inserting new genes into a cell is not a simple process, and many researchers have dedicated their lives to understand how genes can be packaged into organized structures and shipped across the host's cell membrane. Once inside the cell, the new genes must either be integrated into the chromosome or maintained as plasmids in the cytosol, where they express proteins that influence native metabolic networks. Most importantly, recombinant proteins must be regulated in a manner that eliminates the build-up toxic intermediates, prevents pathway bottlenecks, and allows high flow of substrate to product (Peralta-Yahya et al. 2012). This takes careful consideration in the design and construction of genetic components and their subsequent integration and regulation in the cyanobacterium.

9.4.1 Transfer and Recombination of Genes into the Cyanobacterium Genome

The introduction of genes that divert fixed carbon into a desired molecule has been the main approach in engineering cyanobacteria to produce valuable chemicals (Ducat et al. 2011; Ruffing 2011). Ever since the first report of exogenous DNA transfers into a cyanobacterium (Shestakov and Khyen 1970) and the development of recombinant DNA technologies in the 1970s, our knowledge of genetic engineering in cyanobacteria has expanded greatly. Descriptions of recombinant DNA engineering in cyanobacteria are widespread throughout the literature (Golden et al. 1986; Frigaard et al. 2004; Koksharova and Wolk 2002; Eaton-Rye 2004). Most genetic manipulations of cyanobacteria were originally performed to gain knowledge into metabolic, genetic, and photosynthetic processes of these organisms. These studies are now the backbone of research focused on the genetic manipulation of cyanobacteria for applied purposes.

The primary challenge regarding the insertion of DNA into a cyanobacterium is the efficiency of DNA transfer into the cell. Fortunately, several species of unicellular cyanobacteria, such as *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, and *Synechococcus* sp. PCC 7002, are naturally competent and can transport DNA across the cell membrane with moderate to high efficiency (Grigorieva and Shestakov 1982). Consequently, this defining feature makes these cyanobacteria species very popular for genetic research. Other cyanobacterial species, such as *Nostoc punctiforme* ATCC 29133, *Anabaena variabilis* ATCC 29413, and *Anabaena* sp. PCC 7120, have attributes that make them advantageous hosts, due to their ability to produce hydrogen or fix nitrogen. However, DNA insertion into these strains is problematic, due to highly active restriction enzymes that degrade foreign DNA that enters the cell. Mechanisms that protect foreign DNA, such as methylation factors, have been developed for these strains (Elhai et al. 1997). Other transformation techniques, including conjugation and electroporation, have also been developed (Elhai and Wolk 1988; Thiel and Poo 1989; Koksharova and Wolk 2002). Often, selectable markers are incorporated alongside the inserted gene, which helps screen for successful transformants after transformation. These are often antibiotic-resistant markers, which are dependent upon the antibiotic sensitivity of the host, as well as the ability of the host to produce the functional protein product of the antibiotic gene.

The insertion of a gene(s) in a cyanobacterium host can be accomplished using two methods: (1) by integration of the gene(s) into the host chromosome using homologous flanking regions or (2) by expressing the gene(s) outside of the chromosome on a self-replicating plasmid, sometimes referred to as a "shuttle vector" (Wolk et al. 1984). Each of these approaches has its advantages and disadvantages. Although genes that are integrated into the chromosome are more stable in the progeny of transformed lines, the known chromosomal loci that can be disrupted without corresponding negative effects are limited. Chromosomal neutral sites that allow for gene integration and disruption without harmful side-effects to the cell have been identified in cyanobacteria (Clerico et al. 2007). Shuttle vectors harbor the genes-of-interest outside of the chromosome, which preserves chromosomal integrity, and contain replication origins (the site where DNA replication takes place) that are easily recognized by the host cell. However, these plasmids replicate independently of cell division, creating daughter cells with inconsistent plasmid copy numbers. Most shuttle vectors are derived from native cyanobacterial plasmids with uncharacterized plasmid copy numbers, making control of gene expression unpredictable. Antibiotic selection pressure is also required to maintain these plasmids, and this is a concern for scale-up and commercialization.

Gene expression plays a crucial role in developing engineered microorganisms, since it is the regulation of genes that will ultimately influence production of the target compound. Engineered pathway genes can be controlled in several ways (Peralta-Yahya et al. 2012). The number of copies of a pathway gene (gene copy number) can be changed by increasing the number of genes integrated into the genome or by modifying the strength of the origin of replication (site of DNA replication). The rate at which a gene(s) is transcribed to mRNA can be controlled

by changing the strength of the promoter. Promoters can even be designed to be constitutive (always "on") or inducible (turned "on" and "off" during a desired time), giving researchers the power to regulate parts of a pathway, or multiple pathways, independently. Genes can be engineered with synthetic transcriptional terminator sites (TTS) that can control transcriptional efficiency. The strength of the ribosomal-binding site (RBS) upstream of the gene can be predicted using computer modeling, allowing optimized protein translation. Protein turnover (the balance between protein synthesis and degradation) can be regulated by adding a specialized "degradation tag" to a recombinant protein, which marks a protein for destruction. The combinatorial use of these genetic elements allows control over gene expression on the transcriptional, translational, and posttranslational level.

In recent years, the field of synthetic biology has revolutionized genetic engineering and is a crucial tool in designing complex gene components. Synthetic biology is the design and construction of new biological parts, devices, and systems, as well as the redesign of existing biological systems for useful purposes (http://syntheticbiology.org). Genetic components, such as promoters, RBSs, terminators, and replication origins, are organized in the "Registry of Standard Biological Parts," a repository that consists of thousands of genetic parts (available at http://partsregistry.org). These components, also known as "BioBricks," can be assembled using established cloning techniques and used for the rapid construction of engineered microorganisms. Although most of the work in synthetic biology has been performed in *E. coli*, some researchers have dedicated to creating a BioBrick format for cyanobacteria (Huang et al. 2010).

9.4.2 *Demonstrating Limonene Production from Cyanobacteria*

To this date, over 50 cyanobacteria species have completely sequenced genomes, with new cyanobacteria genome projects increasing at a rapid pace. These efforts have revealed orthologs for all known MEP pathway genes in most of the well-studied species (http://genome.microbedb.jp/CyanoBase). Although genome surveys have identified the necessary components to a functional MEP pathway in cyanobacteria, no gene similarities to annotated plant LSs have been found, which explains limonene's absence from volatile profiles from cyanobacteria. This has prompted researchers to investigate the possibility of adding a new carbon sink by inserting a plant LS gene into its genome, thereby channeling natively produced GPP into limonene.

In 2014, a number of research groups showed proof-of-concept demonstrations of limonene production from several different cyanobacteria species using genetic engineering. A (4*S*)-LS from *Picea sitchensis* (Sitka spruce) was linked to a dual *nir-psbA1* promoter and expressed in the filamentous, nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120, using a replicating shuttle vector (Halfmann et al.

2014b). Volatized limonene emitted from the transgenic *Anabaena* were captured using a hydrophobic resin column and analyzed using gas chromatography-mass spectrometry (GC-MS). With expression of the (4*S*)-LS from *P. sitchensis*, the transgenic *Anabaena* yielded 114.3 ± μg limonene L^{-1} during 14 days of continuous growth. Limonene yield was increased 2.3-fold during the same time frame by co-expression of three rate-limiting enzymes (DXS, IDI, GPPS) in the MEP pathway. Optical density and chlorophyll measurements were similar between the wild-type (WT) and transgenic cultures, concluding that limonene's effects on culture growth were minimal to nonexistent. However, an increase in photosynthetic activity was also observed, suggesting limonene acting as an additional sink for carbon fixation.

In both transgenic strains (LS and LS-DXS-IDI-GPPS *Anabaena*), limonene productivity decreased during the duration of the growth trials, presumably from the inability of light to evenly disperse as the cultures became more dense (the "self-shading effect") (Halfmann et al. 2014b). Increasing the light intensity from 50 to 150 μE m^{-2} s^{-1} greatly improved limonene productivity upon initial culture growth (3.6 ± 0.5 μg limonene L^{-1} $O.D.^{-1}$ h^{-1}) but dropped after cell density reached a critical point ($O.D._{700nm} > 1.0$). It is assumed that the drop in productivity is the result of light limitation, which could reduce activity of the MEP pathway by limiting reductants created through photosynthesis, or reduce activity of the psbA1 promoter, which is known to be light-activated.

Kiyota et al. used a similar approach to engineer a limonene-producing cyanobacteria. They expressed a codon-optimized LS from the Chinese medicinal herb *Schizonepeta tenuifolia* in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. Limonene was collected using a gas stripping method, in which outgas from a bubbling culture was released in the octane phase of a cold trap. The system produced limonene at a rate of 41 μg L^{-1} day^{-1} when expressing the LS alone and increased to 56 μg L^{-1} day^{-1} when co-expressing three MEP pathway enzymes (DXS, IPI, CrtE). Sustained limonene production was observed for up to 300 h and decreased to nearly one-third of the initial production rate after 700 h of continuous growth (Kiyota et al. 2014).

A (4*S*)-LS from *Mentha spicata* (spearmint) was expressed in the unicellular cyanobacterium *Synechococcus* sp. PCC 7002, and limonene was collected by applying a dodecane ($C_{12}H_{26}$) overlay on top of the culture (Davies et al. 2014). The hydrophobic layer maintained separation from the culture during growth trials and was able to trap limonene secreted from the cells. Using this methodology, total limonene titers reached 1.7 mg limonene $gDCW^{-1}$ (4 mg L^{-1}) after 96 h. Several factors may attribute to the superior performance of limonene production in this engineered strain. The superior kinetics and high specificity of the (4*S*)-LS from *Mentha spicata* may play a significant role in the fast turnover of GPP to limonene. Also, the dodecane overlay may actively sequester limonene away from the cell, thereby relieving negative feedback pressures exerted by the products and promote the forward reaction of GPP to limonene. Two-phase bioreactors that trap secreted products were also shown to prevent evaporative loss of limonene and other isoprenoids (Bentley and Melis 2012).

Glycogen is a highly branched polymer of glucose and serves as the main carbon and energy reserve in cyanobacteria. The inactivation of glycogen synthesis in several cyanobacteria strains was shown to cause a number of physiological effects during nutrient stress (Gründel et al. 2012; Suzuki et al. 2010). Studies on glycogen-deficient *Synechocystis* and *Synechococcus* mutants showed that under nitrogen depletion, partially oxidized metabolites (α-ketoglutarate, pyruvate, succinate, acetate, and α-ketoisocaproate) were found to accumulate in the culture media (Gründel et al. 2012; Davies et al. 2014). Rerouting these precursors through the MEP pathway would be one strategy to distribute flux from a major carbon sink into limonene synthesis. However, expressing an LS into glycogen-deficient *Synechococcus* sp. PC 7002 resulted in no significant difference in limonene production (Davies et al. 2014). It is therefore presumed that other bottlenecks or regulatory checkpoints in the MEP pathway can prevent carbon flux to limonene, even with the accumulation of precursors in the beginning of the pathway. Interestingly, it was found that these organic acids increased in accumulation with the expression of LS. This mysterious dynamic between limonene synthesis and metabolic overflow in glycogen-deficient cyanobacteria mutants is an interesting area for future research.

9.5 Discussion

Compared to other carbon pathways, cyanobacteria invest a tiny percentage of daily fixed carbon toward isoprenoids, ranging from 2 to 5% of total fixed carbon (Lindberg et al. 2010). Limonene flux of engineered cyanobacteria is even lower, with estimates as low as 0.1% of the total carbon pool (Kiyota et al. 2014). Similar levels of chlorophyll and carotenoids between engineered and WT cyanobacteria suggest that LS activity is a major limiting factor to limonene productivity. To obtain a significant increase in limonene productivity, it may be necessary to express a more optimal LS to synthesize limonene from the GPP pool. Data mining plant genomes can reveal highly active LSs, as well as more suitable enzyme candidates for upstream pathway reactions. Redesigning proteins for higher catalytic activity and specificity is another approach that has seen some success as an engineering strategy (Wen et al. 2009).

There are many potential factors behind the limited allocation of carbon into MEP pathway products in cyanobacteria. These include rapid degradation of unstable intermediates, competition with other carbon pathways that create cell architecture and the photosynthetic apparatus, and the inactivation of enzymatic steps through various host control mechanisms (Kudoh et al. 2014). Increasing the pool of IPP and DMAPP is necessary to provide the needed precursors to isoprenoid products. Expressing the alternative MVA pathway in cyanobacteria that operates in parallel to the MEP pathway can increase flux toward isoprenoid synthesis, since the host organism would not possess the required MVA pathway regulatory elements to control it. This was demonstrated by the expression of a whole MVA pathway in a

Synechocystis sp. PCC 6803, which the cyanobacterium used to produce the C_5 isoprene alongside its native MEP pathway (Bentley et al. 2014). This increased isoprene productivity from ~60 to ~150 μg isoprene L^{-1} over 196 hours of culture growth, an approximate 2.5-fold increase.

Operating whole-engineered pathwaysin microorganisms for chemical production is a formidable task, since most biosynthetic pathways are extremely complex and are regulated at multiple biological levels. Careful consideration of transcriptional and translational efficiencies, enzymatic activities, and pathway cross-talk is needed by the designer to allow for the rapid transformation of the initial substrate to the final product (Keasling 2012). A common misconception is that this can be accomplished by highly expressing all genes in the pathway. However, this is a flawed concept, since all pathway reactions involve enzymes with different kinetics, require different cofactors, are influenced by diverse inhibitory and activating agents, and so on. Expressing a gene at too high a level can erroneously shift metabolites away from the desired product and reduce productivity or in some cases cause the buildup of toxic intermediates that are detrimental to cell maintenance (Keasling 2012). Instead, recombinant enzymes need only to be produced in sufficient amounts that allow for the transformation of intermediates into the desired products at a sufficient rate. This involves coordinating gene expression in a way that takes into account each reaction step from the initial substrate to final product (Kirby and Keasling 2009). Other strategies, such as introducing efflux pumps to prevent feedback inhibition, have also been implemented (Dunlop et al. 2011).

Although additional engineering work has improved isoprenoid production in cyanobacteria, current productivities reported from photosynthetic microorganisms are much lower than engineered heterotrophic bacteria, most notably *E. coli*. For instance, the expression of a heterologous MVA pathway in *E. coli* while co-expressing the *M. spicata* LS resulted in limonene titers reaching 450 mg limonene L^{-1} over 72 h (Alonso-Gutierrez et al. 2013), much higher than any limonene-producing cyanobacteria in the literature (4 mg L^{-1} in 96 h, Davies et al. 2014). This "productivity gap" between engineered cyanobacteria and *E. coli* reflects the infancy of cyanobacteria engineering, since *E. coli* is a popular model organism with a more established bioengineering repertoire. The engineering strategies used in *E. coli* can be adopted for cyanobacteria to increase isoprenoid productivity and titers.

As stated above, *E. coli* has been a highly used model for microbial isoprenoid production, most notably for the production of the sesquiterpene amorpha-4,11-diene, the precursor for the antimalarial drug artemisinin (Martin et al. 2003). Improvements in pathway engineering through optimizing gene codons and copy numbers, alleviation of pathway bottlenecks, and an efficient fed-batch fermentation process boosted amporpha-4,11-diene titers to more than 27 g L^{-1} (Tsuruta et al. 2009). Scientists at the University of California, Berkeley, brought this technology to the commercial level through the biotech company Amyris, which now manufactures artemisinin-based drugs and more recently biofuels from engineered *Saccharomyces cerevisiae* (https://amyris.com/products/fuels/). In the future, we may all benefit from a diverse variety of specialized medicines and fuels that are made exclusively by microorganisms.

9.6 Conclusions

Proof-of-concept demonstrations of isoprenoid production from metabolically engineered cyanobacteria are widespread in the literature, including isoprene (Lindberg et al. 2010), farnesene (Halfmann et al. 2014a), phellandrene (Bentley et al. 2013), squalene (Englund et al. 2014), bisabolene (Davies et al. 2014), and limonene (Davies et al. 2014; Halfmann et al. 2014b; Kiyota et al. 2014). These engineering efforts were successful, thanks to decades of advancements in genomics, molecular biology tools, and recombinant DNA technology. For these biofuel/chemical platform technologies to be successfully commercialized, proof-of-concept studies will need to mature to pilot-scale demonstrations that show the technology can be a cost-effective alternative to petroleum.

The transition from lab-bench to pilot scale can be aided by techno-economic modeling, which analyzes the economic feasibility of industrial-scale cyanobacteria cultivation, harvesting, and chemical extraction from biomass. The Farm-level Algae Risk Model (FARM) was previously used to simulate operation of a commercial limonene-production facility, revealing that photosynthetic limonene productivity would need to reach ~1 mg limonene L^{-1} day^{-1} to be economically viable (Johnson et al. 2016). Advancements in engineering (molecular and industrial) are the likely routes to achieving these rates while also utilizing resources such as natural sunlight, flue gas, and wastewater to be converted into valuable fuels and chemicals. Photobioreactors designed to optimize mass transfer, temperature, nutrients, and solar radiation will also be critical. Furthermore, biomass-biofuel separation techniques must be engineered to collect the biofuel efficiently and alleviate cell toxicity/product feedback. In all, this will require multidisciplinary efforts from molecular biology, biochemistry, biophysics, computational biology, and industrial engineering.

The idea of direct CO_2 conversion into fuels and chemicals has already garnered significant interest from the investment world. Joule Unlimited has brought photosynthetically derived fuels into recognition through the production of *Sunflow*® products, ethanol and diesel fuels produced from engineered algal cells (Berry 2010). Recently, Joule Unlimited announced the development of a 4-acre test site in Hobbs, New Mexico, for planned production of up to 25,000 gallons of ethanol and 15,000 gallons of diesel per acre annually, at an estimated price of $50/barrel (Hepler 2015). Algenol, headquartered in Fort Myers, Florida, confirmed they had exceeded production rates of 9000 gallons of ethanol/acre/year from their 4-acre, outdoor Process Development Unit in Lee County, Florida. This facility previously achieved continuous ethanol production at 7000 gallons ethanol/acre/year in September 2013, up from a projected 6000 gallons ethanol/acre/year (Lane 2013). Company CEO Paul Woods announced that the Direct-to-Ethanol technology allowed the production of ethanol for around $1.00/gallon, using only sunlight, carbon dioxide, and seawater. Whether these companies or similar startups can achieve the same successes with isoprenoids is yet to be seen.

The milestones reached by industry to engineer algae and cyanobacteria to convert inorganic carbon, waste water, and light into diesel and ethanol bring hope for future production of advanced biofuels. However, as noted above, significant improvements in key areas are needed to achieve this goal of using cyanobacteria-based technologies to convert CO_2 into valuable fuels and chemicals.

Take-Home Message

- Limonene, a cyclic C_{10} isoprenoid, is a valuable commodity chemical with applications in solvents, perfumes, flavorings, pharmaceuticals, and biofuels.
- As consumer interest in "all-natural" chemicals continues to grow, attention has turned to utilizing fourth-generation biofuel technologies for limonene production.
- Cyanobacteria can serve as a photosynthetic chassis for synthesizing economically relevant fuels and chemicals. Proof-of-concept studies demonstrate the potential use of genetically modified cyanobacteria to produce a wide range of green solvents, including limonene.
- Future advancements in genetic and industrial engineering may potentially lead to the direct conversion of CO_2 into limonene in industrial facilities.

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