

# Isoprenoid-Based Biofuels: Homologous Expression and Heterologous Expression in Prokaryotes

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Enthusiasm for mining advanced biofuels from microbial hosts has increased remarkably in recent years. Isoprenoids are one of the highly diverse groups of secondary metabolites and are foreseen as an alternative to petroleum-based fuels. Most of the prokaryotes synthesize their isoprenoid backbone via the deoxyxylulose-5-phosphate pathway from glyceraldehyde-3-phosphate and pyruvate, whereas eukaryotes synthesize isoprenoids via the mevalonate pathway from acetyl coenzyme A (acetyl-CoA). Microorganisms do not accumulate isoprenoids in large quantities naturally, which restricts their application for fuel purposes. Various metabolic engineering efforts have been utilized to overcome the limitations associated with their natural and nonnatural production. The introduction of heterologous pathways/genes and overexpression of endogenous/homologous genes have shown a remarkable increase in isoprenoid yield and substrate utilization in microbial hosts. Such modifications in the hosts' genomes have enabled researchers to develop commercially competent microbial strains for isoprenoid-based biofuel production utilizing a vast array of substrates. The present minireview briefly discusses the recent advancement in metabolic engineering efforts in prokaryotic hosts for the production of isoprenoid-based biofuels, with an emphasis on endogenous, homologous, and heterologous expression strategies.

Concerns, like climate change, diminishing fossil fuel supply, energy security, and political instability in petroleum-wealthy regions, have driven global interest toward the production of sustainable and renewable fuels from biological routes specifically from microbial origin. This directs the global interest toward the search for longer-carbon-chain-length ( $C \geq 4$ ) biomolecules as next-generation biofuels having compatibility with the existing fuel infrastructure (1–6). The field of metabolic engineering has provided a wealth of opportunities to expand the scope of biofuels from engineered microbes (1, 5, 7–9). In nature, some of the microbes are capable of decomposing complex sugars into simple sugars, while some others can convert sugars into biofuels natively. The extensive characterization of such hosts and advancement in synthetic biology tools and techniques have enabled researchers to optimize the host's pathway. Various endogenous (the host's genes and pathways), homologous (genes and pathways of other organisms distantly related to the host's), and heterologous (genes and pathways of other organisms that do not correspond in evolutionary origin to the host's) genes and pathways need to be optimized inside a host to enhance biofuel production. The plenteous genetic modifications not only improve substrate utilization, product titers, and yields but also extend the variety of biofuels from microbial hosts (10–17).

Among the vast array of secondary metabolites produced by plants and microorganisms, isoprenoids (terpenoids or terpenes) are the most abundant group of natural products, comprised of more than 40,000 known compounds (18, 19). Isoprenoids are traditionally exploited as a source of fragrances, flavors, and medicines (20). Their tremendous structural and functional diversity (7, 19, 20) provides an alluring motivation to explore biological macromolecules as next-generation biofuels for sustainable economic and industrial growth. Their low hygroscopy, high energy density, and good fluidity at low temperatures place them at the forefront of potentially good diesel and gasoline fuel alternatives (21–23). The present minireview focuses on the engineering of homologous and heterologous metabolic pathways/genes within

the prokaryotic hosts for the production of isoprenoid-based advanced biofuels.

## BACKGROUND

Plants are the major resources of isoprenoids, but limitations such as (i) low growth rates and seasonal production, (ii) soil and land requirements, (iii) low-level and tissue-specific biosynthesis, and (iv) difficulty in harvesting and extraction are the major barriers to the natural production of isoprenoids in large quantities from plants for fuel purposes (23, 24). During chemical synthesis, drawbacks such as stereochemical complexities of isoprenoids, use of hazardous solvents, and environmental concerns restrain their production in large amounts (23, 25). Together, these barriers have led biofuel research interests toward alternative production strategies offered by biotechnology. Biotechnological applications have enabled researchers to develop metabolically engineered microbial factories for the large-scale production of isoprenoid-based biofuels (26) (see Fig. S1 in the supplemental material). Microbes possess several advantages, such as ease in culturing and handling, high growth rates, inexpensive growth medium requirements, genetic traceability, and tractability, which bring them to the forefront of biofuel research (27). Despite the above-mentioned advantages, microbes can also be genetically modified with

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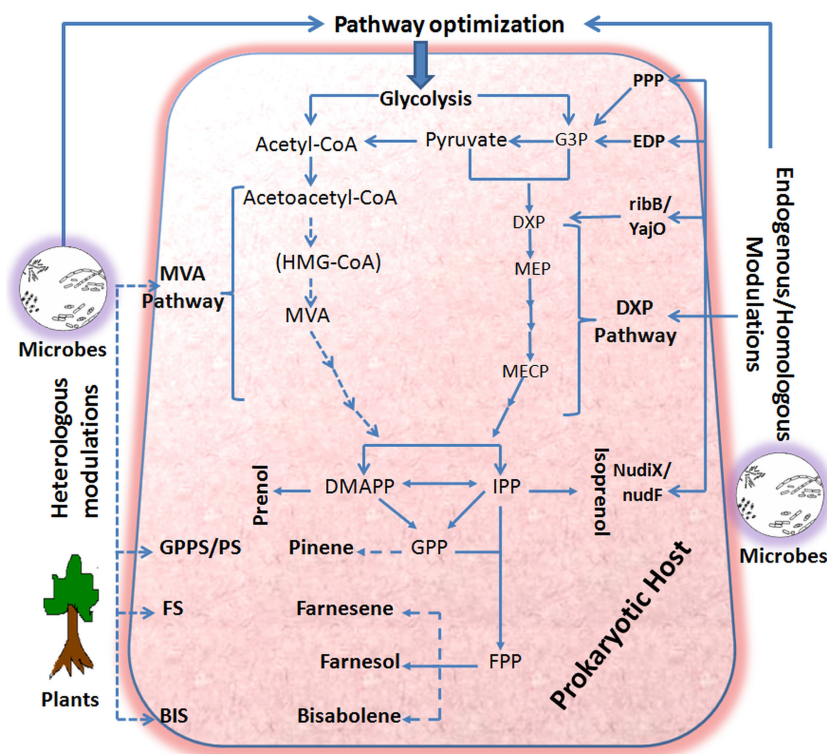
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**FIG 1** Metabolic engineering for the production of isoprenoid-based biofuels in a prokaryotic host. Solid arrows indicate endogenous/homologous pathways/genes, and dashed arrows indicate heterologous pathways/genes in a prokaryotic host. EDP, Entner-Doudoroff pathway; PPP, pentose phosphate pathway; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MVA, mevalonate; G3P, glyceraldehyde-3-phosphate; DXP, deoxyxylulose-5-phosphate; MEP, 2-C-methylerythritol-4-phosphate; MECP, 2-C-methylerythritol-2,4-cyclodiphosphate; IPP, isoprenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GPPS, geranyl pyrophosphate synthase; PS, pinene synthase; FS, farnesene synthase; BIS, bisabolene synthase.

great success rates, and even an entire novel pathway can be introduced utilizing high-throughput synthetic biology tools and techniques (24).

Isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP) are the common precursors for isoprenoid backbone synthesis in all living organisms (28). Almost every prokaryote, with some exceptions, synthesizes its isoprenoids from the deoxyxylulose-5-phosphate (DXP) pathway (29), but some endogenous regulations of the DXP pathway restrict their large-scale production for fuel applications. On the other hand, in the eukaryotic system, the isoprenoid backbone is synthesized from the mevalonate (MVA) pathway by utilizing acetyl coenzyme A (acetyl-CoA) (30–32).

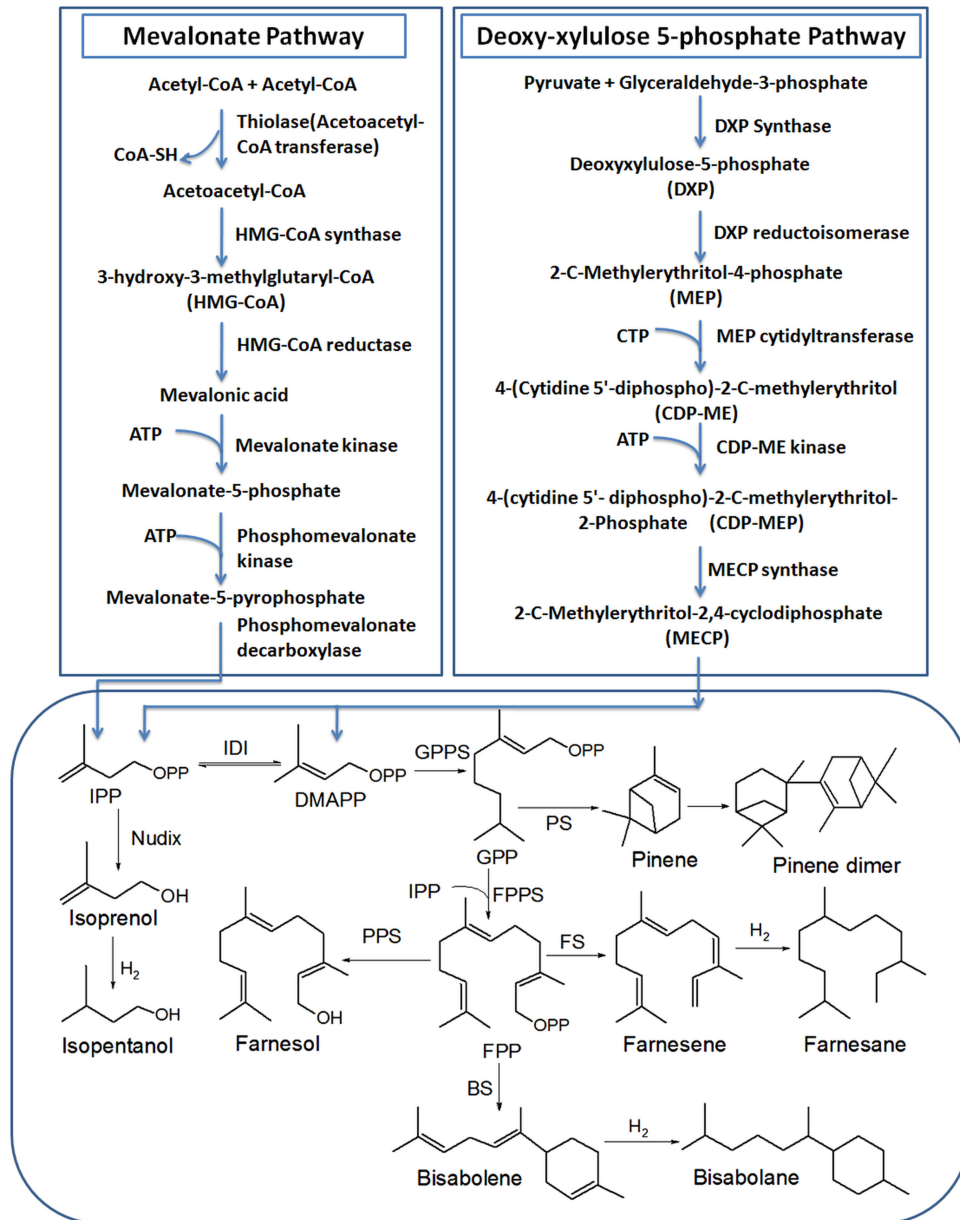
In recent years, enhanced production of isoprenoid-based biofuels has been achieved in microbial systems via tuning an endogenous pathway or engineering a heterologous pathway and genes in new hosts (5, 7, 8, 23). In the prokaryotic systems, to bypass endogenous regulations of the DXP pathway and to ensure overexpression of the isoprenoid backbone, the components of the heterologous MVA pathway have been introduced into their genomes (10–12, 33). Overexpression of key regulatory enzymes of the native DXP pathway has been also utilized to enhance IPP and DMAPP production in prokaryotes (13, 34–37). Similarly, in the eukaryotic system, efforts have been made to enhance isoprenoid production via (i) bypassing the endogenous regulation of the MVA pathway in *Saccharomyces cerevisiae* (38, 39) or (ii) modulating the expression of endogenous/homologous enzymes for

higher production of isoprenoid-based biofuels in yeast (11, 40–42). The metabolic engineering efforts for either endogenous or heterologous gene expression have increased the production of a vast array of isoprenoid-based biofuels or fuel precursors. Figure 1 illustrates the various pathway optimization strategies for the enhanced production of isoprenoid-based biofuels in a prokaryotic host. Stoichiometric studies have shown that the DXP pathway is more competent than the MVA pathway (43); still, the production of isoprenoids by optimizing the DXP pathway cannot go beyond the levels achieved by expressing a heterologous MVA pathway in prokaryotes. In later sections of this minireview, we briefly discuss the strategies for the production of isoprenoid-based biofuels and fuel precursors in prokaryotic hosts.

### ISOPRENOID BIOSYNTHESIS

The MVA and DXP pathways are well-known pathways for the production of a wide range of branched-chain and cyclic metabolites, collectively called isoprenoids, in living systems, e.g., quinones (ubiquinone and plastoquinone), pigments (carotenoids and the side chain of chlorophyll), hormones (gibberellins, brassinosteroids, and abscisic acid), and sterols (cholesterol, ergosterol, and campesterol), as well as other characteristic plant compounds (limonene, pinene, menthol, bacoside, camphor, etc.) (18, 19).

The first-recognized pathway for biosynthesis of isoprenoids is the MVA pathway (30–32), which involves a series of six enzymatic steps to convert acetyl-CoA to IPP (Fig. 2). For many decades, IPP was considered to be formed from the MVA pathway



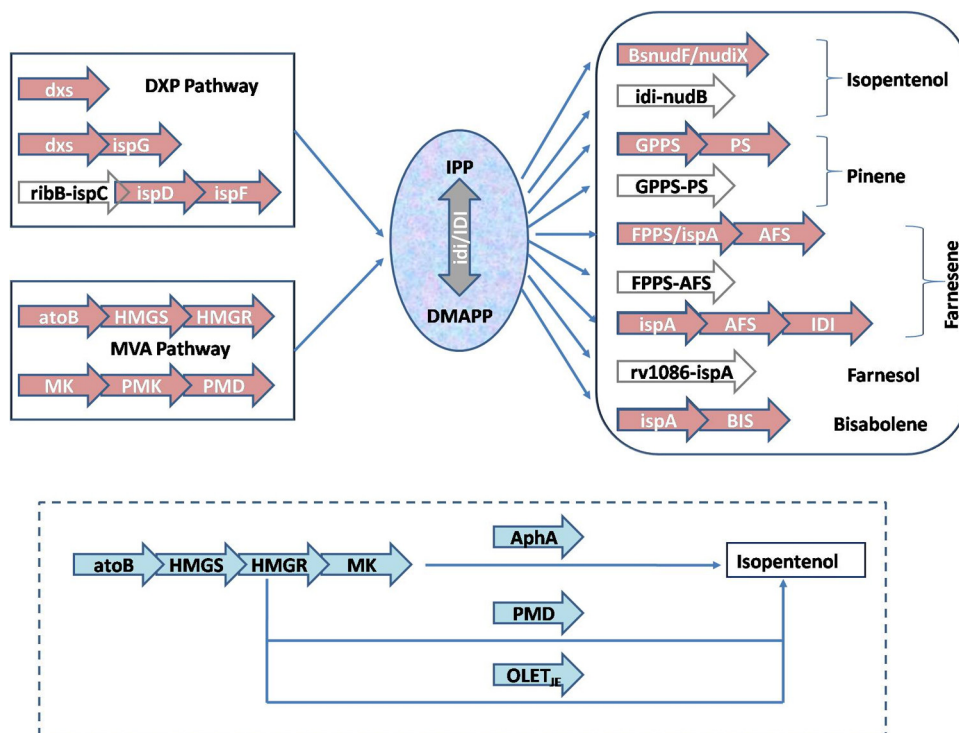
**FIG 2** Metabolic routes for the production of isoprenoid-based biofuels in a living system. The mevalonate (MVA) pathway is from acetyl-CoA, and the deoxyxylulose 5-phosphate (DXP) pathway is from glyceraldehyde-3-phosphate and pyruvate. IPP, isoprenyl diphosphate; DMAPP, dimethylallyl diphosphate; IDI, isoprenyl diphosphate isomerase; nudix, nucleoside diphosphate hydrolases; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GPPS, geranyl pyrophosphate synthase; FPPS, farnesyl pyrophosphate synthase; PS, pinene synthase; FS, farnesene synthase; BS, bisabolene synthase; PPS, endogenous pyrophosphatase.

and was considered ubiquitous in all living organisms. Extensive genomic studies on plants and various eubacteria have led to the discovery of an MVA-independent pathway for IPP and DMAPP biosynthesis (44–48). This is the DXP pathway for isoprenoid production in prokaryotes and chloroplasts of higher plants. The DXP pathway consists of seven enzymatic steps that convert glyceraldehyde-3-phosphate (G3P) and pyruvic acid to IPP and DMAPP (49, 50) (Fig. 2). Though glycolysis is the primary source of the precursor supply for both pathways, there are several other known pathways which also provide precursors for isoprenoid backbone synthesis for, e.g., the pentose phosphate (PP) pathway,

the Entner-Doudoroff (ED) pathway, fatty acid metabolism, and amino acid metabolism (51, 52) (Fig. 1).

**Downstream isoprenoid-based biofuel products.** Based on the number of carbon atoms present, isoprenoid-based biofuels can be subdivided into (i) hemiterpene-based ( $C_5$ ) biofuels, (ii) monoterpene-based ( $C_{10}$ ) biofuels, and (iii) sesquiterpene-based ( $C_{15}$ ) biofuels.

Hemiterpene-based biofuels are synthesized directly from IPP and DMAPP by several downstream enzymes. Various endogenous and homologous phosphatases and pyrophosphatases that can convert prenyl pyrophosphates into respective bio-



**FIG 3** Pathway optimization for the enhanced production of isoprenoid-based biofuels from prokaryotic hosts. Open and filled arrows indicate coexpression and fusion protein expression, respectively. Genes in uppercase letters are heterologous, whereas genes in lowercase letters are endogenous/homologous. The solid-line box shows conventional upstream pathways (MVA and DXP pathways), whereas the dashed-line box shows novel pathways via decoupling of the conventional MVA pathway. *atoB*, acetyl-CoA acetyltransferase gene; *HMGS*, hydroxymethylglutaryl-CoA synthase; *HMGR*, hydroxymethylglutaryl-CoA reductase; *MK*, mevalonate kinase; *PMK*, phosphomevalonate kinase; *PMD*, mevalonate diphosphate decarboxylase; *dxs*, deoxyxylulose-5-phosphate synthase gene; *ispC*, deoxyxylulose-5-phosphate reductoisomerase gene; *ispD*, 2-C-methylerythritol-4-phosphate cytidyltransferase gene; *ispF*, 2-C-methylerythritol-2,4-cyclodiphosphate synthase gene; *ispG*, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase gene; *IPP*, isoprenyl diphosphate; *DMAPP*, dimethylallyl diphosphate; *idi/IDI*, isoprenyl diphosphate isomerase; *nudF*, *B. subtilis* ADP ribose pyrophosphatase gene; *nudIX*, nucleoside diphosphate hydrolases; *ispA*, farnesyl pyrophosphate synthase (*FPPS*) gene; *GPPS*, geranyl pyrophosphate synthase; *PS*, pinene synthase; *AFS*, farnesene synthase; *BIS*, bisabolene synthase; *OLET<sub>E</sub>*, *Jeotgalicoccus* sp. fatty acid decarboxylase.

fuel compounds have been explored in *Escherichia coli* (12, 16, 37, 53, 54).

Long-carbon-chain-length isoprenoid molecules, such as pinene, farnesene, and bisabolene, are not produced by prokaryotic hosts, as the enzymes responsible for their synthesis are not found in prokaryotic systems (7). Such enzymes need to be incorporated from other organisms that act on isoprenyl pyrophosphatases. Monoterpene synthesis requires geranyl pyrophosphate (GPP) as a precursor, but most prokaryotes lack endogenous GPP synthase (*GPPS*) (7). Prokaryotes have the farnesyl pyrophosphate synthase (*FPPS* or *IspA*) enzyme, which is responsible for farnesyl pyrophosphate (*FPP*) synthesis directly from *IPP* and *DMAPP* and is assumed to be a source of *GPP* synthesis in prokaryotes (55). Monoterpene synthase enzymes, such as pinene synthase (*PS*), also need to be expressed heterologously, which can convert *GPP* into monoterpene-based biofuel molecules (Fig. 3).

Sesquiterpene ( $C_{15}$ )-based biofuel synthesis in prokaryotes also requires heterologous enzymes, like farnesene synthase (*FS*) and bisabolene synthase (*BIS*), to convert *FPP* into their respective biofuels, farnesene and bisabolene (Fig. 3). For the production of farnesol (another sesquiterpene derivative) in *E. coli*, endogenous phosphatases and pyrophosphatases have been utilized (33, 56, 57). Later sections briefly discuss strain improvement strategies for the overproduction of isoprenoid-based biofuels and fuel precursors from prokaryotic hosts.

### ENGINEERING MICROBIAL HOSTS FOR THE NONNATURAL PRODUCTION OF ISOPRENOID-BASED BIOFUELS

Most of the research concerned with the production of isoprenoid-based biofuels from prokaryotic hosts has focused on engineering the most-understood model organism, *E. coli*. Strain engineering efforts have provided an array of new opportunities to engineer alternate microbial strains as hosts for the nonnatural production of isoprenoid-based biofuels (13, 15, 58, 59). Engineering alternate microbial hosts also enables researchers to explore their ability to utilize a vast array of carbon sources for biofuel production. Enhanced production of isoprenoids has been achieved through a focused combinatorial approach, multivariate modular pathway engineering (*MMPE*), even without a full understanding of endogenous regulations (35). *MMPE* utilizes partitioning of the overall *DXP* pathway into smaller modules, and components of each module are then tuned separately in order to obtain higher yields of the desired metabolites. Codon optimization has also provided a promising platform for the optimization of nonnative genes to enhance isoprenoid-based biofuel production in microbial hosts (11, 37, 59). However, the application of codon optimization is limited by inherent low enzyme activities and specificities of the isoprenoid pathways in microbial hosts. The work of Leonard et al. has suggested a more promising way to overcome such limitations via the molecular reprogramming pro-



**TABLE 1** Summary of the pathway modulations, engineered microbes, product titers, production rates, and production times of isoprenoid-based biofuels produced from engineered microbes

Isoprenoid(s) by expression type	Engineered microbe	Titer	Rate of production	Time <sup>a</sup>	Reference
<b>Heterologous expression</b>					
Isopentenols	<i>E. coli</i>	~112 mg liter <sup>-1</sup>	NA <sup>b</sup>	43	53
		55 mg liter <sup>-1</sup>	NA	NA	54
		1.3 g liter <sup>-1</sup>	59.3 mg liter <sup>-1</sup> h <sup>-1</sup>	48	12
		2.23 g liter <sup>-1</sup>	NA	48	16
Pinene	<i>E. coli</i>	1.7 mg liter <sup>-1</sup>	NA	NA	10
		0.97 g liter <sup>-1</sup>	4 mg h <sup>-1</sup> g <sup>-1</sup> (DCW)	32	73
		32.4 mg liter <sup>-1</sup>	18.1 mg liter <sup>-1</sup> OD <sup>-1</sup>	NA	74
Farnesol	<i>E. coli</i>	135.5 mg liter <sup>-1</sup>	NA	48	33
Farnesene	<i>E. coli</i>	143.3 mg liter <sup>-1</sup>	NA	NA	57
		380 mg liter <sup>-1</sup>	NA	48	83
Bisabolene	<i>E. coli</i>	1.1 g liter <sup>-1</sup>	NA	96	85
		~912 mg liter <sup>-1</sup>	NA	72	11
<b>Homologous/endogenous expression</b>					
Isopentenols	<i>E. coli</i>	61.9 mg liter <sup>-1</sup>	NA	48	37
Pinene	<i>C. glutamicum</i>	~27 μg g <sup>-1</sup> (DCW)	NA	48	13
Farnesene	<i>Anabaena</i> sp.	~305 μg liter <sup>-1</sup>	~70 μg liter <sup>-1</sup> OD <sup>-1</sup> day <sup>-1</sup>	15 days	59
Bisabolene	<i>S. venezuelae</i>	~10 mg liter <sup>-1</sup>	NA	NA	15
	<i>Synechococcus</i> sp.	0.6 mg liter <sup>-1</sup>	6 μg liter <sup>-1</sup> h <sup>-1</sup>	96	58

<sup>a</sup> In hours, unless otherwise noted.<sup>b</sup> NA, data not available.

cess (MRP) (60). In this approach, the key metabolic nodes are optimized via mutations in either transcription factors or pathway enzymes (17, 60).

In combination with pathway engineering, strategies that reduce metabolic load and improve productivity include (i) localization of pathway intermediates into protein-based synthetic compartments or bacterial microcompartments (61–63), (ii) attachment of proteins (64), RNA (65), and DNA (66) on a synthetic scaffold, (iii) tagging of proteins with localization signals (67), and (iv) harnessing of efflux pumps against product toxicity (68, 69). It is well established that overexpressed heterologous recombinant enzymes form insoluble aggregates having little or no activity (70). These aggregates are known as inclusion bodies. Zhou et al. have reported that recombinant endogenous enzymes of the DXP pathway also form inclusion bodies which impart a negative impact on metabolic flux (71). Their solubility can be increased by several means, like lowering the incubation temperature, overexpressing chaperones, mutagenizing proteins, and using chemicals such as sorbitol (71). Together, utilization of metabolic engineering with other synthetic biology approaches might maximize biofuel titers in a relatively small design space.

**Tuning the endogenous DXP pathway.** It has been established that the DXP pathway is the sole source of isoprenoid supply in most prokaryotic organisms, including blue-green algae (45–48). Overexpression of DXP pathway genes has been reported for the enhanced production of isoprenoids (13, 34–37). Among the isoprenoid-based biofuels, prenol and isoprenol, collectively called isopentenols, are the simplest isoprenoid-based biofuels. The first microbial production of isopentenols was achieved by incorporating components of the heterologous MVA pathway in *E. coli* (53). However, not only is the hemiterpene-based biofuel research concentrated on the expression of the heterologous MVA pathway, but also the native DXP pathway has been optimized in *E. coli* (37).

Incorporation of a codon-optimized synthetic *Bacillus subtilis nudF* (*BsnudF*) gene in *E. coli* W3110 conferred dephosphorylation of IPP and DMAPP into isopentenols (37). Overexpression of native DXP pathway genes (*dxs* and *ispG*) (Fig. 3) significantly increased isopentenol production 3.3-fold over that of the *E. coli* strain harboring *NudF* only. Later, the titer was improved by blocking the Embden-Meyerhof-Parnas (EMP) pathway and activating endogenous pentose phosphate (PP) and Embden-Meyerhof (ED) pathways (37). It is interesting that blocking of the EMP pathway shunts glucose metabolism to the ED and PP pathways and provides a more-balanced DXP precursor (G3P and pyruvate) pool and NADPH supply. Activation of the PP and ED pathways resulted in an additional 1.9-fold increase in the isopentenol titer (37). A final titer of 61.9 mg of isopentenols liter<sup>-1</sup> was reported (Table 1) from 20 g liter<sup>-1</sup> of glucose in a 5-liter-scale batch cultivation system (37).

Monoterpene-based (C<sub>10</sub>) biofuels, such as α-pinene, camphene, and limonene, have viscosities, densities, and a net heat of combustion similar to those of the widely used missile fuel J-10 (21, 72). Microbes do not synthesize pinene naturally, and they require a heterologous PS enzyme to convert GPP into pinene. Most prokaryotes also lack endogenous GPPS and depend on *IspA* for the condensation of IPP and DMAPP into GPP. Therefore, a heterologous GPPS is required to incorporate into their genomes to increase the GPP supply. GPPS and PS enzymes from various sources, such as *Abies grandis*, *Picea abies*, and *Pinus taeda*, have been studied to enhance pinene yield in microbial hosts (13, 73, 74). Both enzymes have been introduced in microbes either with a heterologous MVA pathway (73, 74) or with an optimized endogenous DXP pathway (13). Modulation of the DXP pathway in alternate microbial hosts, such as cyanobacteria, *Corynebacterium glutamicum*, and *Streptomyces* sp., has improved biofuel yields in such hosts (13, 15, 58, 59). Combinatorial gene expression of

heterologous PS and GPPS (from *P. taeda* and *A. grandis*) with native deoxyxylulose-5-phosphate synthase (DXS) and isoprenyl diphosphate isomerase (IDI) improved pinene titers in *C. glutamicum* (13). Twelve strains of *C. glutamicum* were constructed using various combinations of PS and GPPS with either DXS or IDI or both. Coexpression of *P. taeda* PS and *A. grandis* GPPS with both endogenous DXP pathway enzymes (DXS and IDI) resulted in  $\sim 27 \mu\text{g } \alpha\text{-pinene g}^{-1}$  (dry weight of cells [DCW]) (13) (Table 1). Coexpression of PS and GPPS increased pinene production up to a detectable level; however, the fusion protein product of PS and GPPS can also be utilized to enhance pinene titer in alternate hosts.

As with pinene, microbes do not synthesize sesquiterpene-based biofuels naturally. Sesquiterpenes have been proposed as diesel fuels, and their estimated cetane value, cloud point, and energy density are also in the range of those of diesel fuels (2, 7, 15, 75). Recently, metabolic engineering approaches have been utilized in cyanobacteria to exploit their ability to produce isoprenoids from  $\text{H}_2\text{O}$  and  $\text{CO}_2$  (simplest carbon source) (14, 22, 58, 59). The advantages associated with cyanobacteria, such as fast growth compared to that of plants and yeast, high photosynthetic rate, genetic tractability, and sequenced genomes bring them to the forefront of biofuel research. Incorporation of the codon-optimized *fs* gene from *P. abies* in the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 enabled photosynthetic production of farnesene through an endogenous DXP pathway (59). The total photosynthetic accumulation of farnesene was  $\sim 305 \mu\text{g liter}^{-1}$  during 15 days of growth, with specific farnesene productivity at  $\sim 20 \mu\text{g liter}^{-1}$  optical density (OD) $^{-1}$  day $^{-1}$  (59). Further, optimization of the DXP pathway and expression of protein fusion products may improve farnesene titers in cyanobacteria. Biofuel production from cyanobacteria is a slow process, and the yield obtained is also not appropriate for biofuel purposes. However, it requires the least input in terms of growth and production. Insertion of sugar transporter systems in cyanobacteria has overcome diurnal running conditions and has provided an excellent alternative for 24-h chemical production (76). Other constraints, such as obtaining maximum photosynthetic efficiency and minimizing heat generation for the optimization of a photo-bioreactor, can be addressed by (i) providing a saturating amount of light (77) and maintaining culture depth and rate of cell mixing to avoid shelf shading (78), (ii) utilizing hyperthermophilic bacteria (79), or (iii) converting nonphotosynthetically active radiation into usable wavelengths (80).

Bisabolene, a monocyclic sesquiterpene, is a candidate biodiesel fuel and is produced by microbial hosts through the incorporation of a heterologous *BIS* gene from various plant sources (11, 15, 17, 58). Recently, attempts have been made to produce bisabolene by modulating the DXP pathway in different microbial hosts capable of utilizing alternate carbon sources (15, 17, 58). In *Streptomyces venezuelae*, FPP flux was redirected toward bisabolene synthesis via deletion of the geosmin synthase and 2-methylisoborneol (2-MIB) synthase genes in the downstream DXP pathway, with simultaneous overexpression of endogenous FPP synthase 1 (FPPS1) (15). Incorporation of a heterologous *AgBIS* gene facilitated the conversion of accumulated FPP into bisabolene in an engineered host. In order to produce bisabolene from alternate carbon sources, the maximum-bisabolene-producing strain, *S. venezuelae* SZ04, was subjected to different carbon sources, like cellobiose, cellulose, and ionic liquid (IL)-treated

switch grass. Bacteria supplemented with cellobiose yielded a bisabolene titer similar to that of glucose, while low-level production was obtained with IL-treated switch grass, and it was in the order of magnitude of pinene (10). Bacteria supplemented with cellulose failed to improve production over that of the control (15). The euryhaline cyanobacterium *Synechococcus* sp. PCC 7002 also produces its isoprenoids through the DXP pathway from  $\text{CO}_2$  and  $\text{H}_2\text{O}$ . Incorporation of the *AgBIS* gene at a neutral site (termed NSI) between two open reading frames encoding hypothetical proteins in the *Synechococcus* sp. genome produced 0.6 mg of bisabolene liter $^{-1}$  (0.3 mg g $^{-1}$  [DCW]) (58) (Table 1). The production of biofuels might be optimized in such alternate hosts via consolidated bioprocessing (CBP) approaches and utilization of protein fusion products.

**Expressing the heterologous MVA pathway.** Most studies utilizing the MVA pathway to bypass endogenous DXP pathway regulations and to increase the precursor supply for isoprenoid-based biofuel production are focused on engineering well-established prokaryotic host *E. coli* strains. Expression of the MVA pathway has enhanced isoprenoid titers severalfold over titers produced by DXP pathway modulations. Introduction of the MVA pathway in *E. coli* produced toxicity due to the accumulation of isoprenoid precursors (53). To overcome the toxicity associated with IPP and DMAPP accumulation, a library of 19,000 clones harboring *B. subtilis* genomic DNA fragments was screened in *E. coli*. The toxicity was overcome by the expression of *BsnudF*, which encodes an enzyme capable of dephosphorylating prenyl diphosphates into prenyl alcohols. After 43 h of incubation, a final titer at 112 mg of isopentenol liter $^{-1}$  was observed from engineered *E. coli* (53) (Table 1). This study opens up a gateway to explore the heterologous MVA pathway with simultaneous overexpression of bacterial homologous phosphatase and pyrophosphatase superfamilies for the overproduction of isopentenols and other high-yield isoprenoid alcohols in engineered microbes.

Encouraged by the results of the above-mentioned study, Chou and Keasling (54) screened *E. coli*-endogenous nucleoside diphosphate linked to X (NudIX) hydrolase superfamily genes in an engineered *E. coli* strain incorporated with a heterologous MVA pathway. This screening revealed that overexpression of five NudIX hydrolase superfamily genes (*nudB*, *nudF*, *nudI*, *nudJ*, and *nudM*) resulted in a  $>2$ -fold increase in the production of isopentenol compared to the production level in a control that expressed only the MVA pathway genes. Overexpression of *E. coli* NudB (*EcNudB*) resulted in the highest isoprenol production, at 55 mg of isoprenol liter $^{-1}$  from 2 g liter $^{-1}$  of glucose (54). Later, Zheng et al. (12) improved isopentenol titers by introducing components of the entire MVA pathway, including *idi* from *S. cerevisiae* (*idi1*), into *E. coli*. They also overexpressed different phosphatases and pyrophosphatases from various sources in engineered *E. coli* strains to remove pyrophosphate from IPP and DMAPP. Among all enzymes, *B. subtilis* NudF (*BsNudF*) removed pyrophosphate most efficiently and produced the highest yield of isoprenol and prenol in *E. coli* (YY158). To obtain single-isoprenol production, the conversion of IPP into DMAPP was blocked by inactivating the *idi1* gene in *E. coli* (YY158). The resultant strain, *E. coli* YY159, produced 1.3 g of a single isoprenol liter $^{-1}$ , whereas overexpressing *E. coli* NudF (*EcNudF*) with the entire MVA pathway and *idi1* gene in *E. coli* (YY168) produced 0.2 g of isopentenol liter $^{-1}$ , with  $\sim 80\%$  of the total production as prenol (12).

The studies on hemiterpene-based biofuels suggested that a

consortium of homologous/endogenous genes with heterologous pathways or genes could enhance the nonnatural production of isoprenoid-based biofuels at significant levels. In a recent study, the production of hemiterpene-based biofuels was found to be increased by several modifications in endogenous and heterologous enzymes in an engineered *E. coli* strain (16). First, the production level of isoprenol was enhanced by 60% from background level by engineering the Shine-Dalgarno sequence (the ribosomal binding site in the mRNA) of an endogenous *nudB* gene. Engineering the Shine-Dalgarno sequence also elevated protein levels and reduced intracellular IPP accumulation by 4-fold. Further, expression of a heterologous mevalonate kinase (MK) enzyme was optimized by investigating various MK enzymes from different microbes. These modifications enhanced isoprene production to a significant level, and finally, a titer of 2.23 g of isoprenol/liter was obtained (which was ~70% of the pathway-dependent theoretical yield) by using an oleyl alcohol overlay (16). To the best of our knowledge, this is the highest yield achieved for isoprenoid-based biofuel from an engineered strain. The above-mentioned studies have also established NudIX hydrolase superfamily genes as a key player having an important role in the conversion of isoprenoid precursors into biofuels.

Recently, novel MVA-mediated pathways have been successfully assembled in *E. coli* via decoupling of the MVA pathway (81, 82). Kang et al. (81), in their study, recruited phosphomevalonate decarboxylase (PMD) and an *E. coli*-endogenous phosphatase (AphA) to bypass the MVA pathway. PMD produced isopentenol directly from MVA, whereas AphA synthesized isopentenol via an intermediate, isopentenyl monophosphate (IP), from MVA monophosphate (MVAP) (81) (Fig. 3). In another study, a fatty acid decarboxylase from *Jeotgalicoccus* species (OleT<sub>JE</sub>) was introduced into *E. coli* for direct conversion of MVA into isoprenol (82) (Fig. 3). The pathway bypass reduced cellular energetic requirements and escaped the host from IPP-related toxicity. It also eliminated the cellular dependency on NudIX hydrolase superfamily proteins to convert excess IPP and IMAPP into their respective biofuels.

In an attempt to produce pinene, Bokinsky et al. (10) engineered an *E. coli* (MG1655) strain with a heterologous MVA pathway and a downstream pinene-specific enzyme, PS (Fig. 3). Since the authors used IL-treated biomass as a carbon source, the strains of *E. coli* were further engineered separately with heterologous  $\beta$ -glucosidase (*cel3A*) and  $\beta$ -xylosidase (*gly43F*) genes from *Cellvibrio japonicus* to metabolize cellulose and hemicellulose, respectively. A coculture of both the engineered strains produced 1.7 mg of pinene liter<sup>-1</sup> in MOPS-M9 medium containing 3.9% IL-treated switch grass (10). The findings of this study unlock the gateway to explore a consortium of microbes capable of digesting cellulose and hemicellulose, with the engineered microbes capable of converting simple sugar to biofuels.

As IspA quickly takes up endogenous GPP for FPP synthesis in *E. coli*, the excess GPP can also be supplied by expressing heterologous GPPS enzymes from various plant sources. Expression of PS and GPPS (from *A. grandis*, *P. abies*, and *P. taeda*) in various combinations in *E. coli* with a heterologous MVA pathway has been reported to improve pinene titers (73, 74). Coexpression of GPPS from *A. grandis* and  $\alpha$ -PS from *P. taeda* with a heterologous MVA pathway improved the pinene titer in *E. coli* BL21(DE3) (73) (Fig. 3). Later, the culture conditions were optimized to improve the pinene titer in a shake flask (73). A pinene titer at 5.44 mg

liter<sup>-1</sup> was obtained in a shake flask containing 0.25 mmol isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) as an inducer and MD beef extract as an organic nitrogen source at 30°C. Further, in a scale-up study in a fed-batch fermentor, a maximum concentration of 0.97 g of pinene liter<sup>-1</sup> was achieved at 32 h postinduction (Table 1) while a glucose concentration of less than 0.5 g liter<sup>-1</sup> was maintained. The specific productivity of pinene was recorded at 4 mg h<sup>-1</sup> g<sup>-1</sup> (DCW), with a conversion efficiency of glucose to  $\alpha$ -pinene as 2.61% (73).

Despite many attempts to produce pinene from a microbial origin, the titers obtained do not match the orders of magnitude of those of hemiterpenes (12, 16, 37) and sesquiterpenes (11, 33, 83). The possible reasons may be (i) the toxicity associated with the monoterpenes (23), (ii) inhibition of GPPS and PS by GPP, and (iii) the use of magnesium as a cofactor for catalysis (74). Improvement of efflux pumps has shown a potential to overcome product-related toxicity (69, 84) and might be utilized for monoterpene-based biofuels also. Fusion products of two successive enzymes of the isoprenoid pathway have overcome the enzyme inhibition and improved isoprenoid production in *E. coli* (74, 83). To explore the effect of fusion proteins, several GPPS-PS fusion products from *A. grandis*, *P. abies*, and *P. taeda* have been studied in *E. coli* expressing a heterologous MVA pathway (74). The GPPS-PS fusion product (Fig. 3) improved pinene titers compared to those of coexpression systems used in the same study. The highest pinene titer at 32.4 mg liter<sup>-1</sup> with a specific productivity of 18.1 mg liter<sup>-1</sup> OD<sup>-1</sup>, was achieved by expressing the *A. grandis* GPPS-PS fusion product (74). The titer was ~6-fold higher than that in the previous report of Yang et al. (73). Redirecting metabolic flux toward the DXP pathway (15, 37) and expressing the GPPS-PS fusion product (74, 83) can improve the nonnatural production of monoterpene-based biofuels in alternate microbes.

In a study,  $\alpha$ -farnesene (i) was synthesized by incorporating the apple  $\alpha$ -farnesene synthase (*afs*) gene into *E. coli* overexpressing DXS and IDI, (ii) was incorporated with the bottom portion of the MVA pathway (cells were not able to synthesize mevalonate, and mevalonate was supplied externally), or, in a third case, (iii) was incorporated with the entire MVA pathway (cells were able to synthesize mevalonate). Overexpression of DXS marginally increased the background production of  $\alpha$ -farnesene, while an ~5-fold increase was recorded in *E. coli* overexpressing IDI. Later, expression of the bottom portion of a heterologous MVA pathway showed an increase in the yield by ~15-fold, which was enhanced to ~72-fold by the protein fusion product FPPS (IspA)-AFS. Lastly, an approximately 317-fold increase (~380 mg of  $\alpha$ -farnesene liter<sup>-1</sup>) over the initial production was achieved via incorporation of the complete MVA pathway by integration with the FPPS (IspA)-AFS fusion protein (Fig. 3) (83).

Zhu et al. (85) revealed that endogenous IDI plays a key role in  $\alpha$ -farnesene synthesis *in vitro* as well as *in vivo*. Overexpression of IDI with IspA and AFS enhanced the farnesene yield up to ~2,000-fold over that produced by the control strain in *E. coli* incorporated with the MVA pathway (85). A maximum output at 731 mg liter<sup>-1</sup> was obtained 48 h postinduction in a shake flask, which was twice that previously reported (83) under the same conditions. After 96 h postinduction, farnesene production reached ~1.1 g liter<sup>-1</sup> in a shake flask (85). The overexpression of endogenous genes to direct metabolic flux toward FPP production not only enhanced biofuel production (83, 85) but also restored the growth of engineered strains (83). This suggests that a



consortium of endogenous/homologous genes with heterologous genes/pathways can be utilized to overcome the bottlenecks in the production of biofuels more efficiently.

Farnesol (FOH) is another FPP derivative, and its production was first reported in culture broth of squalene synthase-deficient *S. cerevisiae* (86). Early research on FOH production was focused solely on production from *S. cerevisiae* (41, 42, 87, 88). The extracellular fraction and cell lysate of *E. coli* overexpressing endogenous *ispA* and *idi* produce 389  $\mu\text{g}$  of FOH liter<sup>-1</sup> by the action of potato acid phosphatase (56). Incorporation of the heterologous MVA pathway and overexpression of IspA improved the FOH titer by ~350-fold over that previously reported (56) from an engineered *E. coli* strain (33). The engineered strain produced a farnesol titer at 135 mg liter<sup>-1</sup>, and endogenous phosphatases were believed to hydrolyze accumulated FPP to FOH nonspecifically. Identification and optimization of endogenous phosphatases and pyrophosphatases having specificity toward FPP might be utilized to increase the FOH yield in *E. coli*.

Later, Wang and coworkers produced a Z,E isoform of FOH (Z,E-FOH), via expression of a heterologous Z,E-FPP synthase (Rv1086) from *Mycobacterium tuberculosis* in *E. coli* (57). In nature, the chain elongation of the newly formed double bonds can proceed in two possible stereospecific configurations of E and Z. The FPPS catalyzes the condensation of GPP and IPP into FPP in four different potential configurations (E,E, E,Z, Z,E, and Z,Z) (89). *E. coli*-endogenous FPPS (IspA) catalyzes the formation of all E configurations of FPP (E,E-FPP) (55), while Rv1086 catalyzes Z condensation of GPP and IPP into Z,E-FOH in *M. tuberculosis* (90). The expression of Rv1086 in engineered *E. coli* produced 75.3 mg of FOH liter<sup>-1</sup> (E,E-FOH and Z,E-FOH), which was lower than the titer of FOH produced by the overexpression of IspA only. Coexpression of IspA in Rv1086-expressing *E. coli* enhances total FOH production but reduced Z,E-FOH due to the consumption of GPP by IspA. It is well known that prokaryotes lack endogenous GPP synthase; hence, another enzyme, IspA, is responsible for GPP synthesis, which it simultaneously takes up to form FPP (55). To increase GPP availability to Rv1068 the fusion protein IspA-Rv1068 was constructed. This allowed Rv1068 to effectively compete with IspA for GPP and IPP. Z,E-FOH at 115 mg liter<sup>-1</sup> was produced by the engineered strain harboring the IspA-Rv1068 fusion product, which was 80.7% of the total FOH production (57). The strategy of using the fusion protein might also be utilized for the production of monoterpenes in microbial hosts, where efficient GPP synthase is not available. The fusion product of monoterpene synthase with IspA may be an interesting platform for excess GPP supply to overproduce monoterpene-based biofuels. It was also reported that the Z,E- isoform of FPP did not restore the growth of the *ispA* deletion mutant and that it was not metabolized by *E. coli* for the synthesis of its essential isoprenoids (57). Utilization of isoprenoid precursor isomers also reduced the precursors' flux toward other endogenous isoprenoids. This encourages the recruitment of the precursors' isomers to minimize metabolic wastes during biofuel production.

In order to produce bisabolene, *BIS* genes from *Arabidopsis thaliana*, *Pseudotsuga menziesii*, *A. grandis*, and *P. abies* have been screened in the *E. coli* strain carrying the entire MVA pathway (11). *A. grandis*  $\alpha$ -bisabolene synthase (AgBIS) was found to be the highest producer of bisabolene (78 mg liter<sup>-1</sup>), and its codon optimization improved bisabolene yield by ~5-fold in *E. coli*. For further improvement in bisabolene synthesis, the MVA pathway

was divided into (i) an upper module to convert acetyl-CoA into MVA and (ii) a lower module to convert MVA into FPP in *E. coli*. In addition to pathway modulations, codon optimization of hydroxymethylglutaryl-CoA synthase (HMGS), hydroxymethylglutaryl-CoA reductase (HMGR), MK, and phosphomevalonate kinase (PMK) enhanced bisabolene yield in *E. coli*. Lastly, the expression of enzymes of the bottom portion of the MVA pathway was optimized by placing the genes under the control of a strong promoter. The final bisabolene titer was measured at 912 mg liter<sup>-1</sup>, and bisabolene was converted to bisabolane by chemical hydrogenation (11).

The yields obtained through an optimized DXP pathway are not as high as those from the heterologous MVA pathway, as is quite evident from Table 1. However, the efforts to increase the DXP precursors' pool size and bypassing the endogenous regulatory mechanism may contribute to high-level production of isoprenoid-based biofuels. Recently, a novel route was uncovered to enhance the isoprenoid precursors' pool via direct conversion of C<sub>5</sub> sugars to DXP (17). Overexpression of *E. coli*-endogenous *ribB* mutant *ribB*(G108S) and *yajO* genes enabled the direct conversion of ribulose-5-phosphate (Ru5P) to DXP. Expression of the RibB-DXP reductoisomerase (RibB-DXR) fusion product and AgBIS with overexpressed DXP pathway genes (*ispA*, *ispD*, *ispF*, and *idi*) (Fig. 3) produced a maximum bisabolene titer at ~9 mg g<sup>-1</sup> (DCW) (17). The alternate route from pentose to DXP synthesis may be utilized further to circumvent endogenous regulation of the DXP pathway for enhanced production of isoprenoid-based biofuel and fuel precursors. Most of the biofuels need to be hydrogenated before becoming practical biodiesel alternatives. Identification of crystal structures of geranylgeranyl reductase (GGR) from *Sulfolobus acidocaldarius* and its engineering to generate sequence variants (91) opened an innovative approach to *in vivo* reduction of isoprenoid-based biofuels. Enzymatic reduction of double bonds would also eliminate the capital cost associated with the chemical hydrogenation of isoprenoid-based biofuels.

## CHALLENGES AND FUTURE PROSPECTS

Over the past few decades, intensive research has been done to synthesize isoprenoids from biological routes (Table 1). Microbial engineering was done via (i) overexpressing endogenous/homologous genes of the DXP pathway, (ii) introducing components of the MVA pathway or the entire MVA pathway, and (iii) utilizing a consortium of homologous/endogenous and heterologous genes to achieve nonnative production as well as improve titers of isoprenoid-based biofuels (10–12, 15, 83). Despite the commercialization of isoprenoids (75, 92, 93), challenges still persist for achieving higher titers of isoprenoid-based biofuels from biological routes, which can be addressed by utilizing various computational, rational, and combinatorial approaches (63, 94).

While biomass is used for biofuel production, the yield of biofuels critically depends upon the conversion of total sugars released from biomass hydrolysis into the final product. Hemicellulosic biomass hydrolysate generally comprises up to 30% of the pentose sugars of the total carbohydrates; hence, strategies to engineer alternate pathways for xylose catabolism in biofuel-producing microbial strains might enhance the precursors' pool inside the cell (17, 95). Strain engineering efforts with balanced enzyme expression and effective recovery strategies can be utilized to achieve higher yields of the biofuels from microbial hosts. The utilization of cyanobacteria has gained considerable popularity in



recent years due to their ability to produce liquid fuels directly from the least-complex carbon source, i.e., CO<sub>2</sub>, and solar energy (14, 96, 97). However, their nutrient requirements (nitrogen and phosphorus) may place an additional strain on the already heavily loaded fertilizer industry. The utilization of cyanobacteria to generate isoprenoid-based biofuels and fuel precursors is still debatable and in the primary stage of investigation. To date, significant improvements have been made in the titers and yields of isoprenoid-based biofuels in engineered microbes. However, there is a long way to go for their commercialization, as the current energy policy relies upon volume rather than the energy content of the fuel. This requires robust fluxomics and metabolomic tools in coordination with bioprocess techniques and needs integration of other engineering disciplines for the conversion of available feedstock into desired biofuels and fuel precursors.

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