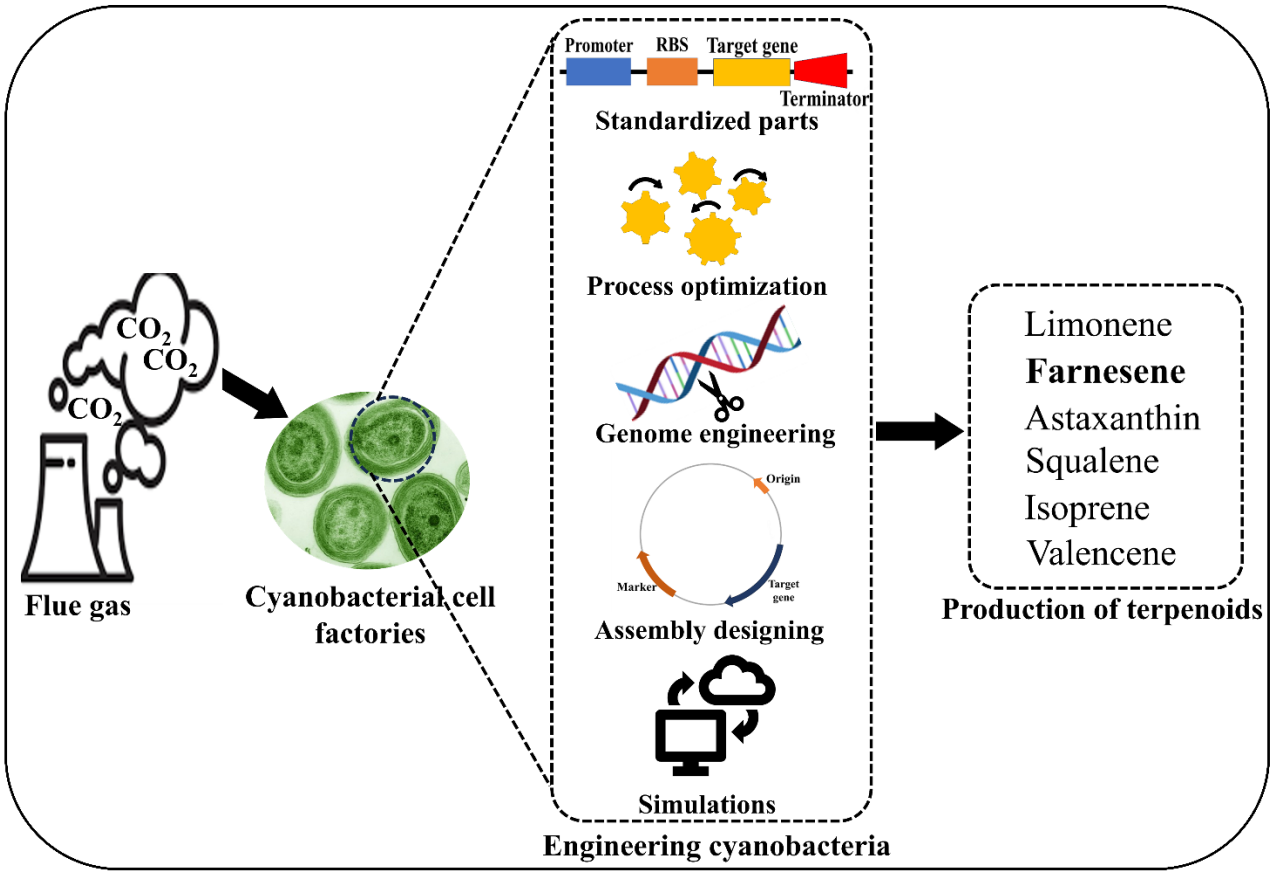
CHAPTER 1

Introduction to carbon dioxide sequestration and terpenoids production by engineered cyanobacteria*



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The global dependency on energy has created a pressing need for clean and sustainable energy resources to fulfil the scarcity of fossil fuels along with a solution for rising environmental issues. A critical imperative is to transition from fossil fuels to renewable energy sources to attain carbon neutrality and fulfil climate commitments on a worldwide scale (De La Peña et al., 2022). According to the United Nations Environment Programme 2020, global greenhouse gas emission has reached 59.1 Gt CO_2 emission, which may cause a 2.8 °C temperature rise till the end of the century (Emissions Gap Report, 2022). The current temperature anomaly is 1.17 °C with the Earth being 1.36 °C warmer than in 1850-1900 (Fig. 1.1(a)) (NASA). The primary concern is to restrict this temperature rise to 2 °C, which can be achieved by worldwide interest towards low-carbon or zero-carbon fuel production. In this regard, the UN General Assembly have already decided 17 sustainable development goals accepted by 193 countries, including affordable and clean energy (goal 7) and climate action (goal 13) (El-Emam and Khamis, 2017). A significant contribution of CO_2 or CO emission is dominated by fossil fuel combustion. It is reported that over 52 years, the global atmospheric CO_2 level has increased by 28.6% from 325 ppm (1970) to 418 ppm (2022) (Fig. 1.1(b)) (NASA).

Employing CO_2 sequestration techniques to generate value-added compounds as alternatives to fossil fuels offers a promising solution for addressing the imperative for clean energy and the escalating issue of global temperature rise. Cyanobacteria are one such organism which sequesters CO_2 to thrive and produce value-added products. In addition, they require light, which is abundant in nature. It is estimated that Earth receives ample sunlight within one and a half hour to fulfil global energy demand for a year (Department of Energy). Utilizing solar energy and CO_2 for the growth of cyanobacteria and the production of desirable compounds can contribute to the carbon-neutral economy.

Terpenoids are one such desirable compound having a wide range of applications which can be sustainably produced from cyanobacteria.

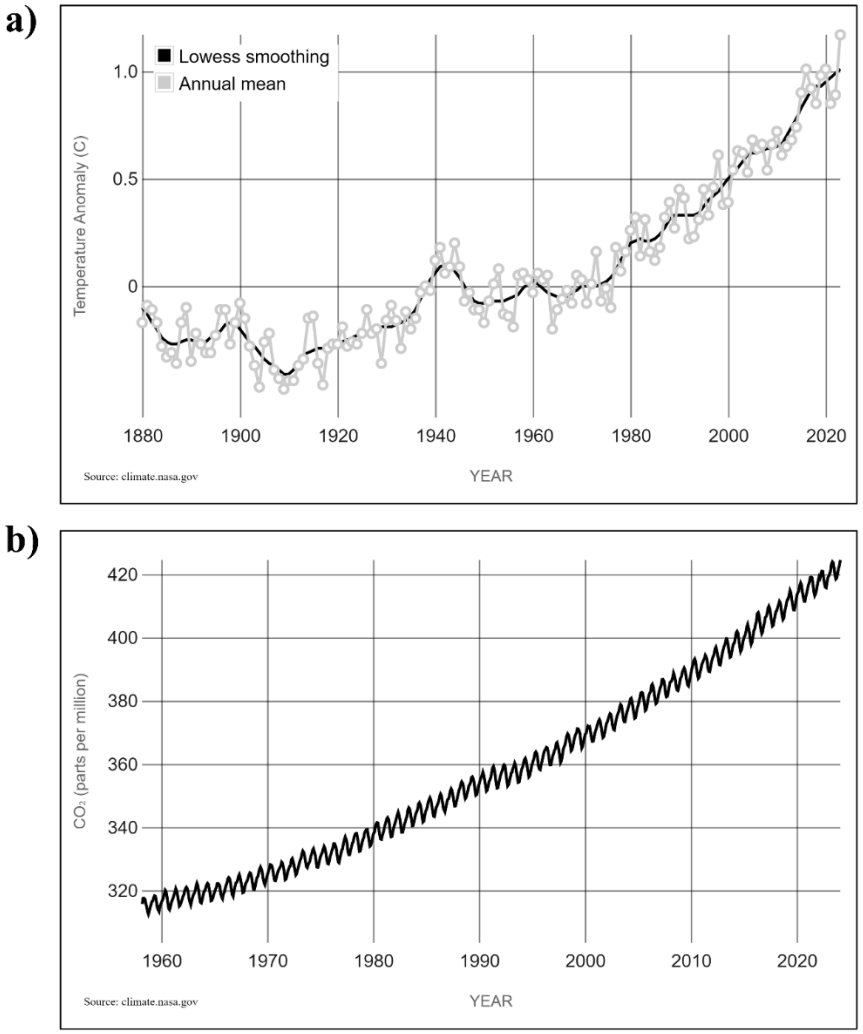

Fig. 1.1 a) Global land-ocean temperature index. **b)** Direct carbon dioxide measurement from 1958-present. Data source: climate.nasa.gov.

Terpenoids, also called isoprenoids or terpenes, are the diverse and largest class of organic compounds known in nature. Plants substantially synthesize them as secondary metabolites with repeating C5 isoprenoid units. Other living organisms such as bacteria (Rabe et al., 2016; Reddy et al., 2020), insects (Darragh et al., 2021; Leonhardt et al., 2010), and fungi (Jakubczyk and Dussart, 2020) also produce terpenoids. Over 80,000 of them have been identified and characterized so far (Pemberton et al., 2017). Such multitudinous molecules have an extensive range of applications too, both biologically and commercially.

Photosynthetic organisms rely on terpenoids as they play an indispensable role in photosynthesis (chlorophylls, carotenoids, rhodopsin). Apart from photosynthesis, they show multifunctional applications in the electron transport chain (plastoquinone), respiration (ubiquinone), membrane stability (sterols), hormones (brassinosteroid phytohormones, gibberellic acid) (Moses et al., 2013). Plants' essential oil comprises volatile terpenoids, which give them their characterized fragrances. Volatile terpenoids exude from the plant's root, stem, leaf, and fruits but chiefy by fowers. Volatile foral terpenoids prompt pollination (entomophily, ornithophily, chiropterophily) (Abbas et al., 2017). Recently, the role of floral scent in *Collinsia heterophylla* (Common name: Chinese Houses, Order: Lamiales, Family: Plantaginaceae) fowers was identified to entice bees for pollination (Larsson et al., 2021). Furthermore, these volatile terpenoids act as defensive and anti-stress compounds against biotic (insects, herbivores) and abiotic stress (temperature, light) (Block et al., 2019; Góngora et al., 2020; Zhang et al., 2019a). Occasionally in tea plants such as Oriental beauty, the distinctive aroma and favor is due to the terpenoids release against attack by tea green leafhopper (*Empoasca onukii* Matsuda) (Liu et al., 2021a).

Terpenoids have ample commercial applications in therapeutics, cosmetics, flavoring, fragrances, agrochemicals, and disinfectants (Ajikumar et al., 2008). Artemisinin, a valuable antimalarial drug, is synthesized by sweet wormwood (*Artemisia annua* L.) (Lopes et al., 2020). Long agricultural lag, fluctuations in abiotic conditions lead to variation in cost and availability of artemisinin. Since the artemisinin and artemisinin combined therapy drugs are estimated to intumesce with the combined annual growth rate of 8.5% from 2017 to 2025 with a market size of $363.8 million in 2017, biotechnological production is the pressing priority (Artemisinin market size). *Saccharomyces cerevisiae* was engineered to produce amorphadiene, a precursor for artemisinin (Westfall et al.,

2012). Aside from being an antimalarial drug, it also aids in renal and respiratory diseases, reviewed by (Cheong et al., 2020; Liu et al., 2018; Xia et al., 2020). Another multibillion dollar drug produced from *Taxus brevifolia* (Common name: Pacifc yew tree, Class: Pinopsida, Order: Pinales, Family: Taxaceae) is Taxol (Paclitaxel). Taxol is a chemotherapeutic drug approved by the Food and Drug Administration which stabilizes microtubule assembly and inhibits cell division (Weaver, 2014). To rely on natural sources for taxol extraction is not eco-friendly as the yield from the yew tree's bark is 0.001–0.005% only, requiring chopping four mature trees for treating a single patient (giving 2 g dose) (El-Sayed et al., 2019). The complexity of the taxol's structure and chemical synthesis makes its synthetic production unfavorable. Therefore, concepts of metabolic engineering help in the semisynthetic production of taxol through microorganisms to cope with these barriers (Abdallah et al., 2019). As individuals are inclining toward nature identical favors and fragrances, their market share was worth $ 30 million in 2017 (Kutyna and Borneman, 2018). Terpenes have characteristic aroma and flavors, such as vanillin (from pods of *Vanilla planifolia*), widely used prominently as a vanilla flavoring substance in bakeries. Similarly, raspberry ketone (from *Rubus idaeus*), cinnamaldehyde (from *Cinnamomum zeylanicum*), limonene (from citrus fruits) are to name some (He et al., 2019; Luziatelli et al., 2019; C. Wang et al., 2019a; Wu et al., 2019). Tetali enlightened the role of terpenes in pharmaceuticals, fragrance, flavors, and biofuels in his review (Tetali, 2019). Terpenes are proven to be a promising substitute (biofuel) for petroleum-based fuels. (Pahima et al., 2019), through computational studies (density functional theory and ab initio quantum chemistry methods), showed that terpenes have all the entailing characteristics of a biofuel. They reckoned enthalpy of combustion, enthalpy of vaporization, enthalpy of formation, cetane number, boiling point, and vapor pressure of various terpenes, namely, pinene, carene, limonene, terpinene, bisabolene, farnesene, and sabinene. The constant increase in

the extraction of value-added products from plants and animals negatively impacts the environment. A sustainable host is required to produce these products that do not compete with food production and proliferate rapidly to meet the demand. One such propitious host is cyanobacteria. Cyanobacteria fascinate researchers as they are photoautotrophic, i.e., they require sunlight and CO_2 as an energy source. Apart from this, C, P, S, N, K, and Fe source is needed, usually provided by BG-11 media in the laboratory (Pattharaprachayakul et al., 2019). They can also be cultivated in wastewater and help in wastewater management (Rueda et al., 2020). They are about 3.5 billion years old and considered to be living fossils (Schopf and Packer, 1987). Since they are the first oxygen-producing organisms, plants originated from them (Rai et al., 2021). It is also considered that the chloroplast inside the plant's cell is the cyanobacterium living inside it, a typical case of endosymbiosis (Ishikawa and Kawai-Yamada, 2019). The methylerythritol phosphate pathway (MEP), which occurs in cyanobacteria, is possessed by chloroplast in plants, giving evidence of this endosymbiotic theory. In addition to this, cyanobacteria own plants like the C2 glycolate cycle (Eisenhut et al., 2006).

Commercially, value-added products are predominantly synthesized by *Escherichia coli* and yeast. Being heterotrophic, they require a carbon source (sugar) for their growth. Shoot up in the production cost of the sugar, it is feasible to use cyanobacteria for the direct production of products from CO_2 (Cheng et al., 2019). Recent advances in the genetic toolboxes for synthetic biology of cyanobacteria expressing heterologous genes became attainable (Sengupta et al., 2018; Sun et al., 2018). Sundry tools like CRISPR/Cas9, promoters, ribosome binding site (RBS), and riboswitches were used in hosts like *Synechococcus elongatus* PCC 7942 and *Synechocystis* sp. PCC 6803, and *Synechococcus elongatus* UTEX 2973 (Chi et al., 2019; Liu and Pakrasi, 2018; Pattharaprachayakul et al., 2020; Werner et al., 2018; Yadav et al., 2021; Yu et al., 2015).

1.1. MEP vs. MVA: Upstream and downstream module of terpenoid production

Though terpenoids seem to show diversity in range and their applications, they all are initiated from 5-carbon moiety isopentenyl diphosphate (IPP) and its isomeric conformation dimethylallyl diphosphate (DMAPP). Isoprene (C_5H_8) is the most basic terpenoid. In accordance with the "isoprene rule and biogenesis of terpenic compound," step-by-step addition of DMAPP to IPP units leads to longer chain terpenoid (Ruzicka, 1953). This indicates that there is a difference of five-carbon isoprene units in terpenes and are classified based on that: hemiterpenoids (C_5), monoterpenoids (C_{10}), sesquiterpenoids (C_{15}), diterpenoids (C_{20}) to triterpenoids (C_{30}), tetraterpenoids (C_{40}), and polyterpenes (>C_{40}). The 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (also called DXP (1-deoxy-D-Xylulose-5-phosphate) pathway) and mevalonate (MVA) pathway account for the generation of IPP and DMAPP, isoprenoid precursors (Fig. 1.2). Plants possess both pathways, MVA employed in the cytosol, whereas MEP in plastids. Individually MVA pathway is operated in the cytosol of eukaryotes, archaebacteria, some bacteria, and the MEP pathway in prokaryotes (bacteria, algae, cyanobacteria). Both the pathways comprise seven enzyme-catalyzed steps to form IPP and DMAPP; however, both pathways' initial reactants differ. MEP initiates with glyceraldehyde 3-phosphate (GAP) and pyruvate, and MVA with two acetyl-CoA molecules. The first step of the MEP pathway comprehends an irreversible reaction, condensation of GAP and pyruvate to form DXP. This step is a rate-limiting step catalyzed by a rate-limiting enzyme DXP synthase (dxs) (Nieuwenhuizen et al., 2015). DXP goes through reductive rearrangement to yield MEP, which is then coupled with cytidine triphosphate (CTP) to form 4-(-cytidine 5'-pyrophospho)-2-C-methyl-D-erythritol (CDP-ME). CDP-ME then undergoes phosphorylation, cyclization, and reductive dehydration to give rise to 4-hydroxy-3-methyl-butenyl 1-diphosphate (HMBPP). Finally, HMBPP generates either IPP or DMAPP with the help of the enzyme HMBPP reductase

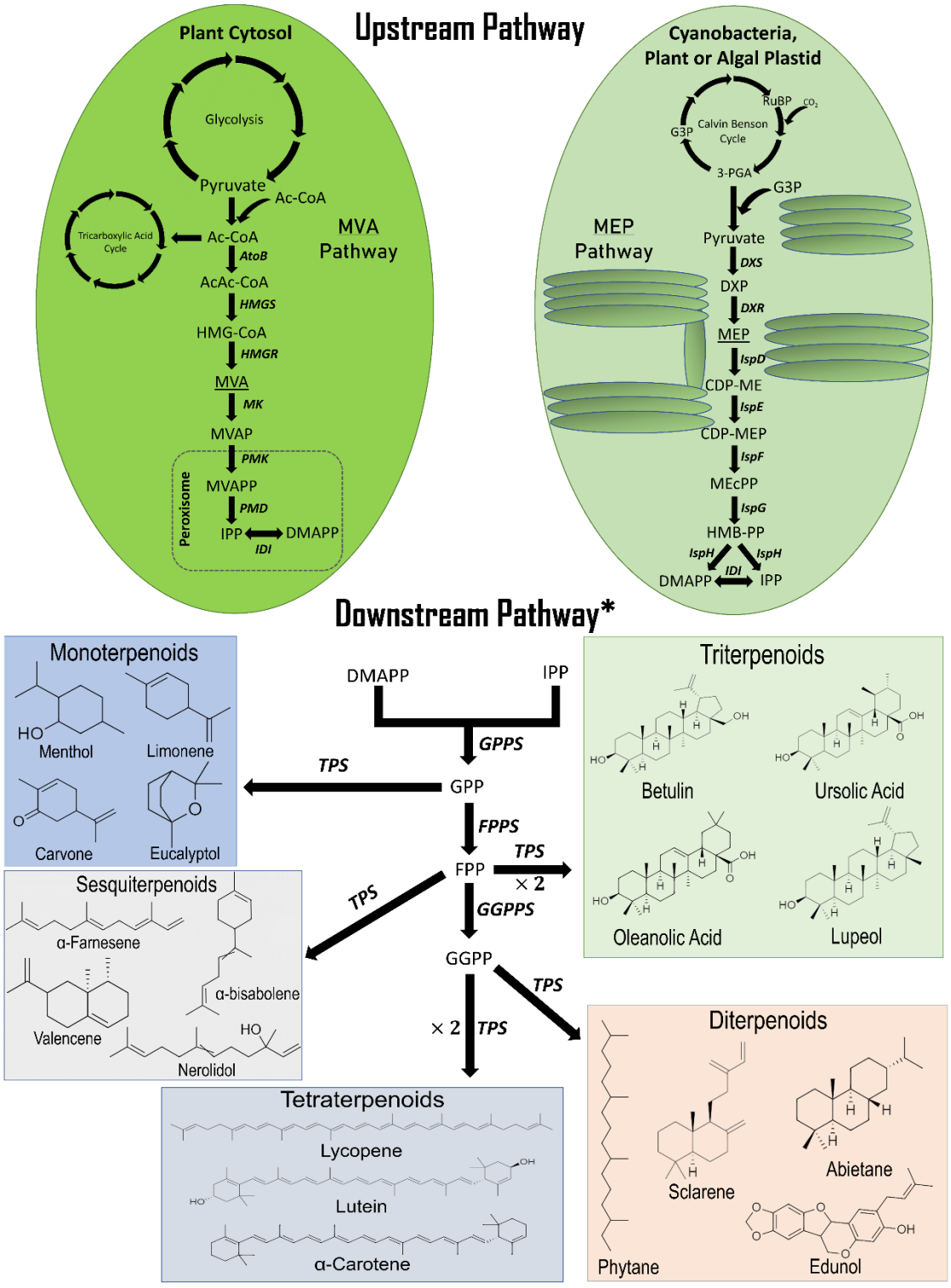

Fig. 1.2. Schematic representation of MVA and MEP pathways with different upstream and common downstream steps, and different classes of terpenoids with their structure generated from IPP/DMAPP pool. Upstream pathway abbreviations – RuBP: ribulose-1,5-bisphosphate; 3-PGA: 3-phosphoglyceric acid; Ac-CoA: acetyl-CoA; AcAc-CoA: acetoacetyl-CoA; HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; MVA: mevalonate; MVAP: mevalonate-5-phosphate; MVAPP: mevalonate-5-pyrophosphate; G3P: glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose 5-phosphate; MEP: methylerythritol-4-phosphate; CDP-ME: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; CDP-MEP: 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; MEcPP:

2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMB-PP: 4-hydroxy-3-methylbut-2-enyldiphosphate; AtoB: acetoacetyl-CoA thiolase; HMGS: HMG-CoA synthase; HMGR: HMG-CoA reductase; MK: mevalonate kinase; PMK: MVAP kinase; PMD: MVAPP decarboxylase; dxs: DXP synthase; DXR: DXP reductoisomerase: IspD: CDP-ME cytidylyltransferase; IspE: CDP-ME kinase; IspF: MEC synthase; IspG: HMBPP synthase; IspH: HMBPP reductase. Downstream pathway abbreviations – DMAPP: dimethylallyl diphosphate; IPP: isopentenyl diphosphate; GPP: geranyl diphosphate; FPP: farnesyl diphosphate; GGPP: geranylgeranyl diphosphate; IDI: isopentenyl diphosphate isomerase; GPPS: GPP synthase; FPPS: FPP synthase; GGPPS: GGPP synthase; TPS: Terpene synthase. * Downstream pathway is common to MVA and MEP pathways and is functional in cytosol and plastid. Besides that, it operates in mitochondria to generate ubiquinone.

(IspH). Therefore, the enzyme isopentenyl diphosphate isomerase (idi), which interconverts IPP and DMAPP, is not crucial for the MEP pathway and is required only to balance IPP:DMAPP ratio. In contrast, MVA commences with the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, which further condensation with the third molecule of acetyl-CoA leads to the production of 4-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). Later, the reduction of HMG-CoA forms MVA, whose twofold phosphorylation leads to the formation of mevalonate pyrophosphate. IPP is produced when the third round of phosphorylation and decarboxylation takes place. The idi is the only common enzyme to both MEP and MVA pathways and helps in the interconversion of IPP to DMAPP (Jin et al., 2020).

Generation of IPP and DMAPP from any of the two pathways nevertheless leads to the same downstream processing in any organism. Sequential head-to-tail condensation of IPP and DMAPP takes place to generate various prenyl phosphate of varying length by 5 carbon units like C_{10} geranyl diphosphate (GPP), C_{15} farnesyl diphosphate (FPP), C_{20} geranylgeranyl diphosphate (GGPP). Afterward, these prenyl phosphates, with the help of terpene synthase (TPS), give rise to numerous monoterpenes (from GPP), sesquiterpene (from FPP), diterpene (from GGPP), triterpene (from two molecules of FPP), and tetraterpene (from two molecules of GGPP) (Abbas et al., 2021; J. Wang et al., 2019b).

To bioengineer cyanobacteria, the MEP pathway is usually targeted for several reasons. First, being the native pathway, it is highly regulated than the MVA pathway (Banerjee and Sharkey, 2014). Feedback inhibition in the MVA pathway is complex making metabolic engineering problematic. Secondly, as the MVA pathway is non-native to cyanobacteria, it poses difficulties in adding the complete pathway with seven genes, each more than 1.5 kb in length (Betterle and Melis, 2019). Moreover, successful expression of the MVA pathway gives the same yield as with MEP pathway engineering (Formighieri and Melis, 2016). Thirdly, MEP is scrutinized as an 'energy-deficient' pathway converting carbon source more efficiently (83%) for precursor IPP synthesis in comparison to the MVA pathway, which utilizes only 56% carbon (Dugar and Stephanopoulos, 2011). Moreover, the MEP pathway gives a theoretical carbon yield of 30.2% compared to the MVA pathway yield of 25.2% (Perez-Gil et al., 2024).

1.2. Engineering upstream MEP/MVA pathway to redirect flux towards terpene precursors

Increased production of a terpenoid is directly proportional to the increase in precursor pool, IPP and DMAPP, which can be attained by metabolic engineering of regulatory enzymes in the MEP/MVA pathway. Upregulation of rate-limiting genes (dxs, IspD, IspF, and idi) and other enzymes of the MEP pathway increases IPP and DMAPP production. Different regulation strategies of the MEP pathway are summarized in a review (Banerjee and Sharkey, 2014). Plethora of research has been conducted in heterotrophic hosts to overexpress these genes to increase the terpenoid yield, which can be taken as a lesson to be applied to phototrophic hosts (cyanobacteria). In one of the studies in *E. coli* to produce levopimaradiene, bottleneck genes *dxs*, *IspD*, *IspF*, and *idi* were incorporated in the chromosome as an operon (Leonard et al., 2010). To increase the copy number of the genes, an operon was inserted in the expression plasmid under the control of the Trc

promoter. Approximately five auxiliary copies of the genes lead to a more than 600-fold increase in levopimaradiene synthesis. Similarly, a 24-fold increase in squalene production was observed when *dxs* and *idi* genes were overexpressed (Liu et al., 2017). Genes order also plays an influential role in metabolic engineering of the MEP pathway (Lv et al., 2013). The *dxs* expression levels were high irrespective of the order, and high isoprene levels were obtained when *dxs*, *DXR*, and *idi* were in an order similar to the native pathway. Apart from using wild-type regulatory genes, their sequences can be modified through error-prone PCR, site-directed mutagenesis, protein engineering, and recombineering (Lv et al., 2016; Volke et al., 2019). Site-directed mutagenesis of Poplar *dxs* reduced its feedback inhibition by IPP, which competes for thiamine diphosphate site on *dxs* (Banerjee et al., 2016). Recently, exogenous *dxs* and prenyltransferase (*PT*) from *Vibrio* sp. Dhg (a fast-growing microorganism) were incorporated in *E. coli* (Kim et al., 2019). The group aimed to find enzymes with higher catalytic efficiencies (1.08-fold for dxs and 1.38-fold for PT). Aside from *E. coli*, *Corynebacterium glutamicum* was engineered by overexpressing IspD and IspF to produce two terpenoids (Lim et al., 2020).

Cyanobacteria being photosynthetic, acts as an excellent source for terpenoids production. Table 1.1 summarizes the recent metabolic engineering of cyanobacteria for terpenoids production. Common cyanobacterial species used as cell factories include *Synechocystis* sp. PCC 6803, PCC 7002, *Synechococcus elongatus* PCC 7942, and recently discovered fast-growing *Synechococcus elongatus* UTEX 2973, PCC 11801, and PCC 11802 (Jaiswal et al., 2020, 2018; Ungerer et al., 2018). Exploiting the native MEP pathway in cyanobacteria is a common strategy, as stated above in the case of heterotrophic hosts. TPS and PT expression (discussed in detail in the next section) do not increase bisabolene production in Synechocystis sp. PCC 6803 (PCC 6803 hereafter) (Rodrigues and Lindberg, 2021). However, overexpression of *dxs* and *idi* doubled the titers in combination with

carotenoids. Sporadically, as IPP and DMAPP pool accumulates with no further conversion to desired products, or pigments, it hinders the cell growth. A cumbersome approach was made by engineering the entire MEP pathway by expressing native MEP genes and non-native MEP genes from *E. coli* in PCC 6803 for comparative analysis to evaluate every step (Englund et al., 2018). In the case of exogenous genes, only *dxs* and *idi* showed increased isoprene production by 14.5 and 3.4-fold, respectively. While in addition to *dxs* and *idi*, *IspG* also showed an increase in isoprene synthesis in the case of endogenous genes (Englund et al., 2018; Gao et al., 2016). The rate of mRNA and protein synthesis of overexpressed *dxs* gene and protein differ by 4 and 1.5-fold than the wild-type strain, respectively (Kudoh et al., 2017). This suggests that a high transcription rate interrupts post-translational protein folding leading to a high soluble to insoluble protein ratio. IPP pool inhibits isoprene synthesis by affecting isoprene synthase (IspS) and dxs activity (by feedback inhibition). This shows the importance of the idi enzyme to convert IPP to DMAPP, leading to an increase in DMAPP/IPP concentration and mitigating the repression on dxs and IspS (Gao et al., 2016). Similar results were acquired by (Chaves and Melis, 2018) by expressing *idi* from *Streptococcus pneumoniae*. The dxs, idi from *E. coli* with farnesene synthase and FPP synthase, increase FPP pool and hence farnesene in *Synechococcus elongatus* PCC 7942 (PCC 7942 hereafter) (Lee et al., 2017; Pattharaprachayakul et al., 2019). The same set of genes with different sources (*dxs* from *Plectranthus barbatus*, *idi* from *E. coli* and squalene synthase from *Botryococcus braunii*) were expressed in PCC 6803 for higher squalene synthesis in comparison to the strain only expressing native squalene synthase (Pattanaik et al., 2020). Analogous to squalene, another triterpenoid that can be used as a fuel precursor is botryococcene, which utilises the same intermediate presqualene diphosphate (PSPP) as squalene. Botryococcene synthase was first characterized in *B. braunii*, Race B, similar to squalene synthase (Bell et

al., 2014; Okada et al., 2004). Since the microalgae *B. braunii* has a slow growth rate, botryococcene production is not feasible using this species (Melis, 2012). The production of botryococcene from cyanobacterial cell factories has not been reported yet and can be a potential prospect for the future.

For *Synechococcus elongatus* UTEX 2973 (UTEX 2973 hereafter), the upregulated *dxs* gene is toxic (Lin et al., 2021). However, *dxs* and *idi* combined expression under lacUV5 promoter optimized by IPTG induction increased limonene synthesis. The *idi* exists in type I and type II forms based on their requirement of divalent metals and reduced flavin mononucleotide and divalent cations, respectively (Thibodeaux and Liu, 2017). Gao et al. (2016) cloned type I *idi* from *Haematococcus pluialis* and *Saccharomyces cerevisiae* and type II *idi* from *Synechococcus elongatus* and *Bacillus subtilis* in PCC 7942. The strain expressing *idi* from *S. cerevisiae* showed the highest isoprene synthase activity and, consequently, high isoprene synthesis (without *dxs* overexpression).

The density of cells present in the production media defines the product titers. The diluted density of cells results in low production of the desired product. Therefore, high-density cultivation (HDC) systems are sought, which accumulate biomass, resulting in higher product formation. Dienst et al. (2020) observed bisabolene production of 7.4 mg/L from *Synechocystis* sp. PCC 6803 (expressing bisabolene synthase) in MC1000 multicultivator system. The production using the same strain reached 179.4 mg/L in HDC. The HDC 6.10 starter kit (CellDEG) utilizes carbonate buffer as a CO_2 source through membrane-mediated technology. The same strain (expressing bisabolene synthase, *dxs*, *idi*, and FPPS) produced 9 mg/L bisabolene in the multicultivator, whereas produced 200 mg/L bisabolene in the HDC system (Rodrigues and Lindberg, 2021).

As seen in the MEP vs. MVA section, the MVA pathway presents some limitations over the MEP pathway to express in a heterologous host. Nonetheless, the expression of

MVA pathway enzymes in heterotrophic and up to some extent in photosynthetic organisms has been explored. Much experimentation has been done by engineering *E. coli* for the non-native MVA pathway, which can be taken as an epitome for engineering cyanobacteria as cell factories for terpenoid production (Navale et al., 2021). MVA pathway genes can be expressed alone as the MEP pathway is already present in the host (*E. coli* and cyanobacteria), or MVA and MEP pathway genes can be overexpressed concomitantly. As MVA is a foreign pathway in prokaryotes, all the six genes have to be expressed in the host, namely *AtoB* (acetoacetyl-CoA thiolase), *HMGS* (HMG-CoA synthase), *HMGR* (HMG-CoA reductase), *MK* (mevalonate kinase), *PMK* (mevalonate 5-phosphate kinase), and *PMD* (mevalonate 5-pyrophosphate decarboxylase). These genes can be expressed under the influence of different promoters like trc and T7 and in plasmids with varying copy numbers (Liu et al., 2019a, 2019b; Nybo et al., 2017). One of the commercially available vectors, ready to use, consisting of all the six genes, was deposited by (Peralta-Yahya et al., 2011) in the addgene plasmid repository (Addgene plasmid # 35151). MVA pathway genes can be taken from several heterologous sources, for instance, *AtoB*, *HMGS*, and *HMGR* genes from *Enterococcus faecalis*, *MK* from *Methanosarcina mazei*, *PMK* from *Streptococcus pneumoniae*, and *PMD* from *Clostridium acetobutylicum* (Liu et al., 2019a). A high concentration of IPP/DMAPP proves to be fatal to the cells, and therefore the expression of MVA pathway genes needs to be regulated (Liu et al., 2019b). One way to reduce gene expression is to express the upper half of the pathway genes in a single polycistron and the other half in another polycistron, in a low copy number plasmid. The highest terpenoid producing strain expresses *AtoB, HMGS, HMGR*, and *idi* under T7 promoter; *MK*, *PMK*, *PMD* promoted by Trc; and terpene synthase expressing individually. Li et al. (2019) showed that amalgamation of MEP and MVA pathways increased cis-abienol titers by 7 and 31-fold, respectively. Another way to regulate the pathway is to use

Table 1.1 Summary of recent advances in metabolic engineering of cyanobacteria for terpenoids production.

Pathway Engineered	Host Strain	Terpenoid synthesised	Characteristic(s) of modified strain	Maximum yield		References
				mg/L	mg/g DCW	
Upstream MEP and Downstream	UTEX 2973	Limonene	Single nucleotide mutated *GPPS* gene, with co-expressed *lims*, *dxs*, and *idi*	16.4[a]	–	Lin et al. 2021
	PCC 6803	Bisabolene	Expresses *Cfdxs*, *sidi*; AgB, EcFPPS	9[b] 200[c]	17[b] –	Rodrigues and Lindberg 2021
		Squalene	*shc* gene inactivated by replacing with *BSS*. Also expresses *Cfdxs*, *idi*, and EcFPPS	5.1[d]	–	Pattanaik et al. 2020
		Astaxanthin	Expresses CrtZ and CrtW with *dxs*	1.089[a]	–	Shimada et al. 2020
		Isoprene	Expresses *idi*, *dxs*, and EgIspS	1.0[e]	2.8[e]	Englund et al. 2018
			Expresses *fni*, and *IspS* fused with *cpcB*	–	12.3[f]	Chaves and Melis 2018
		β-Phellandrene	Expresses *GPPS* and *PHLS*	–	24.0[f]	Betterle and Melis 2019
		Geranyllinalool	Expresses *NaGLS*	–	0.36[a]	Formighieri and Melis 2017
		Lycopene	Expresses *dxs*, *GGPPS*, *Crtb*, and *Crt*I	6[g]	1.48[g]	Taylor et al. 2021
	PCC 7942	Farnesene	Expresses *dxs*, *idi*, *EcFPPS*, and *AFS*, while replacing *ccm* gene cluster with *bicA* and *CA* gene	5.0[h]	–	Lee et al. 2021
		Farnesene	Expresses RBS optimised *AFS* with *dxs*, *idi*, and *EcFPPS*	5.66[a]	–	Pattharaprachayakul et al. 2019
		Farnesene	Co-expression of *dxs*, *idi* and *EcFPPS* with *AFS*	4.6[h]	–	Lee et al. 2017
		Squalene	Expresses *dxs*, *idi*, *EcFPPS* and *SQS*	4.98[a]	–	Choi et al. 2016
		Amorphadiene	Expresses *dxs*, *idi*, *EcFPPS* and *ADS* under trc promoter	19.8[a]	–	Choi et al. 2016
Downstream	PCC 6803	Bisabolene	Optimized RBS for *AgB* and *EcFPPS*	22.2[a]	–	Sebesta and Peebles 2020
			Expresses *AgB* under PetE promoter	7.4[b] 179.4[i]	–	Dienst et al. 2020
		Valencene	Expressed *CnValCS* and *EcFPPS*	9.62[a]	2.88[a]	Matsudaira et al. 2020
		Manoyl oxide	Expresses *CfTPS2* and *CfTPS3*	2[j]	–	Vavitsas et al. 2017
	PCC 7002	Astaxanthin	Expresses *CrtZ* and *CrtW* under psbA2 promoter	3.35[a]	3.0[a]	Hasunuma et al. 2019
Upstream MEP, Downstream, PHB and glycolate synthesis	PCC 6803	Isoprene	Expresses *IspS* gene in place of pta gene; *dxs*, *idi*, *IspD*, *IspE* in place of *phaCE* gene; deleted *glcD1* and *glcD2* gene	0.084[a]	–	Zhou et al. 2021
Downstream and PPP	PCC 6803	Limonene	Expresses *rpi*, *rpe* gene with *GPPS* and *lims*	6.7[a]	–	Lin et al. 2017

Table 1.1 (Continue)
Strains – PCC 6803: *Synechocystis* sp. PCC 6803; PCC 7942: *Synechococcus elongatus* PCC 7942; UTEX 2973: *Synechococcus elongatus* UTEX 2973; PCC 7002: *Synechococcus* sp. PCC 7002.
Abbreviations – *DCW*: dry cell weight; *GPPS*: geranylgeranyl diphosphate synthase; *lims*: limonene synthase from *Mentha piperita*; *EgIspS*: isoprene synthase from *Eucalyptus globulus*; *cfdxs*: DXP synthase from *Coleus forskohlii*; *sidi*: IPP/DMAPP isomerase from *Synechocystis* sp. PCC 6803; *AgB*: bisabolene synthase from *Abies grandies*; *fni*: idi from *Streptococcus pneumoniae*; *cpcB*: β-subunit of phycocyanin; *PHLS*: β-Phellandrene synthase; *NaGLS*: geranyllinalool synthase from *Nicotiana attenuate*; *dxs*: DXP synthase; *GGPPS*: GGPP synthase; *CrtB*: phytoene synthase; *CrtI*: phytoene desaturase; *EcFPPS*: FPP synthase from *Escherichia coli*; *BSS*: squalene synthase from *Botryococcus braunii*; *shc*: squalene hopase cyclase; *CnValCS*: valencene synthase from *Callitropsis nootkatensis*; *CfTPS2*: diterpene synthase TPS2 from *Plectranthus barbatus*; *CfTPS3*: diterpene synthase TPS3 from *Plectranthus barbatus*; *rpi*: ribose 5-phosphate isomerase; *rpe*: ribulose 5-phosphate 3-epimerase; *RBS*: ribosome binding site; *AFS*: farnesene synthase gene from *Malus domestica*; *CrtZ*: β-carotene hydroxylase from *Brevundimonas* sp. *SD212*; CrtW: β-carotene ketolase from *Brevundimonas* sp. SD212; *ADS*: amorphadiene synthase from *Artemisia annua*; *SQS*: squalene synthase from *Saccharomyces cerevisiae*; *ccm*: β-carboxysome; bicA: bicarbonate transporter of PCC 6803; *CA*: carbonic anhydrase; *pta*: phosphotransacetylase; *IspD*: 2-C-methyld-erythritol 4-phosphate cytidyltransferase; *IspE*: 4-diphosphocytidyl-2-Cmethyl-d-erythritol kinase; *phaCE*: polyhydroxybutyrate (PHB) synthesis; *glcD1* and *glcD2*: glycolate dehydrogenase; *PPP*: Pentose phosphate pathway.
Culture conditions – [a]shake flask; [b]MC1000 multicultivator system; [c]high-density cultivation system; [d]flat panel bioreactor; [e]screw cap bottles; [f]one-liter fed-batch bioreactor; [g]25 ml vented culture flask; [h]100 ml duran bottle; [i]high-density cultivation system in two-step semi-batch mode; [j]50 ml glass tube.

integrative vectors and strictly analyze the number of copies of the genes needed for balanced expression, as Hussain et al. (2021) show for lycopene production. The entire MVA pathway for the first time was introduced in cyanobacteria by Bentley et al. (2014) for isoprene production by integrating the pathway genes. The introduction of the MVA pathway instigates a 2.5-fold boost in isoprene yield. Native MEP pathway accompanied by MVA pathway increased flux towards DMAPP and IPP pool, which further enhanced β-phellandrene yield (Betterle and Melis, 2019; Formighieri and Melis, 2016).

1.3. Engineering downstream pathway assisting precursor pool to terpenoid synthesis

It is important to abet IPP and DMAPP pool towards the sink, i.e., desired terpene, to avoid feedback inhibition of upstream pathway enzymes. Downstream processing is the connecting link between precursor (IPP and DMAPP) and final terpene product. It involves two categories of enzyme prenyltransferase and terpene synthase. Prenyltransferase converts DMAPP to GPP, FPP, and GGPP. Prenyltransferase that aids in this conversion

are GPP synthase, FPP synthase, and GGPP synthase, respectively, common to MEP and MVA pathway irrespective of the terpenoid to be produced. GPP, FPP, and GGPP act as an immediate substrate for terpene synthesis, and therefore prenyltransferase engineering is vital. Further, TPS comes into play, converting GPP, FPP, and GGPP into monoterpenes, sesquiterpene, and diterpene.

TPS is usually not present in cyanobacteria and has to be endowed with heterologous expression. The key impediment in using TPS is its low turnover number (Kcat), which spans in the range of 3-4 s^{-1} (Betterle and Melis, 2019). This barrier can be conquered by escalating TPS concentration by overexpressing the TPS gene or using protein fusion constructs (Fig. 1.3). This fusion can be between TPS and any other gene like β-subunit of phycocyanin (CpcB) (Betterle and Melis, 2019; Chaves et al., 2017). Phycocyanin, a copious protein in cyanobacteria, indicates that the gene is under strong expression (promoter, RBS) control (Zhou et al., 2014). Fusing any gene with CpcB will result in its overexpression. Melis's group exploited this approach by creating several fusion constructs with CpcB, for instance, β-phellandrene synthase (PHLS), isoprene synthase (IspS), geranyllinalool synthase (GLS) (Betterle and Melis, 2019; Chaves et al., 2017; Formighieri and Melis, 2017, 2016). CpcB fusion with PHLS ensued overexpression of PHLS and increased its concentration in the cell to 20%, thereby improving PHL yield to 100-fold (Formighieri and Melis, 2015). In consecutive years, the CpcB|PHLS construct was used in conjunction with the co-expression of MVA pathway genes and GPPS (Betterle and Melis, 2019, 2018; Formighieri and Melis, 2016). In continuation to the previous study using CpcB|PHLS fusion construct with entire heterologous MVA pathway gene and GPPS amplified the yield (Formighieri and Melis, 2016). Apart from using native genes for fusion construct, other heterologous genes also show high expression in cyanobacteria (Betterle and Melis, 2018). These heterologous genes include kanamycin (nptI) and chloramphenicol

(cmr) resistance genes. The full-length version of nptI and curtailed version of nptI and cmr were fused with GPPS. CpcB|PHLS with nptI|GPPS gave the highest yield. While the partial cmr sequence from 5' end to 87 nucleotides, i.e., 29 amino acids called cmr29 fused with GPPS, produced about 2.42 mg PHL per gm of DCW. Similarly, nptI|GPPS followed by half of the MVA pathway enzymes resulted in 24 mg PHL per gm of DCW (with CpcB|PHLS and other half MVA pathway enzymes) (Betterle and Melis, 2019). All these strategies resulted in the accumulation of GPPS and PHLS inside the cell factory and increased PHL yield. Other terpenoids such as isoprene and geranyllinalool yield were also escalated by fusion strategy. Chaves et al. (2017) used linker amino acid sequences of varying lengths between CpcB and isoprene synthase (IspS) as there is a decrease in specific activity of IspS by leader sequences of CpcB. Four different linker lengths were used, L7, L10, L16, and L65. CpcB|L7|IspS showed a 27-fold increase in isoprene synthesis than CpcB|IspS, even though only 10% of IspS's activity was retained. Diterpene geranyllinalool (GL) is synthesized as both extracellular and intracellular products with the help of GLS (Formighieri and Melis, 2017). 30-40% GL exude out of the cells and remaining manifests inhibitory effect on PCC 6803 like longer doubling time. CpcB|GLS construct produced up to 390 µg of GL per gm of dcw even with a prolonged doubling time. Cpc operon consists of five genes CpcA, CpcB, CpcC1, CpcC2, and CpcD. α and β subunits of phycocyanin are encoded by CpcA and B, respectively. In contrast, linker polypeptides are encoded by CpcC1, CpcC2, and CpcD, all of which combine to form a phycobilisome, light-harvesting complex. The fusion constructs with CpcB, such as CpcB|PHLS, upregulate when other operon genes are expressed simultaneously (Valsami et al., 2020). Choi et al. (2017a) show that PT and TPS can also be fused by fusing FPPS and squalene synthase in PCC 7942. The engineered strain was further upscaled to be grown in a 6 L photobioreactor.

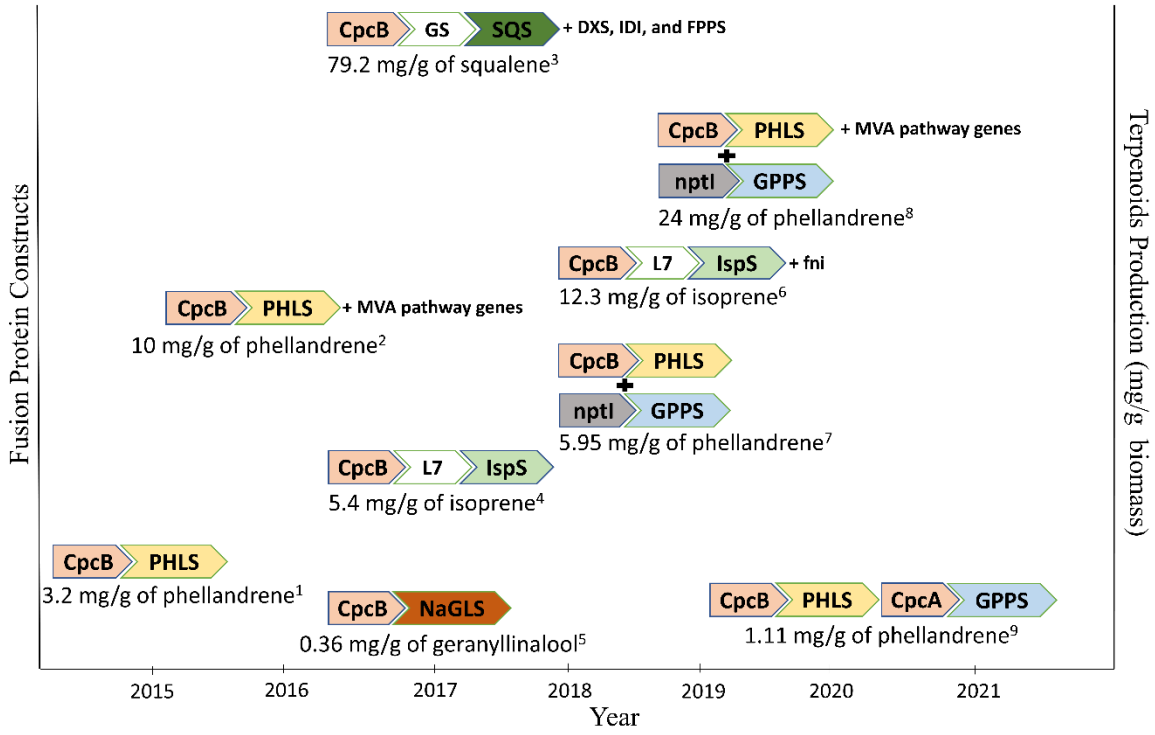

Fig. 1.3. Fusion protein constructs made over the years yielding high terpenoids titer (mg/g biomass). Abbreviations – CpcA: α-subunit of phycocyanin; CpcB: β-subunit of phycocyanin; fni: IPP from Streptococcus pneumoniae; L7: 7-amino acid linker PMPWRVI; PHLS: β-phellandrene synthase; nptI: Kanamycin resistance gene; GPPS: Geranyl diphosphate synthase; SF: Short flexible linker of 4-amino acids, GGGS; NaGLS: geranyllinalool synthase from Nicotiana attenuate. References – [1]Formighieri and Melis, 2015; [2]Formighieri and Melis, 2016; [3]Choi et al., 2017a; [4]Chaves et al., 2017; [5]Formighieri and Melis, 2017; [6]Chaves and Melis, 2018; [7]Betterle and Melis, 2018; [8]Betterle and Melis, 2019; [9]Valsami et al., 2020. The graph is only for representation and not to the scale.

Terpene synthase and prenyltransferase engineering does not limit to CpcB promoter only but also to other promoter sequences, altered ribosome binding sites, codon optimization, and mutation of the genes. The expression of amorphadiene synthase and squalene synthase under the influence of trc promoter was analyzed in PCC 7942 (Choi et al., 2016). The recombinant strain expressing TPS alone showed less yield until the FPPS was not overexpressed. FPPS engineered strains rendered a 12-fold and 50,000-fold increase in amorphadiene and squalene production, respectively (with MEP pathway genes, *dxs*, *DXR*, and *idi*). A nearly similar strain with some further modifications was cultivated in a photobioreactor utilizing industrial flue gas as a carbon source for the generation of squalene (Choi et al., 2020). Evolutionary engineering of PCC 7942 by optimizing RBS

(with the help of RBS calculator) of farnesene synthase increased farnesene synthesis by two-fold (Pattharaprachayakul et al. 2019). RBS affects translation initiation rate (TIR) and hence the protein synthesis. Strain having low TIR shows high farnesene production (about 5.66 mg/L). Similar work in PCC 6803 was done to synthesize bisabolene (7.8 mg/L in five days) by codon-optimized bisabolene synthase and different RBS sequences (Sebesta and Peebles, 2020). Single nucleotide polymorphism mutation in GPPS in UTEX 2973 (having the shortest doubling time of 1.9 hrs) produced limonene at a rate of 8.2 mg/L/day (Lin et al. 2021). Mutation in GPPS combined with a mutation in outer membrane protein B, different RBS sequences for GPPS, and overexpression of MEP pathway genes led to this increased rate of limonene production. Not being a native enzyme in cyanobacteria, TPS has to be taken from a heterologous source such as plants. The source from which TPS is excised determines its specific activity, k_{cat}, and expression. Matsudaira et al. (2020) showed valencene synthesis by employing valencene synthase (*VLS*) gene from three different sources (*Citrus sinensis*, *Vitis vinifera*, and *Callitropsis nootkatensis*). PCC 6803 strains having *VLS* from *C. sinensis* and *V. vinifera* barely showed any signs of valencene production, while *VLS* from *C. nootkatensis* produced 9.62 mg/L of valencene.

Lately, an outbreak of novel coronavirus has led to global pandemic questions about biosafety and environmental risks of recombinant strains. In line with this concern, ion-repressible promoters are used which does not allow cyanobacteria to grow in tap water (Zhou et al., 2019). *Synechococcus* sp. PCC 7002 showed high CO_2 requirement as carboxysome shell protein and carbon concentrating mechanism genes were deleted (Clark et al., 2018). In terms of farnesene production, biocontainment strain was developed by deleting genes encoding for β-carboxysome and carbon concentrating mechanism in PCC 7942 (Lee et al. 2021). The strain produced about 5 mg/L of farnesene and will not grow and produce farnesene at 100% air bubbling.

1.4. Challenges

Terpenoids being complex in structure, pose difficulty in their chemical synthesis. Therefore, the biological synthesis of terpenoids is preferred. Cyanobacteria, which are considered green *E. coli* because they require sunlight and CO_2 as energy and carbon source, are used as cell factories for terpenoid production. A photosynthetic organism has a carbon partitioning issue, in which most of the carbon competes for sugar biosynthesis, biomass cumulation, terpenoid synthesis, and other biosynthetic pathways (Melis, 2013). Lindberg and co-workers demonstrated flux analysis in PCC 6803, showing only 5% of carbon is allotted to terpene synthesis, in contrast to sugar biosynthesis, 80% (Lindberg et al., 2010). Eliminating competing pathways is one of the strategies to increase terpenoids production. Squalene, a triterpenoid, acts as a precursor for sterols and hopanoids in eukaryotes and prokaryotes, respectively. Accumulation of squalene requires inactivation of the gene(s), which further converts it. One such gene is squalene hopene cyclase (*shc*) which converts squalene to hopanoids. Inactivation of *shc* in PCC 6803 leads to accumulation of 5.1 mg/L of squalene (with co-expression of *dxs*, *idi*, and *FPPS* from different sources) (Pattanaik et al. 2020). The MEP pathway is energy-consuming and requires ATP and NADPH generated by photosynthesis. Photorespiration is a competing pathway for this ATP and NADPH and affects terpene production. Deleting glycolate dehydrogenase encoding genes (*glcD1* and *glcD2*) essential for photorespiration has been shown to increase isoprene yield (Zhou et al., 2021). One challenge with blocking photorespiration in the strain is the generation of a high carbon dioxide-requiring (HCR) strain. Surprisingly, Zhou et al. (2021), with impaired photorespiration, do not show an HCR phenotype.

Isoprene and other terpenoids can be toxic to cyanobacterial cells. However, the volatile nature of most of them and extraction with the help of overlay of non-polar solvents

(dodecane, hexadecane, tetradecane) lets easy escape from the cells (Choi et al. 2016; Gao et al. 2016; Pattharaprachayakul et al. 2019; Matsudaira et al. 2020). Prenyl phosphate, FPP, too, is found to be noxious for the growth of cyanobacteria. This can be overcome by increasing the activity or number of copies of TPS (Choi et al. 2016, 2017a). In addition to this, FPP synthase expression under the influence of strong inducible promoters like tic (having two lac operators) keeps low expression levels until induced by IPTG (Sebesta and Peebles 2020). Recently, it was perceived that some tools from genetic toolboxes, like the CRISPR/Cas system, are lethal to cyanobacteria (Pattharaprachayakul et al., 2020). Adopting Cas12 instead of Cas9 proves to be less toxic.

Terpene synthases are slow enzymes; hence, selecting a terpene synthase with high Kcat and low Km is pivotal in terpenoids biosynthesis. The same TPS within different hosts shows a conspicuously distinct activity level (Loeschcke et al., 2017). One possible reason for this could be the codon biasing, for which reason the codon-optimized version of TPS is used (Chaves and Melis, 2018; Betterle and Melis, 2019; Matsudaira et al., 2020; Lin et al., 2021). Moreover, this limitation can also be conquered by fusing TPS with genes under strong promoter influence (explained in the previous section).

To be genetically modified, comprehension of an organism's complete genome sequence is requisite. Cyanobacteria are advantageous in this case as genome information of the common hosts like *Anabaena* sp. PCC 7120, *Synechocystis* sp. PCC 6803, *Synechococcus elongatus* PCC 7942, *Synechococcus* sp. PCC 7002 is available at uniport.org. In comparison to *E. coli*, cyanobacteria have a high doubling time. A newly discovered cyanobacterial strain, *Synechococcus elongatus* UTEX 2973, has the shortest doubling time of 1.9 hrs, which can be further reduced to 1.5 hrs under bright light and is the fastest-growing cyanobacteria (Yu et al. 2015; Ungerer et al. 2918). Being polyploidy in nature, cyanobacteria are arduous to create homozygous strains. CRISPR/Cas comes to

the rescue for this challenge and is efficiently used to produce homozygous mutants (Choi and Woo, 2020).

1.5. Scope and objective of the current study

Despite the challenges faced in engineering cyanobacteria, carbon partitioning, low yield, and toxicity to the cells, the photoautotrophic production of terpenoids is gaining prominence. Recent advances in genetic toolboxes of cyanobacteria by Sengupta et al. (2018) and Sun et al. (2018) make it easier to manipulate genetic pathways towards the compound of interest. Over-expression of bottleneck enzymes of MEP pathway increases DMAPP/IPP pool which further increases terpenoid production. This also reduces carbon competition towards other necessary pathways like pigment formation, sugar synthesis (Melis, 2013). Besides the MEP pathway, introducing a heterologous MVA pathway can also escalate the precursor pool. Expressing the entire MVA pathway with six heterologous genes is laborious, and much work has to be done in this area. The end step in terpenoid synthesis requires terpene synthase, which converts precursors into a different class of terpenoids. Terpene synthases are non-native to cyanobacteria and thereby have to be engineered. Selection of source for terpene synthases has to be chosen wisely with high kcat and codon optimization according to the host is to be done. At times, implementing all these strategies does not give good yield of terpenoids which could be due to the slow growth rate of cyanobacteria. The Discovery of UTEX 2973, PCC 11801, and PCC 11802, having the highest photoautotrophic growth rate, deciphered the issue (Yu et al. 2015; Ungerer et al. 2018; Jaiswal et al. 2018; Jaiswal et al. 2020).

In the present work, fast-growing cyanobacteria *Synechococcus elongatus* UTEX 2973 was employed as a host organism for sustainable farnesene production. The integration vector strategy was utilized to genetically engineer UTEX 2973. For this purpose, three integration vectors were constructed to aid in integrating gene(s) of interest

at the neutral genomic site of UTEX 2973. The first integration vector developed was designed to direct the farnesene synthase gene to the neutral site I (NSI) of genomic UTEX 2973. The second and third integration vectors were designed to direct MEP pathway bottleneck genes *dxs* and *idispA* (*idi* and *ispA* fused together) to the neutral site II and neutral site III of the genomic UTEX 2973. Further, the constructed vectors through triparental conjugation were transformed into UTEX 2973. The modified strains were then characterized and checked for farnesene production.

After the successful lab-scale experiments, to check the economic viability and environmental impacts of the process at a larger scale, a techno-economic analysis and life cycle assessment were performed. In the study, a conceptual plant design of 90 tonnes annual capacity was assumed. Industrial flue gas is the major source of CO_2 which was utilized as the carbon source for genetically engineered cyanobacteria for farnesene production. The farnesene productivity was assumed to be 2.57 g/m^3/day as obtained from lab-scale experimental results. The facility has three main units, namely gas supply, farnesene production and farnesene recovery unit. Aspen Plus software was used for the simulation of the process. On the basis of the simulation, an in-house Excel spreadsheet was generated, which was used to calculate capital expenditure (CapEx), operating expenditure (OpEx), revenue generation and minimum farnesene selling price (MFSP). Based on the results, a sensitivity analysis was done to find out the factors influencing MFSP. Furthermore cradle-to-gate LCA of farnesene was done to assess the environmental impacts associated with the product and process using CCaLC2 LCA software.

Following a thorough review of literature and identified research gap outlined in Chapter 2, the objectives of the study were determined which are:

- **Selection of cyanobacterial strain and construction of integration vector system.**

a) Preparation of integrative vector (pAM2991-*AFS*) for *AFS* gene.

b) Preparation of integrative vector (pBbE1cNSII-*dxs*) for *dxs* gene.

c) Preparation of integrative vector (pBbE1kNSIII-*idispA*) for *idispA* gene.

- **Modification of cyanobacteria for farnesene production.**

a) Genomic Integration of *AFS*, *dxs* and *idispA* genes through triparental conjugation.

b) Characterization of engineered strains.

c) Farnesene production studies by engineered strains.

- **Techno-economic analysis of farnesene production using engineered cyanobacteria.**

1.6. Thesis outline

The thesis has been outlined in six chapters

Chapter 1: The chapter deals with the increasing concern about the rise in greenhouse gases, the role of carbon dioxide in temperature rise, the sequestration of carbon dioxide using cyanobacteria, the production of terpenoids from cyanobacteria, followed by the objectives of the study.

Chapter 2: This chapter focuses on the recent advances in metabolic engineering strategies employed by microbial cell factories for farnesene production. Additionally, it offers insights into the challenges and shortcomings of transitioning engineered strains from lab scale to industry. Further, numerous applications of farnesene and its derivatives are thoroughly investigated in the field of biofuel, to be used for crop protection, as an alternative to thermoplastic elastomers, its pharmacological relevance, and other miscellaneous applications such as skin-whitening agent, flavorant in e-cigarettes, and production of Vitamin E. This is followed by the techno-economic analysis and life cycle

assessment of the farnesene production at the industrial stage. The research gap identified after an extensive review of the literature is also mentioned.

Chapter 3: In this chapter, the construction of three integration vectors is shown. The vectors are constructed according to the host organism, i.e., *Synechococcus elongatus* UTEX 2973, to direct the *AFS*, *dxs*, and *idispA* genes to the genomic neutral site.

Chapter 4: This chapter focuses on the metabolic engineering of *Synechococcus elongatus* UTEX 2973 by transforming the constructed integrative vectors. The vectors were transformed into UTEX 2973 through homologous recombination, which is shown in detail in the chapter. Further, the engineered strains were characterized and checked for farnesene production.

Chapter 5: This chapter deals with the designing and simulation of the conceptual farnesene production plant. The plant utilizes carbon dioxide from the flue gas, and farnesene is produced by engineered cyanobacteria. Further, the techno-economic and life cycle assessment of the plant is done to check the economic and environmental feasibility.

Chapter 6: This chapter outlines the overall conclusion of the thesis and future prospects of the current study.