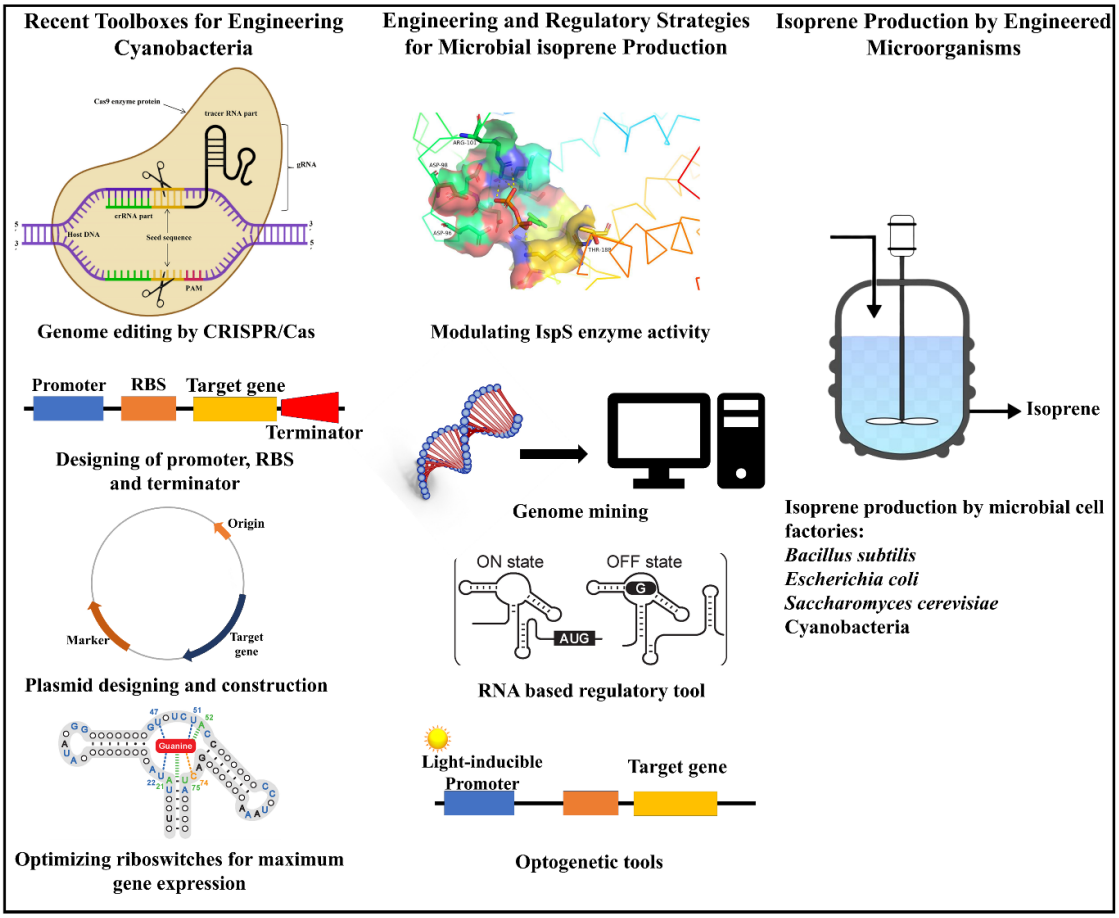
Chapter – 2

A comprehensive review of literature on engineering microorganisms for isoprene production



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2.1 Background

Harnessing solar energy through photosynthesis stands out as a remarkable accomplishment in nature, presenting a potential solution to meet the global energy demand in the future. Cyanobacteria which can fix CO_2 into organic compounds using solar energy are of great significant cellular factories for producing biofuels (Lasry Testa et al., 2019). Apart from relying on sunlight for energy, cyanobacteria require water and specific inorganic nutrients for their growth. Cyanobacteria stand out among photosynthetic organisms as appealing systems for biotechnological applications. Their faster growth rate compared to plants and greater ease of genetic manipulation compared to microalgae provide numerous opportunities to modify complex biosynthetic pathways through synthetic biology approaches (Lin et al., 2021).

2.2 Metabolic pathways for isoprene biosynthesis

The DMAPP and IPP serve as the global precursors for the isoprene and various other isoprenoids, including sterols and carotenoids biosynthesis, irrespective of the originating organisms. As of now, two separate and autonomous pathways for terpenoid biosynthesis have been identified: the MVA pathway and the MEP pathway (Isar et al., 2022a; Kant et al., 2023; Ye et al., 2016). The MEP pathway serves as the principal route for terpenoid production in the majority of bacteria, cyanobacteria, green microalgae, and plant plastids. In contrast, the MVA pathway is predominant in eukaryotes, archaea, and the cytosolic compartments of higher plants. The MEP pathway commences with the utilization of glyceraldehyde-3-phosphate and pyruvate as principal precursor molecules, which undergo condensation to form 1-deoxy-d-xylulose-5-phosphate (DXP). Subsequently, DXP is transformed into MEP and metabolized to generate hydroxy-2-methyl-2-butenyl-4-diphosphate (HMBPP), a crucial intermediate metabolite for the generation of IPP and DMAPP. In this pathway, the enzymes responsible for regulating the reaction rate have

been identified as 1-deoxy-d-xylulose-5-phosphate synthase (DXS) and 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR). Conversely, the MVA pathway is initiated by employing two molecules of acetyl-CoA as the chief precursors, leading to the formation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) subsequently condensation with another acetyl-CoA catalyzed by HMG-CoA synthase, a key performer in isoprene biosynthesis through MVA pathway. Subsequently, HMG-CoA undergoes reduction to mevalonate under the catalytic influence of HMG-CoA reductase enzyme, setting the pace for this pathway. Mevalonate is then subjected to two phosphorylation events and a decarboxylation process, ultimately resulting in the production of IPP, which is interconverted into DMAPP (Rautela and Kumar, 2022). Following the progression through either the MEP or MVA pathway, an IPP isomerase (IDI) is situated downstream, facilitating the reciprocal conversion of IPP and DMAPP. This enzymatic activity has been identified as an additional rate-determining step in the biosynthesis of isoprene. Subsequently, the formation of isoprene occurs through the catalytic removal of pyrophosphate from DMAPP, a reaction catalyzed by IspS (Lindberg et al., 2010).

Due to its well-defined characterization, the MVA pathway is easily amenable to molecular biology interventions and metabolic pathway engineering. As a result, it has been extensively employed in industrial settings for the high-yield production of isoprene in both yeast and bacteria. In contrast, the MEP pathway, though more recently discovered, has received comparatively less explored investigation (Hong et al., 2012). Specifically, the enzymes featuring iron-sulfur clusters in this pathway have not been thoroughly examined at a biochemical level. Nevertheless, analyses pertaining to redox balance and stoichiometry have showed that the MEP pathway achieves energetic equilibrium and tentatively displays greater efficiency compared to the MVA pathway in the transformation of sugars or glycerol into isoprenoids (Kim et al., 2016; Zhao et al., 2013). A schematic

representation of isoprene biosynthesis via MEP and MVA pathway has been presented in Figure 2.1.

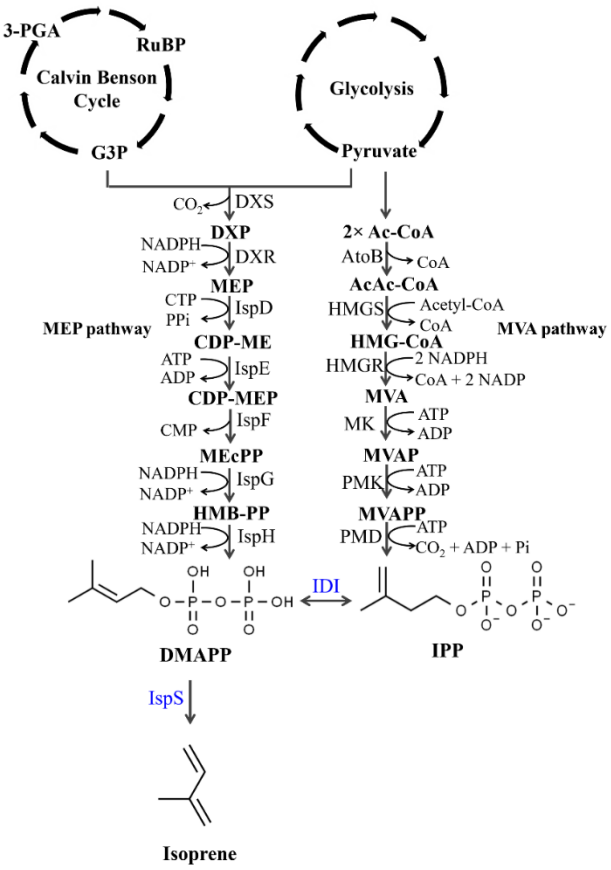

Figure 2.1 Biosynthesis of isoprene through methyl-D-erythritol-4 phosphate (MEP) and mevalonic acid (MVA) pathways. Abbreviations used in MEP pathway, metabolites: RuBP ribulose-1,5-bisphosphate, 3-PGA 3-phosphoglyceric acid,G3P—glyceraldehyde 3-phosphate; DXP—deoxy-D-xylulose-5-phosphate; MEP—methyl-D-erythritol 4-phosphate; CDP-ME—diphosphocytidylyl methylerythritol; CDP-MEP—diphosphocytidylyl methylerythritol phosphate; MEcPP—methyl erythritol-2,4-cyclodiphosphate; HMBPP—hydroxymethylbutenyl diphosphate; DMAPP—dimethylallyl diphosphate; IPP—isopentenyl diphosphate; NADPH—nicotinamide adenine dinucleotide phosphate; CTP—cytidine triphosphate; PPi—diphosphate; ATP—adenosine triphosphate; ADP—adenosine diphosphate; CMP—cytidine monophosphate; Fd_{red}—ferredoxin reduced; and Fd_{ox}—ferredoxin oxidized. Enzymes: DXS—deoxy-D-xylulose-5-phosphate synthase; DXR—DXP reductoisomerase; IspD—CDP-ME synthase; IspE—CDP-ME kinase; IspF—ME-cPP synthase; IspG—HMBPP synthase; IspH—HMBPP reductase; IDI—isopentenyl diphosphate isomerase; and IspS—isoprene synthase; Abbreviations used in MVA pathway, metabolites: 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, Enzymes: AtoB— acetoacetyl-CoA thiolase, HMGS—HMG-CoA synthase, HMGR—HMG-CoA reductase, MK—mevalonate kinase, PMK—MVAP kinase, PMD—MVAPP decarboxylase.

Prompt genome editing using synthetic biology tools leads to its multiple usage in the field of metabolic engineering. The concept of transforming CO_2 into a desirable fuel encouraged the development of genetically engineered cyanobacteria for production of biofuels (Atsumi et al., 2010). Among the initial model cyanobacteria, *Synechocystis sp.* PCC 6803 stood out, with its complete genome being sequenced in 1996 (Kaneko et al., 1996). Various model cyanobacterial strains, including *Synechocystis sp.* PCC 6803, *Synechococcus elongatus* PCC 7942, *Synechococcus sp.* PCC 7002, *Synechococcus elongatus* PCC 11801, *Synechococcus elongatus* PCC 11802 and *Synechococcus elongatus* UTEX 2973 have been used in metabolic engineering and synthetic biology endeavours aimed at the biosynthesis of diverse fuel molecules (Englund et al., 2018; Knoot et al., 2018; Madhu et al., 2023). Certain characteristics of cyanobacteria, such as their capacity for high-density growth, adaptability to non-arable land, ability to thrive in various water sources (including fresh, marine, and wastewater), and their potential to yield both biofuels and economically significant platform chemical products, place them as promising cell factories (Zahra et al., 2020).

2.3 Recent toolboxes for synthetic biology in cyanobacteria

Due to the comparatively limited availability of molecular biology tools for cyanobacteria in comparison to bacteria, there arises a necessity to engineer through a tools of synthetic biology approach. Synthetic biology involves the manipulation and recombination of existing native or foreign tools using various combinations to enhance their effectiveness. Within this framework, several tools such as promoters, riboswitches, ribosome binding sites, and the CRISPR/Cas system have been developed, and a brief overview of these are presented here. Strains like *Synechocystis sp.* PCC 6803 and *S. elongatus* PCC 7942 are commonly utilized as hosts for the evaluation of these genetic tools. It is worth noting, however, that one notable drawback of these strains is their prolonged doubling time.

Recently a research group discovered *Synechococcus.* UTEX 2973, which is the fast-growing cyanobacterial strain having a doubling time of 1.8 h reported to date (Yu et al., 2015). Table 2.1 summarizes the commonly used cyanobacterial strains as hosts.

Table 2.1 Cyanobacterial host organisms used for synthetic biology approach.

Host	Genome size (Year of availability)	Total number of proteins
Anabaena sp. PCC 7120	6.4 Mb (2001)	6206
Synechocystis sp. PCC6803	3.6 Mb (1996)	7446
Synechococcus elongatus PCC 7942	2.7 Mb (2004)	2877
Synechococcus sp. PCC7002	3.0 Mb (2008)	3377
Synechococcus elongatus UTEX2973	2.7 Mb (2015)	2657

2.3.1 Promoters

There are many native and foreign promoters that are used in cyanobacteria and are summarized in Table 2.2. In bacteria, promoters are recognized by the σ factor of the RNA polymerase (RNA P) enzyme and aids in the transcription of the gene of interest. The promoters can be constitutive or inducible. Constitutive promoters transcribe the genes continually in an unregulated way. Whereas inducible promoters are specific to the signals like light, dark, heavy metals, nitrate/nitrite, etc., and are helpful when the intermediate/end products are toxic to the host cells. P_{cpc560} considered as the super-strong promoter was discovered by Zhou et al. 2014. It has the same expression rate as that of the *E. coli* producing functional proteins at a level of up to 15 % of total soluble proteins. It has 14 transcription factor binding sites and two promoters from the C-phycocyanin β subunit (*cpcB*) gene which are assumed to be the crucial factor for its strength. In one of the studies done by Liu & Pakrasi. (2018), promoter cpcB showed the highest expression (sequence

identical to cpc560). They compared 13 different promoters by checking the expression of enhanced yellow fluorescence protein (eYFP). Out of the thirteen promoters, twelve were native, and one was *E. coli* origin. Wang et al. (2018) constructed promoter's library, compared 17 different promoters, and concluded that P_{trc} gives two times better expression than the promoter P_{psbA} (from chloroplast of the flowering plant *Amaranthus hybridus*) and seven times expression than P_{cpcB} ($P_{cpc\ 560}$) and its variants. The expression level was checked in *Synechocystis sp.* PCC 6803 by expressing the ethylene-forming enzyme.

P_{trc2O} and P_{trc1O} are the promoters derived from P_{trc}. P_{trc1O} has a strong lac operator than P_{trc}, whereas P_{trc2O} has two lac operator sites showing efficient repression (Huang and Lindblad, 2013). Markley et al. (2015) constructed a promoter that performs better than trc promoter, giving 48±7-fold expression of *eYFP*. They constructed two orthogonal promoter libraries with the isopropyl β-d-thaiogalactopyranoside (IPTG) induction system, which were tried and evaluated in *Synechococcus sp.* strain PCC 7002. Promoter MB1, MB2, and MB3 were obtained by a change in sequences in the Biobrick promoter J23119. Similarly, 20 different synthetic promoters were assembled by modifying J23119, P_{trc1O}, P_{tic10}, and P_{tac10} promoters, and the intensity of fluorescence was checked in *Synechocystis* and *S. elongatus* UTEX 2973 (Vasudevan et al., 2019). In both cases, J23119 showed the highest fluorescence levels.

Werner et al. (2018) discovered and characterized nineteen native promoters in *Synechocystis* sp. PCC 6803, which is induced by 12:12 hour light and dark cycles (LD). Out of these nineteen promoters, four of the promoters P_{hliC}, P_{rbp1}, $P_{slr0006l,}$ and P_{sigA} show a strong correlation with 12:12 LD cycles when characterized using bacterial luciferase bioluminescent gene. In the same species, metal ions (nickel, cobalt, and zinc) induced nrsB, nrsD, nrsS, coaT, and ziaA promoters were compared with endogenous constitutive promoters (Englund et al., 2016). P_{nrsB} was found to be the most efficient promoter, which

can be regulated and tuned with the help of nickel ions. Kelly et al. (2018) inserted a rhamnose-inducible rhaBAD promoter from *E. coli* to *Synechocystis sp.* PCC 6803 which showed a controlled expression system. P_{synDIF}, a short 48 nucleotides long synthetic promoter in filamentous cyanobacteria for heterocyst-specific expression can be used well for the production of oxygen intolerant enzymes as the promoter gives 10 times more expression after the heterocyst formation (Wegelius et al., 2018). Expression of the promoter changes when it is relocated from the native location to the new one (Albers and Peebles, 2017). P_{psbAII} within the native genomic location of *Synechocystis sp.* PCC 6803 showed 15.8 times increase in the transcript in comparison to only 1.6 times when the promoter is moved to neutral region slr0168. Promoter psbA was employed to regulate the expression of mannitol encoding genes (*mtlD* and *mlp*) in *Synechococcus sp.* PCC 7002, giving a mannitol yield of 1.1 g/L with a productivity of 0.15 g/L/day (Jacobsen and Frigaard, 2014). Huang and Lindblad. (2013) constructed non-inducible promoters and showed that altering a few base pairs can change the strength of the promoters. R40 promoter was used as a template for promoter designing. Specific base pairs changes were done in the R40 promoter at TATAAT site (L12 promoter created whose strength is less than R40) and between the transcription start site and -10 element (L12 promoter having strength more than R40). Three lines of modifications were done between the transcription start site and -10 element creating a total of 19 promoters, L01 to L016, L21 and L22, and L31. The study shows that the L21 promoter has 110 (±1) times strength than L22, opening the possibilities to change the region between −27 and +3 at TATAAT sequence. Bioinformatics tools play an important role in the prediction of promoters in cyanobacteria. Btss finder is one of the means for bacterial promoter prediction, which includes *E. coli* and cyanobacteria. Being novel, it can identify the promoters of different sigma classes of two different phyla. Several native and foreign/synthetic promoters are shown in Table 2.2.

Table 2.2 List of native and foreign promoters used in cyanobacteria.

	Promoter	Gene(s) amplified	Host	Reference
Native	nrsB	PDC, ADH	PCC 6803	Englund et al. (2016)
	cpc560	Ter,eYFP, CrtW	PCC 6803	Diao et al. (2020) Zhou et al. (2014); Liu and Pakrasi (2018)
	hlic, rbp1, slr00061, and sigA	Lux AB	PCC 6803	Werner et al. (2018)
	psbAII	eYFP	PCC 6803	Albers and Peebles (2017)
	A2520, A2579	YFP	PCC 7002	Ruffing et al. (2016)
Foreign or Synthetic	Trc	eYFP, EFE, FaS, dxs, idi, ispA	UTEX 2973 PCC 7942	Markley et al. (2014); Yu et al. (2015); Lee et al. (2017); Wang et al. (2018)
	trc2O	eYFP	PCC 6803	Camsund et al. (2014)
	J23119 and its variants	eYFP	UTEX 2973 PCC 6803	Vasudevan et al. (2019)
	MB1, 2, and 3	YFP	PCC 7002	Markley et al. (2014)
	rhaBAD	YFP	PCC 6803	Kelly et al. (2018)
	synDIF	eYFP	ATCC 29133	Wegelius et al. (2018)
	psbA	M1PDH	PCC 7002	Jacobsen and Frigaard (2014)
	L01 to L016, L21 and L22, and L31	eYFP	PCC 6803	Huang and Lindblad (2013)
	Lac	CscB	UTEX 2973	Song et al. (2018)
	Nir	FaS	PCC 7120	Halfmann et al. (2014)
	conII	eCFP, GFPmut2, yemGFP and YFP	PCC7120 ATCC29133 BL0902 PCC6803	Taton et al. (2014)
	trc1O, lacUV5	CscB	UTEX 2973	Lin et al. (2020)
	vanCC, L03, coaT	Sp^R	PCC 6803	Behle et al. (2020)
	PndbA600	GFP	PCC 7002	Madsen et al. (2021)
	tic2op	AgB	PCC 6803	Sebesta and Peebles (2020)
	cpcG2	T7 RNAP	PCC 6803	Shono et al. (2021)

Abbreviations used: PDC– Pyruvate decarboxylase; ADH– Alcohol dehydrogenase; ter– trans-enoyl-CoA reductase; eYFP– Enhanced yellow fluorescent protein; YFP– Yellow fluorescent protein; EF– Ethylene forming enzyme; FaS – Farnesene synthase; dxs – 1-deoxy-D-xylulose-5-phosphate synthase; idi – isopentenyl diphosphate isomerase; ispA – farnesyl diphosphate synthase; M1PDH – Mannitol 1-Phosphate dehydrogenase; eCFP – Enhanced cyan fluorescent protein; yemGFP – Monomeric yeast-enhanced green fluorescent protein; GFPmut2– Green fluorescent protein; CscB– Sucrose permease; Sp^R – Spectinomycin resistance; CrtW– Carotenoid ketolase; AgB – Bisabolene synthase.

2.3.2 Ribosome binding sites

As the promoters regulate the initiation of transcription, in the same way, ribosome binding sites (RBS) regulate the translation initiation rate of downstream target genes (Kierzek et al., 2001). Upon the initiation of translation, the 3-terminal sequence of the 16S rRNA interacts with the core Shine-Dalgarno (SD) sequence of the RBS through complementary base pairing of nucleotides. Ma et al. (2002) showed in *Synechocystis sp.* 6803 that the 3-terminal sequence of the 16S rRNA is AUCACCUCCUUU and its complementary SD sequence is AAAGGAGGUGAU (core SD sequence underlined). To enhance the yield of 2,3-butanediol in *Synechococcus* 7942, expression levels of the three genes namely adh, alsS, and alsD are coordinated by utilizing four different RBS from *E. coli* (Oliver et al., 2014). Wang et al. (2016) increased limonene synthesis by RBS engineering in *S. elongatus* PCC 7942. The strain L1113 showed limonene production of 32.8 µg/L/OD/day by changing the original RBS of the trc promoter. Similarly, a synthetic RBS introduced in psbA promoter increased the limonene production to 885.1 µg/L/OD/day.

RBS library for cyanobacteria was created by utilizing two RBS sequences from *Synechocystis sp.* PCC 6803 and 8 from BioBrick registry of standard biological parts and predicted by "RBS library calculator" (Englund et al., 2016). The mentioned library was employed to express an *eYFP* with the help of PnrsB, PnrsD, PnrsS, PcoaT, and PziaA promoters which are induced by nickel, cobalt, and zinc metal efflux pumps. More recently, 20 native RBS, which were 22 base pairs long were evaluated. Ptrc1O was selected as the promoter to check the strength of the RBS sequences by the *eYFP* gene. In the same manner, it was also assessed thirteen RBS out of which seven were native of *Synechocystis sp.* 6803, and 6 were from *E. coli*. Codon-optimized GFPmut3, sYFP2, and ethylene forming enzyme were used as the reporter proteins for checking translation efficiency (Thiel et al., 2018). These studies help in the selection of suitable RBSs for overexpression of the end product.

According to a research group, the same RBS can have inconstant translational efficiencies in different microorganisms or different genes in the same organisms (Reeve et al., 2014). RBS calculating tools play an important role which is based on the thermodynamic model to predict the changes in the start codon and 5 untranslated regions in an mRNA transcript. RBS calculator, UTS design, and RBS designer are the majorly used tools to determine translation rates. Each calculator is used efficiently for reverse and forward engineering. RBS designer works by designing RBS sites synthetically on the RNA transcript, while the UTR designer focuses on changing 5-UTR to alter protein expression and predicts translation efficiency (Na and Lee, 2010; Seo et al., 2013). These tools serve an important purpose of generating RBS libraries, but their efficiency can vary. Wang et al. (2018) reported the low efficiency of the RBS library created by the RBS calculator and established a rational RBS design strategy. Likewise, Thiel et al. (2018) also stated that the data predicted by the UTR designer and RBS calculator shows different translation efficiency than the experimental one. In another study, numerous RBS calculated for the bisabolene synthase gene gives 7.8 mg/L titers (Sebesta and Peebles, 2020).

2.3.3 Riboswitches

In comparison with the inducible promoters, riboswitches do not require additional protein factors and are the cis-acting regulatory element that changes the conformation on binding with its ligand controlling TIR (Domin et al., 2017). This makes riboswitches an ideal tool for gene regulation. Additionally, it was illustrated that modified theophylline-responsive riboswitches regulate gene (luciferase) expression more efficiently than inducible promoters (Nakahira et al., 2013). Further, this riboswitch was used in many studies in *S. elongatus* PCC 7942, *Anabaena sp*. PCC 7120, *Leptolyngbya sp*. BL0902, *Synechocystis sp.* 6803 and *Synechocystis sp*. strain WHSyn to check the expression regulation of yellow and green fluorescent protein (Ohbayashi et al., 2016a). The theophylline-responsive

riboswitches used were earlier screened and characterized in the past in Gram-negative proteobacteria (alpha and gamma) and Gram-positive bacteria (Topp et al., 2010). It is also used to regulate intracellular glycogen content (40 to 300% of wild type) in *S. elongatus* PCC 7942 by controlling ADP-glucose pyrophosphorylase. Optimized level of glycogen increases cellular robustness (Chi et al., 2019).

Apart from theophylline-responsive riboswitches, cobalamin-dependent riboswitches work well in *S. elongatus* PCC 7002 as the strain cannot synthesize the cobalamin itself. But this riboswitch cannot work in the strain which synthesizes cobalamin such as *S. elongatus* PCC 7942, *Synechocystis sp.* 6803, *Crocosphaera watsonii* WH8501, and *Synechococcus sp.* WH7803 (Helliwell et al., 2016). Other riboswitches used in cyanobacteria include S-box (SAM), SAM-II (a-proteobacteria) and SAMI/IV-variant riboswitch, thiamine pyrophosphate (TPP)-riboswitch, Glycine riboswitch, SMK box translational riboswitch, purine riboswitch, FMN riboswitch (RFN element), lysine riboswitch, SAH (*S*-adenosyl-l-homocysteine) riboswitch, THF (Tetrahydrofolate) riboswitch Moco (molybdenum cofactor) riboswitch (Singh et al., 2018). Some of the riboswitch's inducers are toxic to the host organism and are key metabolic intermediates, therefore, only theophylline-dependent riboswitch is widely used in cyanobacterial systems.

2.3.4 CRISPR based techniques

The most recent synthetic biology tool is the CRISPR/Cas system, which is marker less. CRISPR/Cas stands for clustered regularly interspaced short palindromic repeats/CRISPR-associated proteins. Its targetability is provided by the single-guide RNA (sgRNA), which is specific to the target genomic site. The sgRNA directs the Cas protein to the target site which cleaves both the strand of the genome. Cyanobacteria being oligoploid and polyploidy shows the difficulty in producing homozygous mutants (Watanabe et al., 2015; Zerulla et al., 2016). CRISPR-based techniques improve this editing efficiency. Wendt et

al. (2016) used CRISPR/Cas 9 system to produce nonbleaching protein A (nblA) mutants of *S. elongatus* UTEX 2973. Once all the copies of the genes are deleted the mutants show visible results (within 1 week) as the nblA serves as a visual reporter gene. These results were verified by increasing the succinate concentration in *S. elongatus* PCC 7942 through glgc knock-out glta/ppc (citrate synthase/phosphoenolpyruvate carboxylase) knock-in by CRISPR-Cas 9 editing (Li et al., 2016).

Cas 9 at higher concentrations showed toxicity in *S. elongatus* UTEX 2973 and *S. elongatus* PCC 7942 cells (Wendt et al., 2016). The quick fix to the problem was the transient expression of Cas 9 through temperature-controlled plasmid. This gave inkling to prospect CRISPR/Cas 12a (also known as Cpf1) (Ungerer and Pakrasi, 2016). Marker less point mutation, a knock-out mutation or a knock-in mutation were generated *S. elongatus* UTEX 2973, *Synechocystis sp.* PCC 6803 and *Anabaena sp.* PCC 7120. The Cas 12 have several merits over Cas 9 which includes, no requirement of tracrRNA to activate crRNA, cost efficiency as it needs only 42 nucleotides RNA which is cheaper to produce, and PAM (Protospacer adjacent motif) sequence is more specific to the cleavage site. A recent application of Cas12a was seen in developing a high-throughput prototyping tool for promoter characteristics (Choi et al., 2021). This gives the opportunity to rapidly characterize promoters by cell-free transcription.

A newer version of the CRISPR/Cas system is CRISPR-interference (CRISPRi) which utilizes the dead Cas9 (dCas9) which binds to the target DNA but has lost the ability to cleave it. This type of system is essential for the genes which are crucial for cell viability and can only be downregulated rather than deleted. Yao et al. (2016) first reported the application of CRISPRi in *Synechocystis sp*. PCC 6803. The sgRNAs were placed in the neutral sites (slr2030-slr2031) due to this 94 % repression was seen in the green fluorescent protein (GFP) protein. CRISPRi can be used for producing carbon-based products as shown

by (Huang et al., 2010). They effectively repressed extrinsic (eYFP) and intrinsic genes (glgc, sdhA and sdhB) to redirect the carbon flow. This laid the foundation for the metabolic pathways in cyanobacteria. Similarly, CRISPRi is being also used to redirect fatty acid flux (Kaczmarzyk et al., 2018). Regulating plsx (phosphate acyltransferase) gene by CRISPRi system fatty alcohol production enhanced to a great extent at 10.3 mg/g dry cell weight (DCW). Modulation of glutamine synthetase (glnA helps in nitrogen assimilation) in *Anabaena sp*. PCC 7120 was shown by Higo et al. (2018). The process is regulated in such a way that ammonium is produced only when dCas inducer is present in the system. Lately, the dCas12a-mediated CRISPR interference system (CRISPRi-dCas12a) was developed in cyanobacteria for repressing genes that are not needed to produce value-added chemicals (repression up to 53-94%). The technique was implemented in *S. elongatus* PCC 7942 to increase squalene production by repressing aconitase (Choi and Woo, 2020).

2.3.5 Vectors

After successfully finalizing the genetic elements like promoters, RBS, riboswitches, etc. a platform is required for taking the gene of interest into the host cell (here, cyanobacteria). For this purpose, vectors come into role, which is a plasmid with properties having antibiotic resistance gene for selection, mobilization elements for transfer, and unique restriction sites for cloning. The heterologous gene can be inserted into the genome of the cyanobacteria or replicate autonomously. The former is done with the help of integrative and the latter with replicative vectors.

Utilizing replicative vectors facilitates the straightforward insertion of a gene of interest into cyanobacteria for bioproduction and various applications (Heidorn et al., 2011). Achieving autonomous gene expression without genomic integration results in higher expression levels. Shuttle vectors, often replicative plasmids, are commonly employed as they can express in two different hosts. Jin and coworkers constructed a shuttle vector for

Synechococcus sp. PCC 6803 utilizing its plasmid. *Synechococcus sp.* PCC 6803's plasmid PCC 5.2 consists of a replicon and combining it with pMB1 (origin of replication of *E. coli*) leads to the formation of shuttle vector pSCB-YFP. Replicative vectors require antibiotic selection/stress to stably maintain them (Jin et al., 2018). Table 2.3 displays a compilation of replicative vectors designed for research applications. The cyanobacterial genome possesses various neutral sites, such as NSI, NSII and NSIII (Ng et al., 2015). The commonly employed approach involves substituting these neutral sites with the gene of interest through homologous recombination (Lee et al., 2017). Another strategy includes integrating the heterologous gene in place of the genes which do have a function in cyanobacteria or do not affect the robustness of the strain. In this case, the heterologous gene utilizes the promoter, RBS, and terminator sequences of the source gene. The significant challenge in integrating the gene of interest into cyanobacteria arises from their high ploidy levels, requiring assurance that each copy contains the desired gene (Heidorn et al., 2011). This is exemplified by the fact that *S. elongatus* PCC 7942 has 3 to 4 genomic copies per cell, while *Synechocystis sp.* PCC 6803 has 218 and 58 genomic copies during exponential and stationary phases, respectively (Griese et al., 2011). Earlier there was no modular cloning (MoClo) system for cyanobacteria, however, plant MoClo with cyanobacteria making CyanoGate system kit were made by (Vasudevan et al., 2019). The kit consists of 96 parts and these can be combined from level 0 to level T to form replicative or integrative vectors. All vectors are submitted at addgene for research purposes (Addgene Kit #1000000146). Commercially available replicative plasmids are listed in Table 2.3 and integrative plasmids have been listed in Table 2.4.

Table 2.3 List of commercially available replicative plasmid.

Plasmid	Size of plasmid (bp)	Heterologous gene(s)	Antibiotic selection	Host	Reference
pAM5187	6195	yem-GFP	Spectinomycin and streptomycin	PCC 7942	Chen et al. (2016)
pFA-sbtA	4107	sbtA	Chloramphenicol	PCC 7942	Desmarais et al. (2019)
PBAD-his6-prkA-pACYC184	4245	prkA	Chloramphenicol	PCC 7942	Parikh et al. (2006)
pAM5084	7443	ecfp-KaiC	Ampicillin	PCC 7942	Cohen et al. (2014)
pCB-SC101-Spe	1900	–	Spectinomycin	PCC 6803	Liu and Pakrasi (2018)
CpcB•PHLS+Cpc	6991	PHLS gene in fusion with CpcB gene	Ampicillin	PCC 6803	Formighieri and Melis (2015)
pEEK2	8657	Kivd and ADH	Kanamycin	PCC 6803	Miao et al. (2017)

Abbreviations used: sbtA– Sodium dependent bicarbonate transporter; prkA– Phosphoribulokinase; ecfp-KaiC– Enhanced cyan fluorescent protein-circadian clock protein kinase KiaC; PHLS– β-phellandrene synthase; CpcB– C-phycocyanin beta chain; Kvid – α-ketoisovalerate decarboxylase; ADH – Alcohol dehydrogenase.

Table 2.4 Integrative plasmids available commercially.

Plasmid	Size of plasmid (bp)	Integration site	Heterologous gene	Host	Reference
KFP08	9688	NS 2	SigF2 epitope	PCC 7942	Fleming and O'Shea (2018)
KFP14	7765	NS 1	RpoD6 epitope	PCC 7942	Fleming and O'Shea (2018)
gCOTS-pyl	14137	NS 2	Pyrrolysyl tRNA synthetase	PCC 7942	Chemla et al. (2017)
pAM1573	8414	NS 2	Cm resistance	PCC 7942	Andersson et al. (2000)
pEERM3 Km	3919	NS 1	CfTPS2andCfTPS3	PCC 6803	Englund et al. (2015)
pEERM1 Km	3728	psbA2	CfTPS2 and CfTPS3	PCC 6803	Englund et al. (2015)
pEERM6 Km	3946	sqs	CfTPS2 and CfTPS3	PCC 6803	Englund et al. (2015)
pAM2991	7993	NS 1	LdpA	PCC 7942	Ivleva et al. (2005)
SPS-6803	11841	NS 2	SPS	PCC 6803	Abramson et al. (2016)

Abbreviations used: NS– Neutral site; sqs– Squalene synthase; CfTPS2 and CfTPS3 – *Coleus forskohlii* terpene synthase 2 and 3; LdpA– Light-dependent period A; SPS– Sucrose phosphate synthase.

2.4 Transformation techniques

Ease in the genetic modification process has been a very essential necessity for cyanobacterial strain development using synthetic biology tools for metabolic engineering applications. Three procedures are being widely used for the insertion of target genes into cyanobacterial host cells:

transformation, conjugation, and electroporation (Vioque, 2007). The efficiency of transformation in cyanobacteria depends on biochemical and physical barriers of the host

which varies from species to species (Stucken et al., 2013). Transformation also depends on the size, structural organization, and concentration of the target DNA used in the engineering process (Nagarajan et al., 2011). Transformation can be done by either an integrative plasmid or by a replicative plasmid. DNA transfer by integrative plasmid employs the foreign DNA incorporation into the genomic DNA of host cells by the process called homologous recombination (Heidorn et al., 2011). Whereas, replicative plasmids replicate and express independently along with foreign DNA in the host cell (Wang et al., 2013). These two types of plasmids have been well developed for the transformation of cyanobacteria. It has already been demonstrated the possibility of using linear DNA segment in *Synechocystis sp.* PCC 6803 for metabolic engineering applications (Nagarajan et al., 2011). The *S. elongatus* PCC 7942 was first time engineered with linear DNA fragment using ethylene diamine tetra acetic acid (EDTA) as DNases inhibitor (Almeida et al., 2017). Some cyanobacterial strains like *Synechocystis sp.* PCC 6803, *S. elongatus* PCC 7942, and *S. elongatus* PCC 7002 are naturally competent to take foreign DNA (Johnsborg et al., 2007; Lindberg et al., 2010). The attribute of natural competency is not common in some other strains. Non-competent strains can be transformed by a well-developed method called tri-parental conjugation, which employs helper, conjugal, and cargo plasmids (Yu et al., 2015). DNA transmission from *E. coli* to nitrogen-fixing cyanobacterial strains of *Nostoc* and *Anabaena* have been genetically manipulated using tri-parental mating (Ruffing, 2011). A study successfully applied the tri-parental conjugation process in *S. elongatus* UTEX 2973 with the help of helper and conjugal plasmids (Yu et al., 2015).

2.5 Engineering and regulatory strategies for microbial isoprene production

2.5.1 Modulating IspS enzyme activity

In plants, *IspS* is located into the plastids and catalyzes the conversion of DMAPP into isoprene (Lindberg et al., 2010). Notably, despite the widespread occurrence of biosynthetic

routes for IPP and DMAPP, the isolation and purification of isoprene synthases have thus far been accomplished exclusively in plant species. The first *IspS* gene sequence was successfully isolated from the plant *Populus alba* × *P. tremula* by (Miller et al., 2001). After that *IspS* gene from various plants including *Pueraria montana*, *Eucalyptus globulus*, *Populus tremoides*, *Ficus septica*, *Populus canescens*, *Casuarina equisetifolia*, and *Metrosideros polymorpha* were identified and isolated (Li et al., 2018; Ye et al., 2016; Yeom et al., 2018). Microorganisms such as bacteria, yeast, archaea, cyanobacteria, and green microalgae lack *IspS* gene. However, plant-origin *IspS* could be heterologously expressed in microbial systems for sustainable production of isoprene. The *IspS* from different plants have been expressed in the microbial system and their enzyme kinetic parameters like Michaelis constant (K_m), and enzyme turnover number (K_{cat}) have been determined (Table 2.5). Moreover, enzyme assay studies indicated that most of the IspS enzymes have low affinity and low activity for DMAPP substrate due to their high K_m and low K_{cat} values (Li et al., 2018). The IspS enzymes with higher K_{cat} and lower K_m values are needed for efficient and elevated microbial production of isoprene to fulfill worldwide isoprene need. In recent years, researchers have focused on enhancing the IspS enzyme activity and affinity, searching for new IspS enzymes, directed evolution, in-silico mutation designs, and other metabolic modulating strategies for improved isoprene production (Li et al., 2018; Yeom et al., 2018). Most of the terpene synthases consist of two conserved domains, α and β at their active site. A third domain γ is also found in some terpene synthases. In a study, IspS enzyme was engineered with the insertion of a heterologous GFP at the site of γ domain of IspS while preserving the enzyme activity. Thus, inserting the GFP domain improved the kinetic parameters of the IspS enzyme K_m and K_{cat} from 0.19 mM, 0.29 per S to 0.18 mM, and 0.37 per S, respectively (Gonzalez-Esquer et al., 2021). The catalytic

properties of isoprene synthases isolated from different plants have been presented in Table 2.5.

Table 2.5 Isoprene synthase (*IspS*) from different sources and their kinetic parameters.

IspS source	K_m for DMAPP (mM)	K_{cat} (per S)	Reference
Pueraria montana	7.7	0.088	Sharkey et al. (2005)
Populus alba	15.9	0.034	Oku et al. (2022)
Populus tremoides	8.0	1.7	Silver and Fall (1995)
Ficus septica	3.4	0.011	Oku et al. (2022)
Eucalyptus globulus	0.2	0.195	Sharkey et al. (2013)
Populus canescens	2.45	--	Schnitzler et al. (2005)
Casuarina equisetifolia	0.30	0.015	Oku et al. (2015)
Metrosideros polymorpha	8.11	0.35	Yeom et al. (2018)

2.5.2 Genome mining

Genome mining is a combined process of bioinformatics investigations to find out the biosynthetic pathway of enzymes, secondary metabolites, and bioactive natural products and the characterization of their chemical and functional interactions (Albarano et al., 2020). Traditional screening of terpene synthase genes has been used for decades to identify novel isoprene synthases from plants. However, traditional approaches are time-consuming and economically not viable (Männle et al., 2020). In recent years advances in next-generation genome sequencing, synthetic biology tools, and gene mining technologies have improved the rate of genomic data acquisition and reduced the operating cost. Genomic DNA analysis technology upgrade has revolutionized the search for new unexplored

biosynthetic gene clusters (BGC), and novel biosynthetic pathways, that synthesize novel metabolites, and other bioactive compounds in microorganisms (Tracanna et al., 2017). Mining of genes having the conserved domains could be done using DIAMOND, NCBI BLAST, or HMMer tools. Gene/protein sequences taken from genome annotation of different organisms could be aligned using the Clustal Omega sequence alignment tool to find out conserved domains (Maurya et al., 2019).

2.5.3 RNA based regulatory tools

Complete knockout of the competing pathway may exert detrimental effect on the cell viability since MEP pathway substrates are directly associated with the central metabolic pathway of the cell. Alternatively, expression of genes associated with side reactions could be downregulated without completely abolishing the metabolite level. In recent years, artificial antisense RNA (asRNA) based regulatory tools have been used in regulation of gene expression in heterotrophic microorganisms (Kang et al., 2014). RNA based regulation has been also implemented in cyanobacterial cells *Synechocystis sp*. PCC 6803 (Ohbayashi et al., 2016b; Sakai et al., 2014). Rodrigues et al. (2023a) used Hfq-MicC regulatory system for enhancing isoprene production by downregulation 6 genes involved in competing pathway in *E. coli*. RNA scaffold mediated regulation was used for the improved isoprene titre in *E. coli* (Liu et al., 2022).

2.5.4 Optogenetic tools

Optogenetics is a powerful tool that controls the gene expression with light. Gene expression can be controlled using light sensitive protein to activate or repress gene expression whenever required (Takano, 2016). To use optogenetics to control isoprene production in cyanobacteria, researchers could introduce light sensitive genes that are specific to the light sensitive isoprene biosynthesis pathway. One potential approach is to use a light sensitive transcription factor that binds to the promoter region of the gene in

isoprene biosynthesis pathway, activating their expression in response to light (Chen et al., 2020). Alternatively, a light sensitive RNA switch that controls the translation of the mRNA, could be used. Optogenetics could also be used to optimize isoprene production by controlling the timing and intensity of light exposure as well as by controlling the expression of other genes involved in photosynthesis and cellular metabolism (Liu et al., 2022). Optogenetics has great potential as a tool for controlling isoprene production in cyanobacteria, and could help to develop more sustainable and environmentally friendly processes.

2.6 Isoprene production by engineered microorganisms

Irrespective of the chassis organism's species, the development of production strains for bio-based chemicals typically involves one or more of the following strategies:

1. Metabolic pathway engineering, which entails introducing a heterologous pathway or engineering an endogenous pathway to establish the target biosynthesis route.
2. Enzyme engineering, involving the engineering, design, and discovery of key enzymes to improve rate-limiting steps in the biosynthesis pathway.
3. Repression, encompassing the down-regulation or deletion of key enzymes in competing pathways to reduce or eliminate carbon flow loss.
4. Balancing, which entails engineering related metabolic pathways to establish the necessary energy and cofactor balance, as well as achieving a balanced supply and conversion of key intermediates.

In the fermentation-based process for producing isoprene, the pivotal focus lies in developing a microbial cell factory that is physiologically streamlined to efficiently guide the conversion of sugars, ideally from cost-effective sources like lignocellulosic feedstocks. This engineered metabolic pathway is designed to yield isoprene at the highest possible

rates. Typically, the construction of isoprene-producing microorganisms involves the expression of an exogenous *IspS* gene and the modification of an isoprenoid precursor pathway. Since microorganisms lack *IspS* gene, plant *IspS* genes have been utilized for the microbial production of isoprene. The synthesis of isoprene has been engineered in a diverse range of microbes by introducing a modified plant *IspS* gene. In addition to endeavours in gene discovery and protein engineering to acquire a suitable *IspS* for constructing a productive biosynthetic pathway, extensive attention has been dedicated to engineering the upstream pathways responsible for precursor delivery, namely, the MVA and MEP pathways. The initiatives in MEP pathway engineering have been centered on refining and harmonizing the expression of pathway enzymes. This has been achieved through techniques such as gene overexpression, the employment of various promoters, and the incorporation of gene homologs sourced from diverse microbial origins (Ajikumar et al., 2010; Zhao et al., 2013). The prokaryotic hosts *B. subtilis*, *E. coli*, and the cyanobacterium *Synechocystis*, as well as the eukaryotic host *S. cerevisiae*, have undergone engineering for the purpose of isoprene production.

2.6.1 *Bacillus subtilis*

B. subtilis has been recognized for its natural ability to produce isoprene. Although isolating the *IspS* from this organism has posed challenges, research has demonstrated the involvement of the native MEP pathway in isoprene synthesis by *B. subtilis*. To enhance isoprene production in *B. subtilis*, two potentially rate-limiting enzymes in the MEP pathway, DXS and DXR, were overexpressed. It was observed that overexpression of DXS led to an increase in isoprene yield, while overexpression of DXR did not bring about any change. This suggests that the reaction catalyzed by DXS is the rate-limiting step in isoprene synthesis by *B. subtilis* (Xue and Ahring, 2011). Vickers and Sabri (2015) revealed that overexpressing kudzu *IspS* can lead to an increase in isoprene concentrations, reaching

up to 1.2 mg/L. Moreover, a chimeric strain of *Bacillus subtilis* demonstrated an isoprene production concentration of 1.434 mg/L, which was higher than the wild-type strain's production of 0.388 mg/L.

2.6.2 *Escherichia coli*

To increase the isoprene yield in *E. coli*, endeavours have focused on both enhancing the expression of native MEP pathway genes and introducing foreign MEP/MVA pathway genes from different organisms. In *E. coli*, isoprenoids are naturally synthesized through the Embden-Meyerhof pathway (EMP) in conjunction with the MEP pathway. Consequently, the MEP-dependent isoprene biosynthesis pathway, established upon the introduction of a heterologous *IspS*, can be divided into two modules: (1) EMP module that generates pyruvate and G3-P from sugar substrates. (2) MEP module that produces isoprene as the final product (Isar et al., 2022b; Kant et al., 2023). The efficiency of the MEP pathway is not only constrained by the involved reactions but is also influenced by the availability of the starting materials (pyruvate and G3-P), which plays a role in regulating this route. To enhance isoprene production, strategies in metabolic engineering could be devised, focusing on both the substrate-feeding module and the MEP module as engineering targets, respectively. The overexpression of the native DXS and DXR genes in an *E. coli* transformant, coupled with the introduction of a *IspS* gene of *P. nigra*, led to an improvement in isoprene production from 94 mg/L to 160 mg/L. Furthermore, the heterologous expression of *B. subtilis* DXS and DXR resulted in a further enhancement of isoprene production, reaching 314 mg/L. The superior efficiency of *B. subtilis* DXS and DXR in enhancing isoprene production compared to the native enzymes may be attributed to differences in their protein structures. This suggests that enzymes from different sources might exhibit varying performance in the same host (Zhao et al., 2013). In a study aimed at broadening the range of feedstocks for isoprene biosynthesis, an *E. coli* mutant strain

with a blocked Leloir pathway was employed as the host. This strain exclusively directed D-galactose towards the DD pathway, which was complemented by the introduction of a *Pseudomonas syringae* dehydrogenase encoding gene (gld), responsible for converting D-galactose into D-galactonate. In addition to the engineering of sugar metabolism and the heterologous expression of *P. alba IspS*, the endogenous MEP pathway bottlenecks, *DXS* and *IDI*, were overexpressed to enhance precursor supply. This effort resulted in an isoprene production of 264 mg/L from galactose (Ramos et al., 2014).

2.6.3 *Saccharomyces cerevisiae*

The yeast *S. cerevisiae* serves as a eukaryotic model organism for genetic engineering and is a promising cell factory for various biotechnological applications. It naturally utilizes the MVA pathway to produce essential isoprenoids crucial for cell growth and metabolism. Since yeast lack the inherent capability for isoprene production, the introduction of an exogenous *IspS* gene is necessary to catalyze the conversion of DMAPP into isoprene, thereby enabling isoprene synthesis in *S. cerevisiae*. To simultaneously increase the supply of acetyl-CoA and enhance the MVA flux, a comprehensive push-pull-restrain strategy was devised to engineer the native MVA and acetyl-CoA pathways in an *IspS* expressing *S. cerevisiae* strain for increased isoprene production (Lv et al., 2014). This engineering scheme can be divided into two main components: the up-regulation of precursor supply in the acetyl-CoA module and the MVA pathway through the overexpression of acetyl-CoA synthase, acetoacetyl-CoA thiolase, and tHMG1 (push-strategy); and the increase of the isoprene branch flux via the overexpression of *IDI* and the introduction of two copies of *IspS* (pull-strategy). Additionally, the competing pathway was down-regulated by weakening the promoter strength of the farnesylpyrophosphate synthetase gene (restrain-strategy). By concurrently modifying the galactose regulatory network through the deletion of GAL80 (a transcriptional repressor involved in regulating transcription in response to

galactose), and engineering the carbon source by using glycerol-sucrose as the carbon source, the final strain achieved an accumulation of 37 mg/L of isoprene under aerobic conditions. This represented an approximately 782-fold increase compared to the parental strain harbouring only one copy of *IspS* (Lv et al., 2014).

2.6.4 Cyanobacteria

In their natural state, cyanobacteria do not naturally produce isoprene. Cyanobacteria lack the isoprene synthase gene responsible for catalyzing the conversion of DMAPP to isoprene, which constitutes the ultimate step in isoprene synthesis. While they possess the MEP pathway for synthesizing a range of terpenoid molecules, cyanobacteria can be engineered to incorporate the *IspS* gene from higher plants. This modification enables them to undergo photosynthetic isoprene production. Heterologous expression of the *IspS* gene from a plant has been the strategy of many researchers to produce isoprene in the cyanobacterial system. The pioneer successful cyanobacterial isoprene production was documented by a research group in year 2010, who incorporated the *IspS* gene from the plant *Pueraria montana* into *Synechocystis sp.* PCC 6803, regulated by the light-responsive PsbA2 promoter (Lindberg et al., 2010). This led to an isoprene yield of 50 μg/g DCW. In a separate study, another research team employed intermittent CO_2 supplementation in engineered *Synechocystis sp.* PCC 6803 and achieved a yield of 120 μg/g DCW within a closed cultivation vessel over a 192-hour culture period (Bentley and Melis, 2012). Furthermore, when the isoprene synthase gene was expressed in conjunction with enzymes from the MVA pathway, there was a notable 2.5-fold increase in isoprene yield (Bentley et al., 2014). In another study, *S. elongatus* PCC 7942 was by overexpressing *IDI* in combination with isoprene synthase which resulted in 1.26 g/L isoprene production (Gao et al., 2016). A comparative presentation of isoprene production using engineered microorganisms has been shown in Table 2.6.

Table 2.6 Microbial isoprene production and corresponding volumetric productivities in different production hosts.

Microbial host	Engineering strategy	Production conditions	Cumulative production	Productivity (µg/L/h)	Reference
Synechocystis sp. PCC 6803	*IspS* gene integration under native P_{psbA2} promoter	Closed vessel cultivation	50 µg/g DCW (24 h)	--	Lindberg et al. (2010)
Synechocystis sp. PCC 6803	*IspS* gene integration under native P_{psbA2} promoter in host	Fed-batch cultivation in a closed system	0.00015 g/L (192 h)	0.78	Bentley and Melis (2012)
Synechocystis sp. PCC 6803	*IspS* and MVA pathway enzymes were expressed	Fed-batch cultivation in a closed system	250 µg/g DCW (192 h)	~ 1.53	Bentley et al. (2014)
Synechocystis sp. PCC 6803	*IspS* expressed under P_{psbA2} promoter	Closed system, alkaline, and saline conditions	0.00012 g/L (96 h)	1.25	Chaves et al. (2015)
Synechocystis sp. PCC 6803	*IspS* gene inserted in pVZ325 replicative plasmid under various promoters	Closed and open cultivation system, Plasmid-based expression.	93 µg/g DCW (24 h; closed system) 336 µg/g DCW (24 h; open)	1.2 4.2	Pade et al. (2016)
Synechococcus elongatus PCC 7942	*IspS*, *DXS*, *IspG* and *IDI* genes were cloned under P_{psbA2}, P_{trc} and P_{cpcB} promoters	Open cultivation system, aerated with 5% CO_2	1.26 g/L (504 h)	2500	Gao et al. (2016)
Synechocystis sp. PCC 6803	*IspS*, *IDI* and *DXS* gene integration	Open cultivation system,	0.0016 g/L (96 h)	16.6	Rana et al. (2022)
Yarrowia lipolytica	*IspS* and MVA pathway expression	17 ml sealed vials	0.0005 g/L (72 h)	6.94	Shaikh and Odaneth (2021)

Microbial host	Engineering strategy	Production conditions	Cumulative production	Productivity (µg/L/h)	Reference
Saccharomyces cerevisiae	*IspS* and MVA pathway expressed	5-L fed-batch cultivation	2.53 g/L (120 h)	21050	Lv et al. (2016)
E. coli BL21 (DE3)	*IspS* and MVA pathway expressed	5-L fed-batch cultivation	4.5 g/L (40 h)	112500	Cheng et al. (2021)
E. coli BL21 (DE3)	*IspS* and MVA pathway expressed	5-L batch 5-L fed-batch 300-L fed-batch	4.84 g/L (25 h) 22.29 g/L (54 h) 25.2 g/L (72 h)	193000 412770 350000	Lee et al. (2020)
Pantoea ananatis	*IspS* and MVA pathway expressed	1-L fed-batch cultivation	2.5 g/L (48 h)	52080	Nitta et al. (2020)
E. coli BL21 (DE3)	ptsG gene deleted. *IspS, galP* and *glk* genes expressed	250 ml shake flask	0.6652 g/L (48 h)	13850	Guo et al. (2019)
E. coli BL21 (DE3)	*IspS* and MVA pathway genes expressed	20 ml sealed vials	0.587 g/L (36 h)	16305	Liu et al. (2019)
E. coli JM109 (DE3)	*Pta* and *PoxB* genes deleted. *IspS* and MVA pathway genes expressed	20 ml sealed vials	0.092 g/L (44 h)	2091	Liu et al. (2019)
Bacillus subtilis	*IspS* gene expressed	10 ml sealed vials	0.0014343 g/L (48 h)	29.88	Gomaa et al. (2017)

IspS: isoprene synthase, *DXS*: deoxyxylulose 5-phosphate synthase, *IspG*: 4-hydroxy-3-methylbut-2-enyl diphosphate synthase, *IDI*: isopentenyl diphosphate isomerase, MVA: mevalonic acid pathway, *galP*: galactose permease, *glk*: glucokinase, *ptsG*: phosphotransferase system, *Pta*: phosphate acetyltransferase, *PoxB*: pyruvate oxidase B.

2.7 Techno-economic assessment of microbial isoprene production

In current scenarios, environmental impacts like global warming, acidification, and greenhouse gas emission are at their peaks because of complete dependency on fossil fuels. A transition to carbon-neutral fuels is required to reduce these environmental impacts and achieve the aims of the Paris Agreement. This motivates researchers to search renewable and sustainable alternatives of fossil-based fuels. When creating such products or processes, inventors must consider potential environmental effects in addition to technical and financial factors to ensure the sustainability of processes and products. Techno-economic analysis (TEA) examines a technology's technical performance and economic efficiency.

It is well-recognized that converting lab-scale technologies into a commercial product is an expensive, time-consuming, and risky enterprise. Techno-economic analysis helps in making decisions in such cases by providing probable production and capital cost of a given process at a commercial scale with process description (Xin et al., 2016). In biorefineries, TEA-based comprehensive process and plant-label models are frequently carried out for process optimization, scaling-up combining different technologies to integrate bioprocess (Murthy, 2022; Wenzlick and Siefert, 2020). Two renewable approaches for isoprene production by autotrophic (Microalgae/cyanobacteria) and heterotrophic (Bacteria) organisms were evaluated for material efficiency and economic efficiency using green metrics (Matos et al., 2013). In his study, they observed that the theoretical production of isoprene from microalgae is very low for commercial purposes. However, the market value of microalgae biomass can make the processes economically feasible. But in this case, the production of isoprene would represent a minor coproduct with a low impact on the market when compared with other high-value compounds. While isoprene production by bacteria at 10 tonnes years^{-1} scale cost at least 59560 $/tonnes (at maximum theoretical production),

which is higher than the current bio-isoprene (2500 to 5000 $/tonnes). Another research group evaluated isoprene production cost for chemical (Sumitomo process) and biological (engineered *E. coli*) process using green metrics and observed slightly higher production cost for bacterial (5,308 $/tonne) than chemical process (4,646.7 $/tonne) (Morais et al., 2015). Another research group estimated the $1.5/L minimum selling price for 1,4-dimethyl cyclooctene (Jet-A_{eq}) from isopropanol derived from bioconversion of sorghum biomass, when the supply chain and process are optimized, Raney nickel catalyst is used for hydrogenation with 18.3 g CO_2/MJ GHG footprint. For the economic feasibility of isoprene-based fuel, there is a need for process optimization and pathway engineering to gate maximum theoretical yield (Matos et al., 2013). To obtain economic viability, microalgal biofuel production can be coupled with higher-value non-fuel co-products production to achieve drastic reductions in capital costs.

2.8 Identified research gaps

Taking into account the insights gathered from the literature review, as well as the imperative to establish a sustainable process for photosynthetic isoprene production using genetically engineered cyanobacteria, the following research gaps have been identified.

- There have been no documented comprehensive investigations utilizing fast growing genetically modified cyanobacteria for the production of isoprene. Such a study could provide valuable insights into the feasibility and efficiency of utilizing engineered cyanobacteria for this purpose, potentially opening up new avenues for sustainable isoprene production methods.
- So far, there has been no reported strategy aimed at inhibiting the side reactions of the MEP pathway using a suitable inhibitor, for maximization of isoprene production. This suggests an unexplored path in the field, as researchers have yet to investigate the potential benefits of employing an inhibition strategy to optimize

isoprene yield by blocking side reactions within the MEP pathway. This area of study holds promise for advancing the efficiency of isoprene production methods.

- There has been a lack of efforts in systematically optimizing the production process parameters to enhance isoprene production in engineered cyanobacteria. This indicates an unexploited potential for research and development, as researchers have yet to focus on the crucial aspect of optimizing production conditions to maximize isoprene yields. Exploring this avenue could lead to significant advancements in the efficiency of isoprene production using engineered cyanobacteria.
- As of now, there are limited comprehensive economic assessment conducted on the process of isoprene production using engineered microbial systems. This signifies a notable gap in the research landscape, as the economic viability of such a production method remains unexplored. Conducting an economic assessment would provide valuable insights into the feasibility and potential cost-effectiveness of employing engineered microbial systems for isoprene production, contributing to a more thorough understanding of its practical applicability.