





**SPECIAL ISSUE ARTICLE**

# Photoautotrophic production of renewable ethylene by engineered cyanobacteria: Steering the cell metabolism towards biotechnological use

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**Abstract**

Ethylene is a volatile hydrocarbon with a massive global market in the plastic industry. The ethylene now used for commercial applications is produced exclusively from non-renewable petroleum sources, while competitive biotechnological production systems do not yet exist. This review focuses on the currently developed photoautotrophic bioproduction strategies that enable direct solar-driven conversion of CO<sub>2</sub> into ethylene, based on the use of genetically engineered photosynthetic cyanobacteria expressing heterologous ethylene forming enzyme (EFE) from *Pseudomonas syringae*. The emphasis is on the different engineering strategies to express EFE and to direct the cellular carbon flux towards the primary metabolite 2-oxoglutarate, highlighting associated metabolic constraints, and technical considerations on cultivation strategies and conditional parameters. While the research field has progressed towards more robust strains with better production profiles, and deeper understanding of the associated metabolic limitations, it is clear that there is room for significant improvement to reach industrial relevance. At the same time, existing information and the development of synthetic biology tools for engineering cyanobacteria open new possibilities for improving the prospects for the sustainable production of renewable ethylene.

## 1 | COMMERCIAL SIGNIFICANCE AND PRODUCTION OF ETHYLENE: NEED FOR RENEWABLE INDUSTRIAL SOLUTIONS

Fossil resources continue to be extensively utilized for the manufacture of hydrocarbon fuels and different organic materials for human consumption, despite the severe environmental impact on the climate and global ecosystems. Due to the sheer volume of the use, which currently corresponds to over 4000 Mt of crude oil per year (IEA 2020), the detachment from petroleum-based products presents one of the grand challenges of humankind. Renewable energy sources such as wind and solar power now provide competitive alternatives for the fuel and

energy sectors through the electrification of energy supply and transportation. However, we also need to find sustainable replacements for petrochemicals and derived materials, including solvents, plastics and synthetic fabrics, which constitute over 10% of the total crude oil demand (IEA 2018; for more on the subject see Levi & Cullen 2018).

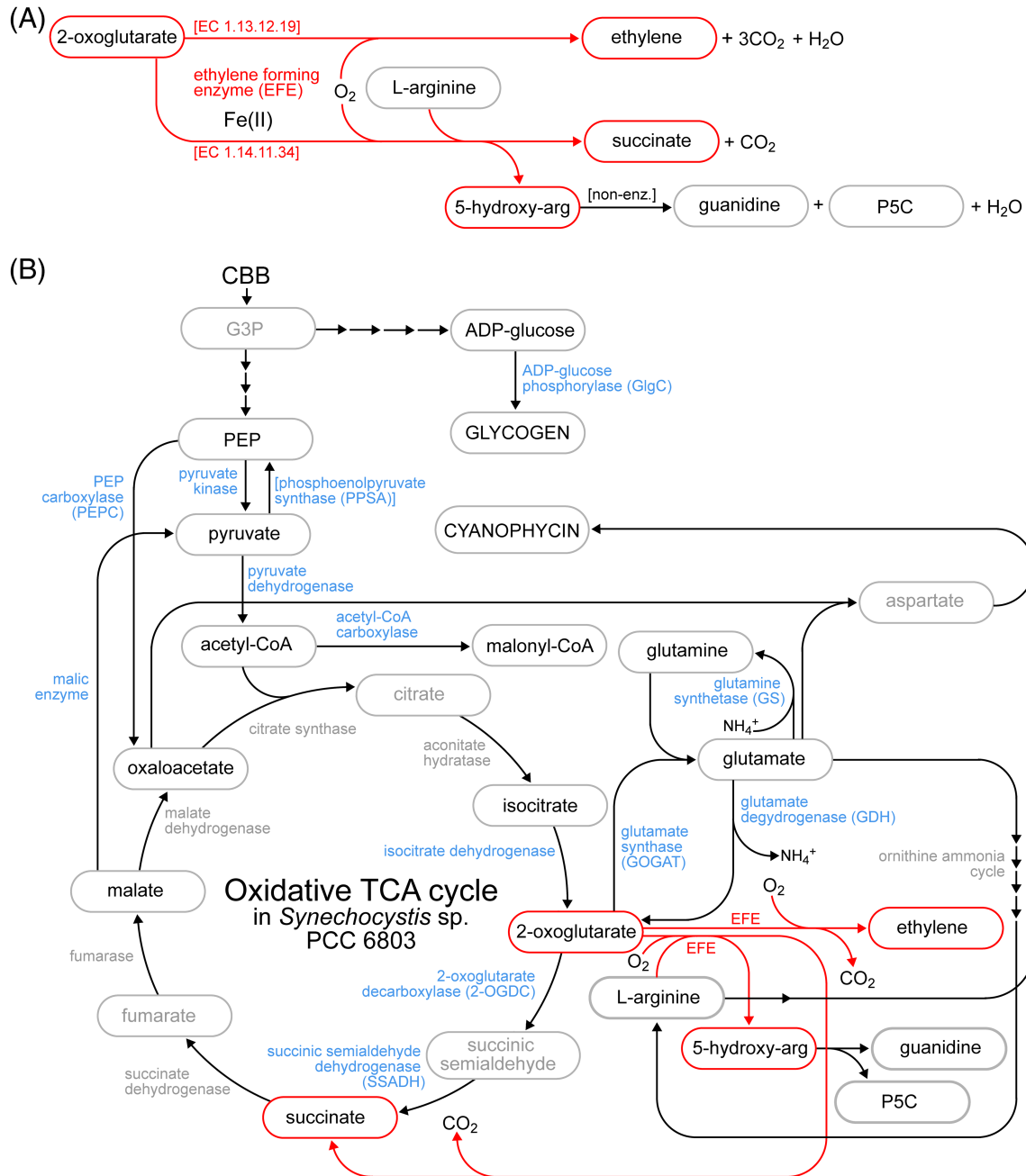
The most abundant industrial petrochemical is the light olefin ethylene (C<sub>2</sub>H<sub>4</sub>), which is globally used in chemical manufacturing at multimillion ton annual scale (Fernandez et al. 2018). Ethylene is primarily used as a precursor for producing polyethylene (PE), which is the most common plastic in the market, found for example in plastic bottles and other forms of packaging. Current large-scale ethylene production relies on steam cracking of petroleum-

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derived materials, which requires high energy input and is one of the largest single CO<sub>2</sub>-emitting processes in chemical industry. Although advanced reactor technologies are being developed to reduce the energy intensity and carbon emissions (Amghizar et al. 2020), the process is still based on oil, while alternative sustainable biotechnological production systems do not exist. This scenario does not only apply to ethylene but describes the global

challenge in general. There is an urgent demand for entirely new carbon-neutral strategies to supply renewable precursors to the manufacturing industry, in parallel to the development of new products such as biodegradable plastics to replace those now in use (Pellis et al. 2021). Ultimately, this calls for complementary industrial solutions based on readily available sustainable sources of energy, carbon and catalysts.



**FIGURE 1** The reactions catalyzed by the ethylene-forming enzyme (EFE) from *Pseudomonas syringae*. (A) Representation of the two parallel EFE-catalyzed reactions (red arrows) showing the substrates, cofactors and products. (B) A simplified nonstoichiometric representation of the heterologous EFE reactions (red arrows) integrated as part of the metabolic network of the cyanobacterium *Synechocystis* sp. PCC 6803 engineered to produce ethylene from 2-oxoglutarate. The metabolic intermediates discussed in the text are shown in black font, and the corresponding enzymes in blue font. Enzyme cofactors and side reactions have been omitted for clarity. Arg, arginine; ADP, adenosine diphosphate; CBB, Calvin-Benson-Bassham cycle; CoA, coenzyme A; P5C, 1-pyrroline-5-carboxylic acid; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid

## 2 | BIOTECHNOLOGICAL SYSTEMS USING PHOTOAUTOTROPHIC CYANOBACTERIA AS ETHYLENE-PRODUCING HOSTS

There are at least two biological enzyme-catalyzed processes that release ethylene, which offer alternatives for the development of novel biotechnological production systems. Plants and some algae produce ethylene as a hormone involved in the control of developmental processes and stress responses (Binder 2020; Ju et al. 2015). This pathway proceeds in two consecutive steps from S-adenosyl-L-methionine (SAM) via 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene, catalyzed by the enzymes ACC synthase (ACS) and ACC oxidase (ACO), respectively (Bleecker & Kende 2000; Houben & van de Poel 2019). Although the system has been linked with possible constraints including limited abundance of SAM or the release of cyanide as a side product, it has been shown to be applicable for heterologous ethylene production (Jindou et al. 2014) and offers a target for further engineering. The current review is focused on another strategy based on microbial *ethylene forming enzyme* (EFE). This enzyme is found in several plant pathogens including the Gram-negative bacterium *Pseudomonas syringae*, which in nature produce ethylene to interfere with the infected plant's normal ethylene signaling. This is expected to render the plant more susceptible to damage, which is advantageous for the invading pathogen (Weingart et al. 2001). The ethylene-forming reaction catalyzed by EFE uses the tricarboxylic acid (TCA) cycle intermediate 2-oxoglutarate (2-OG) as the primary substrate and is therefore tightly coupled to the cellular carbon/nitrogen metabolism, as discussed in detail in the following sections.

Heterologous expression of EFE has been applied to produce ethylene in several heterotrophic microorganisms including *Escherichia coli* (Fukuda et al. 1992a, 1992b; Lynch et al. 2016) and *Saccharomyces cerevisiae* (Pirkov et al. 2008; Johansson et al. 2014; Johansson et al. 2017; see review Eckert et al. 2014). These systems are fundamentally restricted by the dependence on carbohydrate starting materials, which typically derive from relatively limited sources of biomass, thereby setting a limit to the upscale potential. An alternative strategy is to express EFE in photosynthetic cyanobacteria to achieve direct solar-driven production of ethylene from CO<sub>2</sub> (Eckert et al. 2014; Figure 1), which as a process is energetically much more favorable in comparison to any system that uses plant biomass as starting material. The concept is founded on long-term biochemical research on cyanobacterial metabolism and molecular engineering, which has branched off towards the development of novel biotechnological applications (Angermayr et al. 2015; Hagemann & Hess 2018; Luan et al. 2020; Luan & Lu 2018). Because ethylene is a volatile gas, it requires a culture system with a closed gas circuit but enables end-product harvest without the need for separation of biomass or extraction from the growth medium. In addition, since ethylene diffuses spontaneously out from the cell and into the culture headspace, potential problems with product toxicity and end-product inhibition are avoided. Although the overall approach is very attractive, there are still many biological and technological hurdles that need to be overcome in order to allow the transition towards efficient large scale

production systems (Markham et al. 2016). For this to be possible, it is necessary to understand the function of the cyanobacterial host at molecular level, and to be able to extrapolate the current knowledge towards new metabolic engineering and cultivation strategies. This review focuses on the development of cyanobacterial ethylene production systems and provides a perspective to different aspects of integrating the heterologous EFE pathway as part of the cyanobacterial metabolic network.

The research on cyanobacterial ethylene production dates back to the end of the 1980s and is primarily based on the expression of heterologous EFE (Figure 1) that originates from the Gram-negative plant pathogen *P. syringae* *pv. phaseolicola* PK2. Although the field is quite dispersed, a majority of the work has been carried out in *Synechocystis* sp. PCC 6803, in addition to several case-studies in *Synechococcus elongatus* PCC 7942 and in *Synechococcus elongatus* PCC 11801. Associated literature on the cyanobacterial ethylene production systems that rely on EFE have been summarized in Table 1 (see also supplementary table S1 in Carbonell et al. 2019). While providing a general overview of the expression and cultivation strategies used in the field, the listing underlines the diversity and related difficulties in direct qualitative or quantitative comparison between parallel studies. Altogether, it is clear that even the best systems currently produce ethylene only at very low levels (Table 1), demonstrating the need for prominent improvement to enable the transition towards biotechnological use.

## 3 | EFE STRUCTURE AND FUNCTION: BASIS FOR TWO SEPARATE REACTIONS AND IMPLICATIONS ON ETHYLENE PRODUCTION

The native EFE from *P. syringae* *pv. phaseolicola* PK2 is a soluble monomeric enzyme (350aa; 39 445 Da; Martinez & Hausinger 2016) that shares sequence homology and overall fold with dioxygenases in the Fe(II)/2-OG oxygenase superfamily. Extensive molecular studies including biochemical functional characterization, 3D-crystallography, structure-based mutagenesis and kinetic analysis have provided detailed information on the complex EFE-catalyzed reaction, the catalytic mechanism and structure-function interactions (Martinez et al. 2017; Martinez & Hausinger 2016; Zhang et al. 2017). EFE is a unique enzyme both in the 3D fold and function, as it shares structural characteristics with both 2-OG oxygenase subgroups I and II, with a distinct active site topology, and essentially, the unusual ability to produce ethylene (Martinez et al. 2017).

EFE catalyzes a bifurcated reaction with two distinct conversion reactions that essentially compete with one another (Fukuda et al. 1992a, 1992b; Martinez & Hausinger 2016; Figure 1A). In the ethylene-forming reaction [EC 1.13.12.19], which is unique and of specific interest in this review, the enzyme catalyzes a four-electron oxidation of 2-OG into ethylene and three equivalents CO<sub>2</sub>. The reaction requires molecular oxygen as co-substrate, and is dependent on Fe(II) and L-Arginine (L-Arg) as cofactors. Notably, L-Arg is not consumed in this reaction (Martinez & Hausinger 2016), in contrast to

**TABLE 1** List of engineered cyanobacterial ethylene production systems described in literature

Engineered strain	Genes expressed	Cultivation conditions	Culture vessel	Cultivation mode	Sampling vial	Maximum productivity	Reference
<i>Synechococcus</i> sp. PCC7942 R2-SPc ΔpUH25	1 × <i>efe</i>	OD ≈ NA, 1 × BG11, 30°C, ambient, 162 μmol photons m <sup>-2</sup> s <sup>-1</sup>	NA	Batch	NA	≈25 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> (≈25 nL ml <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> )	(Fukuda et al., 1994)
<i>Synechococcus</i> sp. PCC7942 R2-SPc ΔpUH24	1 × <i>efe</i>	OD ≈ NA, 1 × BG11, 25°C, ambient, 100 μmol photons m <sup>-2</sup> s <sup>-1</sup>	E-flasks	Batch	2 ml in 64 ml flask	≈323 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> (≈323 nL ml <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> )	(Sakai et al., 1997)
<i>Synechococcus</i> sp. PCC7942 R2-SPc	1 × <i>efe</i>	OD ≈ NA, 1 × BG11, 28°C, 200 ml min <sup>-1</sup> 1% CO <sub>2</sub> , 26 μmol photons m <sup>-2</sup> s <sup>-1</sup>	50 ml in 700 ml Loux bottle	Batch	NA in 16 ml test tube	≈0.8 mol kgDW <sup>-1</sup> h <sup>-1</sup>	(Wang et al., 2000)
<i>Synechococcus</i> sp. PCC7942 R2-SPc ΔpUH24	1 × <i>efe</i>	OD <sub>730</sub> ≈ 1.0, 1 × BG11, 28°C, ambient, 4.4 × 10 <sup>-4</sup> J cm <sup>-2</sup> s <sup>-1</sup>	50 ml in 500 ml E-flasks	Batch	1 ml in NA sealed tube	≈451 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> (≈451 nL ml <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> )	(Takahama et al., 2003)
<i>Synechocystis</i> sp. PCC 6803 ΔpsbAII	2 × <i>efe</i>	OD <sub>730</sub> ≈ 15.0, 5 × BG11, 30°C, 5% CO <sub>2</sub> , 20 mM NaHCO <sub>3</sub> , 600 μmol photons m <sup>-2</sup> s <sup>-1</sup>	35 ml in 250 ml E-flasks	Batch	2 ml in 13 ml Hungate tube	≈250 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup>	(Ungerer et al., 2012)
<i>Synechocystis</i> sp. PCC 6803	1 × <i>efe</i>	OD <sub>750</sub> ≈ 0.5, 1 × BG11, 30°C, 1% CO <sub>2</sub> , 100 μmol photons m <sup>-2</sup> s <sup>-1</sup>	250 ml E-flasks	Batch	1 ml in 10 ml serum bottle	≈170 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> (≈170 nL ml <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> )	(Guerrero et al., 2012)
<i>Synechococcus elongatus</i> sp. PCC 7942	1 × <i>acs</i> , 1 × <i>aco</i>	OD <sub>730</sub> ≈ 1.0, 1 × BG11, 27°C, 100 μmol photons m <sup>-2</sup> s <sup>-1</sup>	50 ml in 250 ml E-flasks	Batch	10 ml in 20 ml vial	≈3.4 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> (≈3.4 nL ml <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> )	(Jindou et al., 2014)
<i>Synechocystis</i> sp. PCC 6803	1 × <i>efe</i>	OD ≈ NA, 1 × BG11, 30°C, 5% CO <sub>2</sub> , 50 μmol photons m <sup>-2</sup> s <sup>-1</sup>	NA	Batch	2 ml in 13 ml Hungate tube	≈718 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup>	(Xiong et al., 2015)
<i>Synechocystis</i> sp. PCC 6803 Δogdc Δssadh ΔphaAB	2 × <i>efe</i> , 1 × <i>kgfP</i>	OD <sub>730</sub> ≈ 0.1, 5 × BG11 (1 × nitrate), 30°C, 5% CO <sub>2</sub> , 200 μmol photons m <sup>-2</sup> s <sup>-1</sup> , 1 mM 2-OG	200 ml in 250 ml E-flasks	Semi-continuous	1 ml in 10 ml sealed bottle	≈858 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup>	(Zhu et al., 2015)
<i>Synechocystis</i> sp. PCC 6803	2 × <i>efe</i>	OD <sub>680</sub> ≈ 1.0, 5 × BG11, 30°C, 1% CO <sub>2</sub> , 100–200 μmol photons m <sup>-2</sup> s <sup>-1</sup>	NA ml in Flat-panel PBR	Quasi-continuous	-	≈0.26 mmol gDW <sup>-1</sup> h <sup>-1</sup>	(Zavřel et al., 2016)
<i>Synechocystis</i> sp. PCC 6803 ΔpsbAII	1 × <i>efe</i> , <i>xyIA</i> , <i>xyIB</i>	OD <sub>730</sub> ≈ 0.1, 1 × BG11, 30°C, 5% CO <sub>2</sub> , 20 mM NaHCO <sub>3</sub> , 50 μmol photons m <sup>-2</sup> s <sup>-1</sup> , 50 mM xylose*	NA	Batch	2 ml in 13 ml Hungate tube	≈109 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup> (≈945 μl l <sup>-1</sup> h <sup>-1</sup> )	(Lee et al., 2015)
<i>Synechocystis</i> sp. PCC 6803 Δogdc Δssadh ΔntcA	4 × <i>efe</i>	OD <sub>730</sub> ≈ 0.1, 1 × BG11, 30°C, bubbled with air, 50–100 μmol photons m <sup>-2</sup> s <sup>-1</sup>	NA	Batch	1 ml in 10 ml sealed bottle	≈2463 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup>	(Mo et al., 2017)
<i>Synechocystis</i> sp. PCC 6803	1 × <i>efe</i>	OD <sub>730</sub> ≈ 0.5, 1 × BG11, 30°C, 1% CO <sub>2</sub> , 220 μmol photons m <sup>-2</sup> s <sup>-1</sup>	NA ml in 1 L flat glass bottle	Batch	4 ml in 20 ml vial	≈195 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup>	(Veetil et al., 2017)
<i>Synechococcus elongatus</i> sp. PCC 7942	1 × <i>efe</i>	OD <sub>750</sub> ≈ 0.1, 1 × BG11, 30°C, 1% CO <sub>2</sub> , 50 μmol photons m <sup>-2</sup> s <sup>-1</sup>	100 ml in 250 ml E-flasks	Semi-continuous	1 ml in 10 ml vials	≈140 μl l <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup>	(Carbonell et al., 2019)
<i>Synechocystis</i> sp. PCC 6803	1 × <i>efe</i> , 3 × <i>pepc</i> <sup>a</sup> , 1 × <i>pepc</i> <sup>b</sup>	OD <sub>730</sub> ≈ 0.1, 1 × BG11, 30°C, ambient, 20 μmol photons m <sup>-2</sup> s <sup>-1</sup>	50 ml in NA ml E-flasks	Batch	2 ml in 6 ml vial	≈0.83 μg ml <sup>-1</sup> OD <sup>-1</sup> h <sup>-1</sup>	(Durall et al., 2020)

TABLE 1 (Continued)

Engineered strain	Genes expressed	Cultivation conditions	Culture vessel	Cultivation mode	Sampling vial	Maximum productivity	Reference
<i>Synechococcus elongatus</i> sp. PCC 11801	1 × <i>efe</i>	OD <sub>730</sub> ≈ 0.05, 1 × BG11, 38°C, 50 mM NaHCO <sub>3</sub> , 1% CO <sub>2</sub> , 20 μmol photons m <sup>-2</sup> s <sup>-1</sup>	50 ml in NA ml E-flasks	Batch	20 ml in 120 ml Hungate glass bottles	≈338 μmol gDW <sup>-1</sup> h <sup>-1</sup>	(Sengupta et al., 2020)
<i>Synechocystis</i> sp. PCC 6803	1 × <i>efe</i>	Chl ≈ 33 μg, 1 × BG11, 30°C, 200 mM NaHCO <sub>3</sub> , 35 μmol photons m <sup>-2</sup> s <sup>-1</sup>	3 ml in 23.5 ml vials	Semi-continuous	23.5 ml vials	≈0.84 μmol mg Chl <sup>-1</sup> h <sup>-1</sup>	(Vajravel et al. 2020)
<i>Synechocystis</i> sp. PCC 6803	1 × <i>efe</i>	Chl ≈ 26 μg Chl, 1 × BG11, 30°C, 200 mM NaHCO <sub>3</sub> , 35 μmol photons m <sup>-2</sup> s <sup>-1</sup>	1% alginate thin films (3 × 1 cm strips) in 23.5 ml vials	Continuous	23.5 ml vials	≈1.28 μmol mg Chl <sup>-1</sup> h <sup>-1</sup>	(Vajravel et al. 2020)

Note: Most strategies base on the expression of heterologous ethylene-forming enzyme (EFE) from *Pseudomonas syringae* to enable direct photoautotrophic production of ethylene. The numerical values and units are presented as in the original publications, but with the light intensities converted to SI units for consistency. The maximum productivity values are given also in ml<sup>-1</sup> ml<sup>-1</sup> OD h<sup>-1</sup> (in brackets) when possible. The listed gene abbreviations and the corresponding encoded enzymes: *aco*, aminocyclopropane carboxylate oxidase; *acs*, 1-aminocyclopropane-1-carboxylate synthase; *efe*, ethylene-forming enzyme; *kgfp*, 2-OG permease; *pepc*, phosphoenolpyruvate carboxylase; *xyIA*, xylose isomerase; *xyIB*, xylokinase.

Abbreviations: E-flask, Erlenmeyer flask; NA, Not available data; Photobioreactor, PBR All the experiments were performed under photoautotrophic mode, unless indicated by an asterisk (\*).

<sup>a</sup>Native gene.

<sup>b</sup>*Synechococcus* sp. PCC 7002 origin.

what has been anticipated earlier, which may have important metabolic implications for future engineering. In the alternative second reaction (EC 1.14.11.34) which is typical to 2-OG oxygenases, EFE catalyzes the C5-hydroxylation of L-Arg into 5-hydroxy-L-Arg, with concomitant oxidative decarboxylation of 2-OG into succinate. Subsequently, the resulting 5-hydroxy-L-Arg decomposes nonenzymatically into the two final products guanidine (Wang et al. 2019) and L-Δ-1-pyrroline-5-carboxylic acid (P5C). The ethylene-forming reaction and the hydroxylation of L-Arg are thus separate events, and represent two alternative scenarios (Martinez & Hausinger 2016). The ratio between ethylene and succinate synthesis has been reported to be 2:1 (Fukuda et al. 1992a, 1992b; Martinez & Hausinger 2016), but this is likely not fixed, and depends on the exact reaction conditions. Combined with molecular structural information, this could provide an opportunity for enhancing ethylene production by attenuating the unwanted L-Arg hydroxylation by structure-based enzyme engineering, or by optimizing the process conditions.

EFE is composed of 10 α-helices and two β-sheets that form a distorted double-stranded β-helix (DSBH) core, with an unusually hydrophobic active site pocket at the interface of the secondary structure elements. The enzyme undergoes extensive structural rearrangement upon L-Arg binding by induced fit mechanism (Zhang et al. 2017), which is essential for the catalysis, and involved in determining the reaction specificity between ethylene and succinate formation. In the proposed complex substrate recognition pattern, the 2-OG substrate is first coordinated to Fe(II) in the active site, followed by L-Arg binding, which results in conformational changes in the active site topology, and the alignment of the different reacting species (Martinez et al. 2017). The atypical ability to produce ethylene appears to be linked with changes in metal coordination, dioxygen binding site, and the hydrogen bonding pattern around the substrates. Ultimately, the L-Arg in the active site is aligned in a configuration that does not favor C5-hydroxylation (off-line configuration) and enables the ethylene-forming reaction to take place. Consistent with alternative binding modes between the substrates, the ethylene-forming reaction shows substrate inhibition in regards to both L-Arg and 2-OG (Zhang et al. 2017), emphasizing the intramolecular factors dictating the outcome, and the complexity of the reaction when it comes to optimizing ethylene production. While this obviously reflects on the interpretation of the kinetic parameters, the apparent  $K_M$  values for both 2-OG and L-Arg are in the micromolar range, whereas the apparent  $K_{cat}$  for ethylene formation has been reported to be around 2 s<sup>-1</sup> (Martinez & Hausinger 2016). Notably, the catalytic efficiency  $K_{cat}/K_M$  is significantly higher for ethylene formation in comparison to L-Arg hydroxylation (Martinez & Hausinger 2016), which further supports the view of the ethylene reaction being the predominant pathway in EFE.

The ethylene-forming reaction consumes equimolar amounts of 2-OG and is thus metabolically connected to a number of endogenous pathways branching off from the TCA cycle, including the biosynthesis of derived amino acids (glutamate, glutamine, proline in addition to L-Arg) and purine metabolism (guanidine). Importantly, as 2-OG provides the carbon skeleton for nitrogen assimilation and serves as an

indicator for the intracellular carbon/nitrogen balance in cyanobacteria (Fokina et al. 2010), the use of this intermediate pool for ethylene biosynthesis may affect central metabolic flux rates and intracellular equilibria such as ATP/NADPH ratio – with potential downstream effects at the cellular level. Some of the associated key considerations are discussed in more detail in the following sections from the viewpoint of ethylene production and biotechnological limitations.

### 3.1 | Ethylene and EFE as the limiting factors in heterologous cyanobacterial production systems

Ethylene in itself appears not to be toxic to cyanobacterial cells. Adverse physiological effects that would restrict the development of biotechnological systems have not been reported in *Synechocystis* or *Synechococcus* even at high atmospheric ethylene concentrations (Carbonell et al. 2019; Ungerer et al. 2012; Vajravel et al. 2020). However, cyanobacteria have been shown to harbor an active plant-like ethylene receptor protein (SynEtr1 encoded by *slr1212* in *Synechocystis*; Rodríguez et al. 1999) that has been linked to various cellular functions. Ethylene has been shown to affect, for example, cyanobacterial phototaxis, pilus biogenesis and biofilm formation (Lacey & Binder 2016), as well as cellular fatty acid composition and growth properties (le Henry et al. 2017). Transcriptomic profiling further reveals the range of potential ethylene-induced responses in cyanobacteria (Lacey et al. 2018), which reflects the complexity of the interactions that may become relevant for future generations of engineered ethylene-producing strains.

With the objective of maximizing ethylene production in cyanobacteria, the expression of EFE must first be optimized to ensure that the ethylene-forming reaction per se would not be the primary system limitation. To accomplish this, various approaches have been evaluated to (1) increase the *efe* gene dosage per cell, and to (2) improve EFE expression efficiency at the level of transcription and translation. In the most common expression strategies *efe* has been integrated in the host chromosome, with the introduction of two (Ungerer et al. 2012; Zhu et al. 2015) or more (Mo et al. 2017; Xiong et al. 2015; Zhu et al. 2015) gene copies to increase the intracellular EFE concentration. As an alternative, RSF1010-based replicative plasmids have also been used for expressing EFE in cyanobacteria (Carbonell et al. 2019; Veetil et al. 2017). Although the plasmids offer a convenient strategy for cyanobacterial engineering in general (Ferreira et al. 2018), the partition mechanisms and copy number-associated effects that directly affect expression system stability and potential use in larger scale (Million-Weaver & Camps 2014), are not comprehensively understood. To maximize the expression at transcriptional level, EFE has been expressed under the control of several different promoters (Guerrero et al. 2012; Wang et al. 2018; Zhu et al. 2015), and multiple copies of the same promoter (Veetil et al. 2017). The expression systems have also been optimized in regards to translation by using alternative RBS elements (Thiel et al. 2018; Veetil et al. 2017; Wang et al. 2018), and by altering the

upstream 5' UTR sequences (Wang et al. 2018; Xiong et al. 2015) to promote more efficient protein synthesis. Modification of EFE by fused affinity tags that enable extraction (Guerrero et al. 2012; Ungerer et al. 2012), and codon optimization to prevent species-specific translational limitations (Carbonell et al. 2016; Veetil et al. 2017), have not had notable effects on ethylene productivity in the systems tested so far. It appears that in some of the engineered cyanobacterial systems the amount of EFE is no longer the primary restriction in ethylene production, as demonstrated by cases where simple increase in the enzyme concentration provides no further improvement (Durall et al. 2020; Wang et al. 2018). However, as the ethylene forming reaction is dependent on the local substrate concentrations and associated EFE substrate inhibition kinetics (Zhang et al. 2017), the efficiency of 2-OG conversion to ethylene may again become the critical bottleneck as the substrate availability continues to be improved.

### 3.2 | Steering the carbon flux towards the TCA cycle and the EFE substrate 2-OG

The availability of required substrates and enzyme cofactors is a major consideration in the development of any biotechnological system. In the case of the heterologous EFE pathway, ethylene biosynthesis is dependent on the efficient flux of cellular resources towards the oxidative TCA cycle intermediate 2-OG, while the associated regulatory and house-keeping functions must be maintained to ensure the well-being of the host. Importantly, photoautotrophic cyanobacteria do not use the TCA cycle as the primary source of cellular chemical energy like heterotrophic organisms, and the flux through 2-OG is mainly limited to the anaplerotic reactions needed for photoautotrophic growth. This is reflected on the native carbon distribution in the cell, which may severely restrict the availability of 2-OG for ethylene production. However, the cyanobacterial metabolic network appears to be relatively flexible and adjustable to changing environmental and genetic conditions (Xiong et al. 2015), as seen, for example, in strains that are able to efficiently shift between different trophic modes depending on the availability of light, CO<sub>2</sub> and carbohydrate substrates. This plasticity is also reflected on our ability to tailor the strains (Xiong et al. 2015), yet while being beneficial, it also allows the host cell to effectively buffer against the targeted changes, thereby complicating rational engineering. Hence, individual changes may have wide-ranging effects on the cellular metabolic network, and the interactions must be realized when synchronizing between the carbon fixation and the downstream steps towards the 2-OG, while blocking the native pathways that potentially compete with EFE.

2-OG is the primary substrate for EFE, and the only carbon-based precursor needed for ethylene biosynthesis (L-Arg only serves as a cofactor in the ethylene-forming reaction; Figure 1A). As a proof of concept, supplementation of 2-OG in the growth medium has been shown to increase ethylene production in engineered *E. coli* (Lynch et al. 2016) and in cyanobacteria expressing EFE (Carbonell et al. 2019; Zhu et al. 2015). The use of extracellular 2-OG by



cyanobacteria, however, appears to be limited by inefficient penetration through the cell membrane, as demonstrated by enhanced ethylene production after the introduction of heterologous 2-OG permease (KgtP) from *E. coli* (Zhu et al. 2015). In analogy, as the native carbon flux to the TCA cycle is stringently regulated by the cell, metabolic engineering strategies that funnel cellular resources down towards 2-OG are likely to improve ethylene production (see Figure 1B for the reactions described in the text). In the simplest scenario, this is observed by merely over-expressing EFE, which alters the cellular carbon flux distribution, and steers resources in the direction of the 2-OG more effectively than in the WT (Xiong et al. 2015). This pull towards 2-OG also results in concurrent upregulation of the carbon fixation reactions in the upper part of the metabolism, thereby affecting the entire pathway from the CBB cycle to the ethylene-forming reaction (Xiong et al. 2015). The flux to the TCA cycle in *Synechocystis* has been shown to increase by the overexpression of phosphoenolpyruvate carboxylase (PEPC), an enzyme that catalyzes the irreversible carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate (Hasunuma et al. 2016; Hasunuma et al. 2018). This detour allows the cell to fix more carbon, and to bypass the primary oxidative pathway through pyruvate (pyruvate kinase) and acetyl-CoA (pyruvate dehydrogenase complex), which are strictly influenced by the energy level and the redox status of the cell. In accordance, the introduction of additional copies of PEPC has been shown to significantly increase the production of ethylene in EFE-expressing *Synechocystis* strains, which is expected to be directly linked with increased availability of 2-OG (Durall et al. 2020). Ethylene production may further be enhanced by the simultaneous introduction of heterologous phosphoenolpyruvate synthase (PPSA from *Synechococcus*; Durall et al. 2020), which catalyzes the conversion of pyruvate into PEP. In context with the endogenous malic enzyme that converts malate to pyruvate, PPSA may support the flux via the PEPC-route back to the TCA cycle and 2-OG. Alternatively, the flux via the main pyruvate dehydrogenase route may be reinforced by limiting the native pathways that use acetyl-CoA as substrate, and hence compete with citrate synthase for the entry of carbon into the TCA cycle. Such endogenous pathways include the storage compound polyhydroxybutyrate (PHB) which effectively drains the carbon away from 2-OG and ethylene under nitrogen limitation, and fatty acids required for the production of membrane lipids when the cells are growing. Accordingly, blocking the activity of acetyl-CoA carboxylase that directs carbon towards malonyl-CoA for de novo fatty acid biosynthesis, has been shown to improve ethylene production in cells overexpressing PEPC (Durall et al. 2020). The flux towards ethylene may also be enhanced by engineering the TCA cycle steps, by overexpressing isocitrate dehydrogenase that is responsible for the conversion of isocitrate to 2-OG, or by inactivating the subsequent competing native steps (Lynch et al. 2016; Zhu et al. 2015) catalyzed by 2-OG decarboxylase (OGDC) and succinic semialdehyde dehydrogenase (SSADH) that together form succinate in cyanobacteria (Zhang & Bryant 2011).

In context with the high substrate demand, it should be noted that the EFE-catalyzed reaction releases three equivalents of CO<sub>2</sub> in each reaction cycle from five-carbon 2-OG into ethylene (Figure 1A),

which makes the overall pathway very carbon-inefficient (Eckert et al. 2014). Although this is mechanistically a wasteful reaction, the released CO<sub>2</sub> is destined to be recycled back to be fixed by RuBisCO in the CBB cycle, and is thus not lost from the system, unlike in heterotrophic host organisms that are unable to fix CO<sub>2</sub>. In this respect, the EFE pathway via 2-OG appears well compatible with the photoautotrophic machinery, although the true net impact of the CO<sub>2</sub> release cannot be quantitatively assessed before the dynamic interactions with the surrounding metabolism have been mapped out.

### 3.3 | Ethylene production via 2-OG in context with nitrogen metabolism in cyanobacteria

Besides the carbon flux, photoautotrophic ethylene production from 2-OG is intimately linked with cellular nitrogen metabolism and various regulators in the reaction network. At the precursor level, 2-OG is the substrate for glutamate, and therefore serves as the carbon skeleton for the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle, which is the main ammonium assimilation pathway in cyanobacteria (see review Zhang et al. 2018). Restricted availability of 2-OG would therefore limit the process, with potential impact on ethylene production as well as the maintenance of native carbon/nitrogen homeostasis, that may cause many indirect secondary effects.

At regulatory level, 2-OG plays a key role as a signaling molecule indicating nitrogen starvation in the cell (Zhang et al. 2018). In this cascade, 2-OG is bound by NtcA, the global nitrogen regulator in cyanobacteria, which triggers various downstream cellular responses when nitrogen is scarce (Zhao et al. 2010). At this common interface between ethylene biosynthesis and regulation of nitrogen metabolism, the expression levels of NtcA have been shown to inversely correlate with ethylene production in *Synechocystis* strains expressing EFE (Mo et al. 2017). This is expected to be related at least in part to the increased flux towards glutamine biosynthesis via GS, as promoted by NtcA, which drains the flux away from 2-OG and EFE. In parallel, ethylene biosynthesis is likely to be affected by the global signal transduction protein P<sub>II</sub> that also regulates nitrogen metabolism in cyanobacteria (Scholl et al. 2020). P<sub>II</sub> is involved in sensing the overall energy and carbon/nitrogen status of the cell (Watzet et al. 2019), and modulates the activity of a number of interaction partners including NtcA and PEPC in response to 2-OG and ATP/ADP levels. Under energy-rich conditions when nitrogen is abundant, and the 2-OG levels are down, P<sub>II</sub> forms a complex with PEPC, which results in the loss of ATP-induced native inhibition and stabilizes the enzyme (Scholl et al. 2020), thereby promoting the carbon flux towards 2-OG through PEPC.

The introduced ethylene pathway is interconnected with the cellular carbon/nitrogen metabolism also via L-Arg, which is regulated in response to nitrogen availability. The biosynthesis of L-Arg is dependent on N-acetyl-L-glutamate kinase (NAGK), an enzyme catalyzing the committed step in cyclic arginine synthesis, that is regulated by the same P<sub>II</sub> control circuit that modulates the activity of NtcA and PEPC. When nitrogen is abundant and 2-OG levels are low, the

system upregulates the production of L-Arg, and further, enhances the flux towards the cyanobacterial nitrogen reserve cyanophycin (multi-L-arginyl-poly-L-aspartic acid; Maheswaran et al. 2006). Although L-Arg is required as a cofactor for ethylene synthesis and consumed in the competing branch of the EFE reaction, it is likely to limit ethylene production less than the availability of 2-OG. This is consistent with the observations that the supplementation of L-Arg in the culture medium has not had any clear positive effect on ethylene production in *Synechococcus* (Carbonell et al. 2019) or *Synechocystis* (Durall et al. 2020), while in heterotrophic *Saccharomyces cerevisiae* L-Arg addition actually reduced the yield (Johansson et al. 2014).

The intracellular signals associated with nitrogen limitation, whether caused by conditional factors or genetic modifications that increase the 2-OG levels, also link to the central carbon metabolism, and trigger the accumulation of glycogen reserves (Aikawa et al. 2012; Yoo et al. 2007). In this regulatory circuit, the buildup of 2-OG alters the crosstalk of P<sub>II</sub> with another two interactors, PirC (PII-interacting regulator of carbon metabolism) and PGAM (2,3-phosphoglycerate-independent phosphoglycerate mutase), consequently redirecting the cellular carbon flux towards glycogen storage (Orthwein et al. 2021). In context with ethylene production, the inactivation of glycogen biosynthesis by the deletion of *glgC* (*slr1176*; encodes ADP-glucose pyrophosphorylase) prevents this, and leads to the accumulation and excretion of 2-OG during nitrogen starvation in *Synechocystis* (Gründel et al. 2012; Carrieri et al. 2012; Carrieri et al. 2015). However, the higher 2-OG levels in the  $\Delta$ *glgC* background do not necessarily lead to higher ethylene productivity when coupled with the expression of EFE (Veetil et al. 2017), underlining the complexity of the metabolic interactions. This may be linked to the fact that glycogen is a cellular energy buffer crucial for native cyanobacterial physiology. Disturbances in the native regulation may alter the cellular photosynthetic capacity (Gründel et al. 2012), NADP(H), cofactor redox balance (Shinde et al. 2020), and the ATP/ADP energy charge ratio (Cano et al. 2018), which are likely to affect the overall metabolism of the cell. In addition, the  $\Delta$ *glgC* strains exhibit reduced stress tolerance towards different environmental fluctuations including light stress and nitrogen limitation (Gründel et al. 2012), which may restrict the prospects for biotechnological use (see review Luan et al. 2019).

The ethylene pathway also generates nitrogen-rich guanidine (CH<sub>5</sub>N<sub>3</sub>) as a side-product in the competing EFE-catalyzed reaction via 5-hydroxy-L-Arg (Figure 1). As a toxic compound, guanidine must be removed either by degradative pathways that recycle nitrogen back into use, or by transport out from the cell (Nelson et al. 2017). The expression of EFE in *Synechocystis* and in N<sub>2</sub>-fixing *Anabaena* sp. PCC 7120 has been shown to result in the accumulation of significant amounts of guanidine in the culture medium, suggesting that endogenous conversion reactions in these cyanobacterial hosts do not effectively break down the product (Wang et al. 2019). While this promotes wasteful efflux of fixed nitrogen from the cell, it allows guanidine to be extracted as a parallel target chemical of commercial interest, thus increasing the industrial potential of the EFE systems under development (Wang et al. 2019). The guanidine metabolism in

prokaryotes is not yet comprehensively understood (Nelson et al. 2017; Salvail et al. 2020; Wang et al. 2019), and possible engineering strategies to enzymatically recycle the product to enhance ethylene production remain to be explored. The other product formed in the breakdown of 5-hydroxy-L-Arg alongside guanidine is P5C, which is a native metabolic intermediate at the intersection of glutamate and proline biosynthesis (Wang et al. 2019). Excess of P5C may alter the abundance of these amino acids in the cell, and due to the complex role of proline in a range of cellular processes (Christgen & Becker 2019), could result in unexpected physiological effects in strains expressing heterologous EFE at high levels.

### 3.4 | Cultivation strategies for ethylene production in cyanobacteria

Besides the genetic background, the culture conditions have a significant effect on the production of ethylene by engineered cyanobacteria. As ethylene biosynthesis via the EFE pathway is dependent on the metabolic flux down to the TCA cycle and 2-OG, conditional factors that alter the metabolic state of the host cell directly influence the amount of the available resources funneled to the target pathway. As photosynthetic organisms are not primarily dependent on the TCA cycle as a catabolic pathway for generating biological energy equivalents (ATP and NADPH), and mainly use it to obtain amino acid precursors for protein synthesis, the overall flux towards 2-OG under different trophic conditions (photomixotrophy and photoautotrophy) can be severely limited (Wan et al. 2017). The flux ratios together with the total efficiency of the light conversion and CO<sub>2</sub> fixation, which directly influence ethylene production, are therefore determined by external factors such as light, and the availability of carbon and nitrogen. These factors, as experienced by individual cells in the system, are dependent on the specific cultivation setup and equipment used for ethylene production and differ from one published study to another (Table 1).

In most published cases, ethylene production by cyanobacterial hosts has been studied in lab-scale liquid batch cultures in Erlenmeyer flasks (Table 1). In these systems, the aeration, and hence the gas transfer between the headspace and the medium, is facilitated simply by mixing. This has a direct impact on the distribution of CO<sub>2</sub> to the cells, which typically limits optimal photoautotrophic production. In more advanced photobioreactor systems, gas may be actively pumped into the medium, which can improve the availability of carbon in the culture. The carbon dioxide concentrations typically vary between ambient and 5% CO<sub>2</sub> in the gas phase, while bicarbonate is in many cases supplemented as an additional source of inorganic carbon. In this context, also the temperature (20–30°C), the medium pH (7.5–8) and buffer capacity affect the solubility and hence the carbon uptake efficiency. While higher CO<sub>2</sub> concentrations boost the cellular carbon uptake in general, the impact on ethylene production is not necessarily linear, and dependent on combined conditional and genetic effects. The carbon partitioning is affected by the overall energy status of the cell, and increased CO<sub>2</sub> availability may promote biomass accumulation, the flux towards storage compounds, or the excretion of organic



acids through overflow metabolism at the expense of ethylene, if the system is not in balance (Cano et al. 2018; Gründel et al. 2012). It is important also to note that the availability of oxygen, which is essential for the EFE-catalyzed reaction as well as for the oxidative TCA cycle, is likely to be less restricted in oxygen-producing photoautotrophic cyanobacteria as compared to heterotrophic production systems, in which ethylene production is clearly limited by the O<sub>2</sub> solubility and transfer through the medium (Johansson et al. 2013).

The performance of photoautotrophic production systems is also critically affected by the light conditions. Apart from the incident light source that determines the quality and quantity of incoming radiation, the distribution of light to individual cells is the sum of many parameters. These include cultivation vial dimensions, the length of the light path, culture optical density and mixing, that consequently influence the level of absorption, self-shading and light fluctuation. As in most cases in cyanobacterial research in general, ethylene production has been studied mainly under constant white light. The use of moderate growth light appears to promote ethylene production over higher light intensities (Ungerer et al. 2012). This may reflect the level of direct photodamage and ROS-induced stress, and the allocation of resources for maintenance functions that restricts the optimal use of cellular resources for ethylene biosynthesis under increased illumination, but also possible direct effects on EFE expression. In comparison to suspension cultures, the light conditions can be more effectively controlled in respect to temporal fluctuations and homogeneity in solid-state culture systems where the cells are static and entrapped in thin layer polymer matrix (Vajravel et al. 2020).

In addition to CO<sub>2</sub> and nitrogen, the chemical composition of the rest of the culture medium (Table 1) affects cell growth and the overall productivity. While most cyanobacterial ethylene production systems are based on BG11 medium, the use of concentrated medium has been shown to increase productivity (Ungerer et al. 2012; Zhu et al. 2015). As no single component could be attributed to the effect, the limitation appears to be a sum of multiple nutrient-associated factors (Zhu et al. 2015), which are not directly related to carbon or nitrogen. Although most production strategies rely on culture conditions under which the cells operate in fully photoautotrophic mode, the effect of supplemented carbohydrate substrates that promote mixotrophic growth has also been studied. Generally, it appears that the availability of sugars in the cyanobacterial cultures enhances ethylene production. However, the effect is likely associated with faster growth and resulting increase in the total biomass in the culture, rather than improvement at the level of single cells resulting from increased respiratory flux towards the TCA cycle (Lee et al. 2015). At the same time, transition towards glycolytic metabolism may induce a number of systemic metabolic effects such as downregulation of photosynthesis (Solymosi et al. 2020), which may not be desired when developing photoautotrophic production platforms. Once stable production has been established, the conditions can be maintained by a continuous or semi-continuous cultivation mode, where the medium is refreshed to maintain optimal nutrient status for prolonged ethylene production (Carbonell et al. 2019; Vajravel et al. 2020; Zavřel et al. 2016; Zhu et al. 2015).

Long-term system stability is essential for the development of any biotechnological application and determined by the genotype of the host strain in context with the cultivation conditions. As ethylene does not induce any acute toxicity effects in cyanobacteria, and EFE expression does not cause major metabolic imbalance despite the use of 2-OG as substrate, the engineered production strains appear to be generally relatively stable. Ethylene production has been maintained at steady levels for many weeks in step-wise batch cultures in *Synechococcus* (Carbonell et al. 2019) and in *Synechocystis* (Zhu et al. 2015), but the long-term stability and performance in optimized continuous flow suspension cultures still needs to be systematically evaluated. Solid state systems seem to be a prominent option for production, as the entrapment of cells in hydrogel polymer matrix restricts cell growth and provides extra stability, thus allowing the cells to maintain efficient photosynthetic activity and ethylene biosynthesis for extended periods (Vajravel et al. 2020). As growth in this setup is suppressed, possible constraints associated with nitrogen availability and regulation via 2-OG are likely less critical in comparison to actively dividing cells.

### 3.5 | Quantitative monitoring of ethylene production

Ethylene is a highly volatile compound that spontaneously diffuses out from the producer cell, and eventually into the culture headspace. This alleviates the need for chemical extraction from the culture medium, which is beneficial for the development of a continuous bioproduction process but requires a closed system for product collection and isolation. The most conventional strategy used for quantitative ethylene analysis is to transfer an aliquot of the producer culture into a sealed small-scale vial, followed by incubation and the analysis of the gas-phase, typically using a gas chromatograph fitted with a flame ionization detector (GC-FID). It is noteworthy that this type of a two-phase procedure does not necessarily give precise information on the actual main culture, and that the exact analytical setup significantly affects the absolute calculated productivities, which together complicate meaningful quantitative comparisons between published studies. A more advanced system combines cultivation in a photobioreactor with a membrane-inlet mass-spectrometer (MIMS), which enables high-resolution analysis of ethylene (Zavřel et al. 2016) directly from the headspace. This type of a strategy may aid process parameter optimization during system upscale, as it allows real-time monitoring of long-term continuous production, and would be convenient for large scale industrial processes.

## 4 | CONCLUSIONS ON FUTURE POTENTIAL AND STEPS TOWARDS POTENTIAL EXPLOITATION

Years of basic enzyme-level research on EFE, and parallel development of photoautotrophic expression systems to produce ethylene,

have provided a wealth of information on how the pathway functions, and how the catalytic reaction integrates as part of the cyanobacterial metabolic network. Accumulation of biological information has also allowed us to acquire a rather detailed understanding on the role and regulatory interactions of 2-OG in the cyanobacterial cell. In addition to being an important biosynthetic building block for the cell, 2-OG plays a central regulatory role at the intersection of cellular carbon, nitrogen and energy metabolism. EFE on the other hand is a relatively complicated enzyme which catalyzes two overlapping reactions, one which produces ethylene from 2-OG, and one which directs carbon away from the target pathway. The engineering challenge is to synchronize the efficient metabolic flux towards 2-OG and the expression of EFE in a balanced manner, as an integral part of a highly malleable metabolic framework, which has been optimized for millions of years to acclimate to fluctuations. Even though we have not yet reached photoautotrophic ethylene production levels which would be relevant for industrial applications, there has been a significant increase in knowledge and synthetic biology technologies that allow us to determine what to do and how to do it. Besides the ethylene-specific knowhow, the research has accumulated general information on cyanobacterial engineering and cultivation techniques, that can be useful when designing production systems for other volatile target metabolites such as hydrogen or isoprene.

The progress towards biotechnological photoautotrophic platforms to convert CO<sub>2</sub> directly into ethylene requires the development of continuous systems for step-wise upscale and process optimization. In this transition from research laboratories to industry, the prospects of utilizing both suspension cultures as well as solid-state cultures need to be further systematically explored. Besides allowing more accurate evaluation of the system performance when the conditions are not in constant change, continuous production strategies also enable the fine-tuning of individual culture parameters for different strains. In addition, the use of direct analytical methods, such as MIMS or commercial probes for quantitating volatile organic compounds, would give access to more uniform data, more reliable comparative analysis, as well as system automatization, when compared to strategies that rely on separate analytical cultivation steps.

As for the biology, the current molecular-level knowledge on cyanobacterial ethylene production, combined with access to advanced synthetic biology tools, provides means for developing next-generation EFE expression systems with optimized gene dosage, transcription and translation. Although it is difficult to estimate how much the EFE expression levels ultimately need to be pushed up, as more effective strain-specific engineering strategies emerge to steer the cellular carbon flux towards 2-OG, the improvement of EFE catalytic performance and the substrate availability are expected to proceed hand in hand. Due to the intimate role of 2-OG and L-Arg in cellular carbon/nitrogen metabolism, this will need to take into account the host organism as a whole, including the complex regulatory networks that govern the biosynthetic and catabolic equilibria. In order to achieve sufficient photon conversion efficiencies, we must fuse the ethylene-specific engineering steps with different strategies

to increase the overall photosynthetic carbon fixation with flux analysis and metabolomics to pinpoint the next metabolic bottlenecks. In the light of the current knowledge, the potential needs to be evaluated together with EFE side products such as guanidine, which need to be effectively recycled back to use, unless extracted in parallel for added commercial value. For all this, profound exploitation of computer-based modeling and bioinformatics will be necessary to interpret the complex data, and to design and evaluate further metabolic modifications.

There is room to critically improve the photoautotrophic production of carbon-based target chemicals such as ethylene in engineered cyanobacterial hosts. Reaching these novel industrial solutions, however, will require resources and persistent long-term multidisciplinary collaboration across different fields of fundamental biological research, applied biosciences, and industrial technology development.

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## AUTHOR CONTRIBUTIONS

All authors contributed to the planning, writing and revision of the manuscript.

## DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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