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Doing synthetic biology with photosynthetic microorganisms

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Abstract

The use of photosynthetic microbes as synthetic biology hosts for the sustainable production of commodity chemicals and even fuels has received increasing attention over the last decade. The number of studies published, tools implemented, and resources made available for microalgae have increased beyond expectations during the last few years. However, the tools available for genetic engineering in these organisms still lag those available for the more commonly used heterotrophic host organisms. In this mini-review, we provide an overview of the photosynthetic microbes most commonly used in synthetic biology studies, namely cyanobacteria, chlorophytes, eustigmatophytes and diatoms. We provide basic information on the techniques and tools available for each model group of organisms, we outline the state-of-the-art, and we list the synthetic biology tools that have been successfully used. We specifically focus on the latest CRISPR developments, as we believe that precision editing and advanced genetic engineering tools will be pivotal to the advancement of the field. Finally, we discuss the relative strengths and weaknesses of each group of organisms and examine the challenges that need to be overcome to achieve their synthetic biology potential.

1 | INTRODUCTION

Recent advances in synthetic biology (SB) have facilitated the release of new bio-based products from photosynthetic microbes on the market. The key drivers of these recent developments are new techniques and methods for the genetic manipulation of organisms, the drop in cost of bioengineering, and changes in consumer attitudes that now favor products produced in more sustainable ways. However, the synthetic biology business landscape has largely overlooked photosynthetic microbes.

Photosynthetic microbes can be found in almost every conceivable ecosystem and can grow cheaply using CO_2 as sole carbon source. These unique characteristics are, at least in theory, of great interest to the industry (Fabris et al., 2020). SB applications to micro-algal bioengineering could be of crucial importance to our efforts to meet some of the large global challenges such as facilitating the transition to a low-fossil carbon society (Ng et al., 2020). Production systems that use solar energy, water, and CO_2 as building blocks to produce carbon-based fuels and chemicals may potentially add to climate change mitigation. However, algae or cyanobacteria are rarely used as host organisms for production. This is often attributed to the limited availability of genetic engineering tools and the slow growth rates of photosynthetic microbes. The first SB tools for cyanobacteria and algae appeared several years after their heterotrophic counterparts, while microbial fermentation is a process that has evolved and been optimized over millennia (Salque et al., 2013). However, the narrative that algae and cyanobacteria can replace yeast and bacteria as metabolite producers has led to unfair comparisons and required autotrophs to compete under unfavorable conditions, when in fact their major forte is the sustainability provided by direct photosynthetic bioproduction (Leister, 2019).

SB facilitates both basic and translational research of microalgae. SB tools can help to solve complex biology questions, characterize components unique in different organisms, and incorporate biological parts from different organisms within an engineering framework. Microalgae have a wealth of promising features including the use of sunlight as an energy source, genetic and metabolic robustness, energy management, redox regulation, and incorporation of protein complexes and electron chains in membranes. They also produce many natural metabolites not found in other organisms, and they use unique organelles, such as the carboxysomes and pyrenoids (Singh et al., 2017). Therefore, microalgal SB focuses on three major approaches: (a) development of characterized genetic parts that allow the bioengineering of photosynthetic microbes, (b) metabolic engineering for the bioproduction of desirable metabolites especially metabolites that are challenging to produce in heterotrophic production hosts, and (c) fundamental understanding of cellular mechanisms.

Stemming from experiences from the diverse research on photosynthetic microorganisms, within and outside the NordAqua consortium (https://nordaqua.fi), this review aims to provide an overview of the SB practices used for photosynthetic microbes. We introduce the techniques developed for their bioengineering

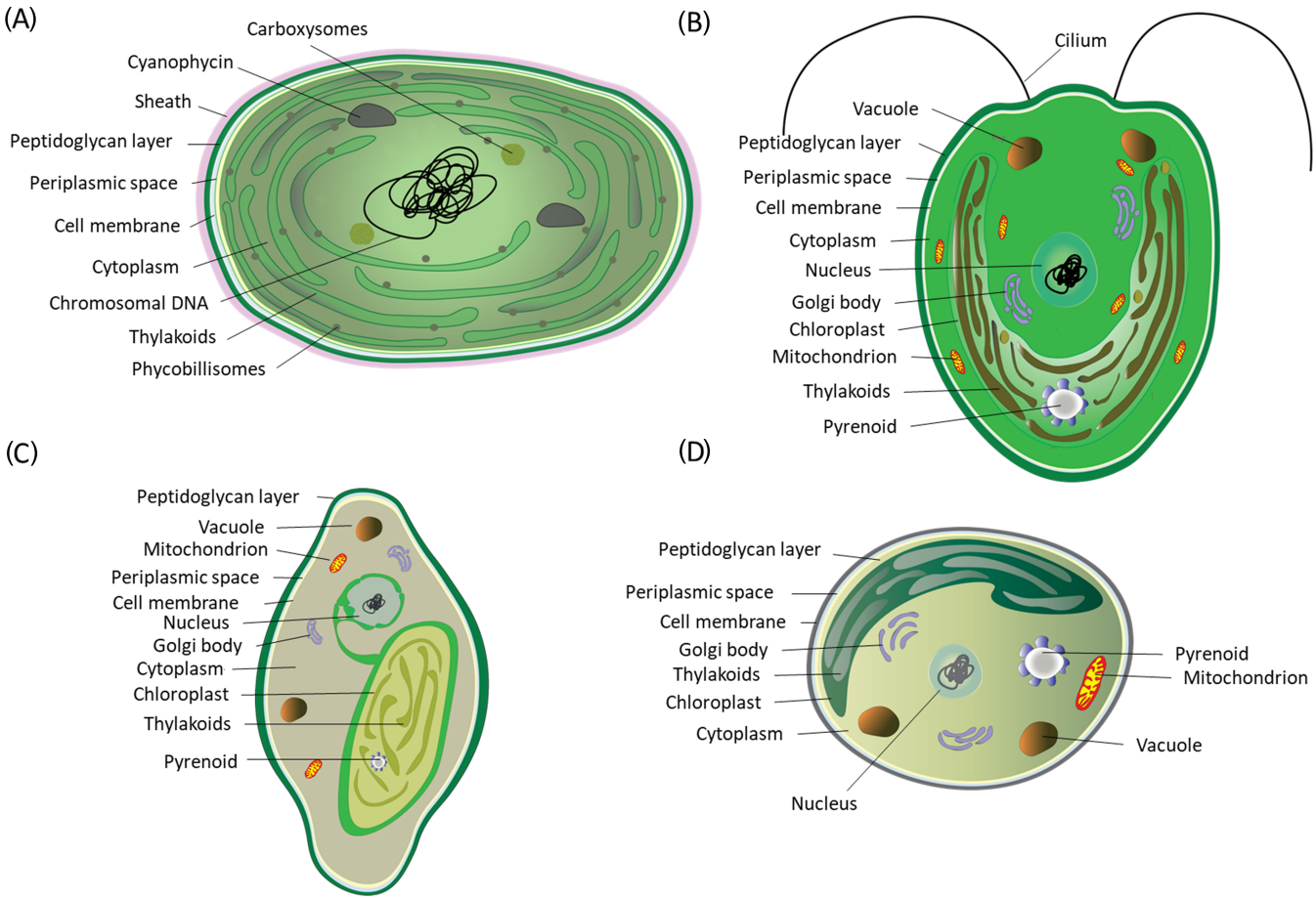

FIGURE 1 Schematic representation of the cell structures of the four groups of photosynthetic microbes discussed. (A) Cyanobacteria, (B) chlorophytes (e.g. *Chlamydomonas* sp.), (C) diatoms, and (D) eustigmatophytes (e.g. *Nannochloropsis* sp.)

and compare the strengths and weaknesses of the most popular SB tools used in research and early commercial approaches. We focus on the broad groups of cyanobacteria, chlorophytes (green algae), eustigmatophytes (*Nannochloropsis* species) and diatoms (Figure 1), and provide an overview of the transformation techniques (Figure 2), SB tools (Figure 3), and available community resources for each group. The model species of each group are where most SB development emerge and are the best characterized, even if they are not necessarily ideal bioproduction hosts. We especially highlight the CRISPR developments in each group, as we believe that the developments on advanced genetic engineering tools will shape the future of SB using photosynthetic microorganisms. The successful adoption of advanced, standardized genetic engineering is a prerequisite for the wide community and industry acceptance.

2 | THE STATE-OF-THE-ART OF GREEN SYNTHETIC BIOLOGY

2.1 | Cyanobacteria

Cyanobacteria are the original photoautotrophs and the "inventors" of oxygenic photosynthesis more than 2.5 billion years ago (Schirrmeister et al., 2013). Some cyanobacteria are unicellular and grow as single cells. Others have developed multicellular characteristics, where they grow in filaments which may or may not be branching (Boone & Castenholz, 2001). Cyanobacteria occupy many ecological niches, and their activities contribute substantially to total oxygen production and carbon dioxide sequestration and nitrogen fixation on the planet, and it is estimated that the marine cyanobacteria *Prochlorococcus* and *Synechococcus* alone are responsible for 25% of

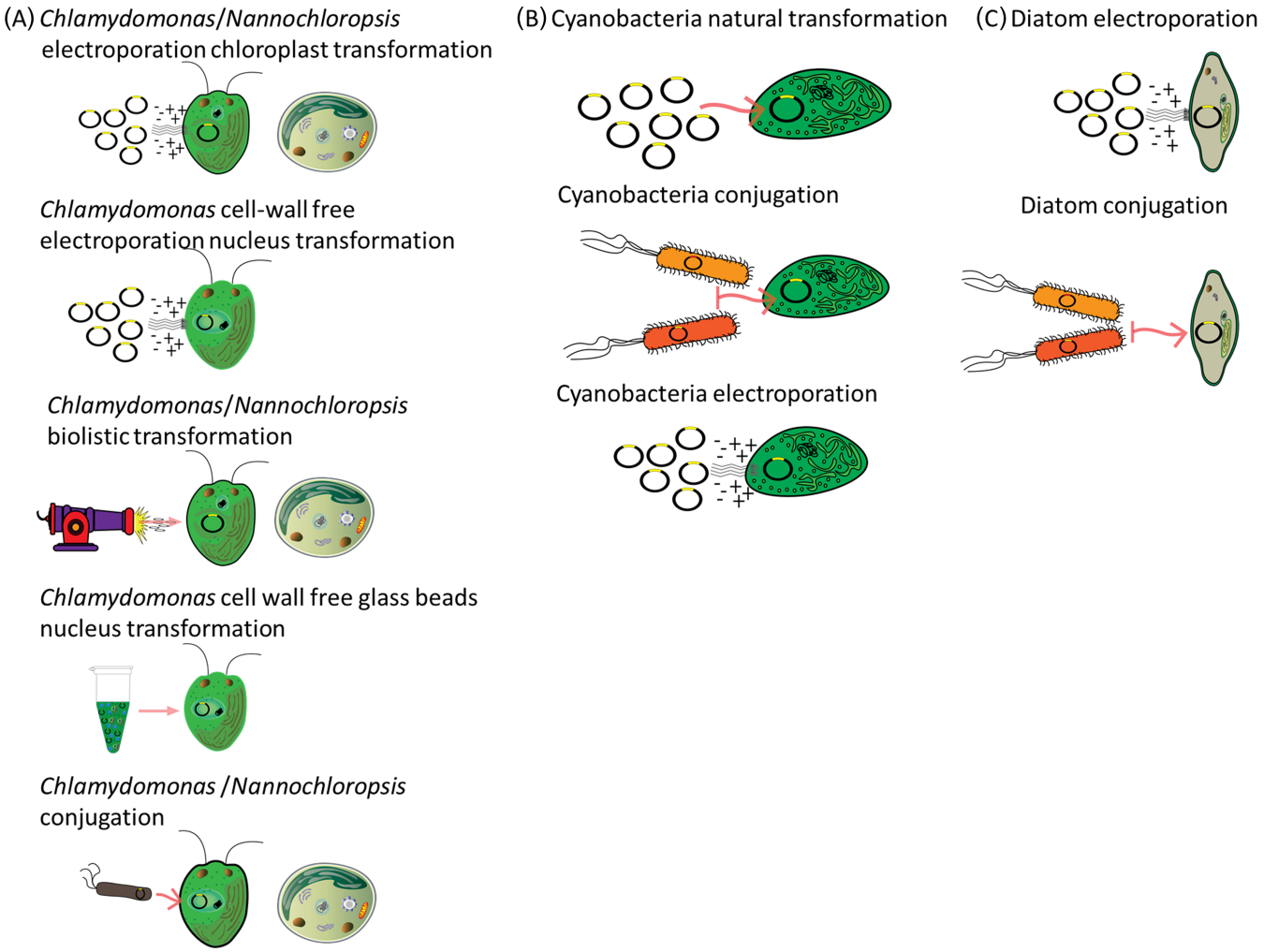

FIGURE 2 Schematic overview of common genetic transformation techniques used to genetically engineer photosynthetic microbes. (A) For chlorophytes (*Chlamydomonas*) and eustigmatophytes (*Nannochloropsis*): Electroporation and biolistic bombardment can be used for chloroplast targeted transformation for both *Chlamydomonas* and *Nannochloropsis*, while electroporation or vortexing with glass beads can be used to modify the nuclear genome of *Chlamydomona*. Bacterial conjugation or Agrobacterium-mediated transfer can also be used to introduce DNA into these cells. (B) For cyanobacteria: Natural transformation or conjugation can be used to transfer DNA for integration in the chromosome or as replicating plasmids. Plasmids can also be transferred via electroporation. (C) For diatoms: Electroporation and bacterial conjugation are examples of techniques that can be used to introduce DNA in diatoms. Agrobacterium-mediated transfer or biolistic bombardment may also be used

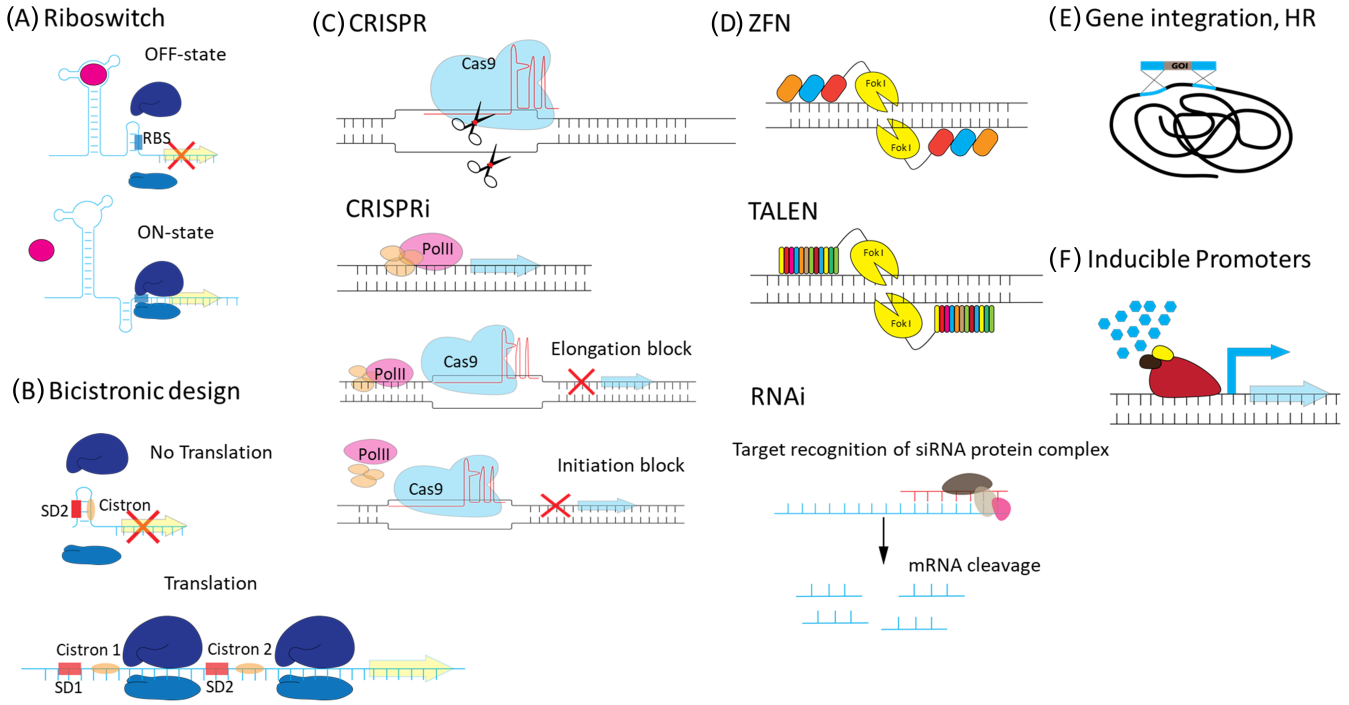

FIGURE 3 An overview of common synthetic biology tools and strategies for the bioengineering of photosynthetic microbes. (A) Riboswitches are structural regulatory sequences on mRNA that can bind small signal molecules to activate or inactivate the expression of a protein. The example in the figure is a Riboswitch where the RNA structure shields the RBS, in the OFF-state, and thereby restrict ribosome binding and translation. (B) Bicistronic design (BCD) alleviates issues in translation activation caused by mRNA structures formed in the interface of the 5′ untranslated region and the expressed gene when the construct is monocistronic. (C) CRISPR/Cas complexes can be used for precise genome engineering, while CRISPRi makes use of inactivated Cas mutants to instead block the expression of genes. (D) Zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) are programmable, sequence-specific nucleases that can be used for genome engineering. (E) Homologous recombination (HR) allows targeted insertion or inactivation of DNA sequences in the genome. (F) Inducible promoters are needed for strict control of production pathways and regulatory circuits in the engineered cells

the net primary production in the oceans (Flombaum et al., 2013; Zehr, 2011), demonstrating the great capacity for productivity by these photosynthetic microorganisms.

Cyanobacteria were the first photosynthetic microbes to have their genomes sequenced (Kaneko et al., 1996). Their genome range in size from 1.4 to 12 Mb and can be arranged in a circular chromosome with up to 13 extrachromosomal elements. The model cyanobacteria have well-established genetic systems, completely sequenced genomes, validated gene models, and curated genome annotations that allow the construction of high-quality genome-scale metabolic network models (Gudmundsson et al., 2017). Although cyanobacterial strains show promising traits, including the efficient production of biomass or bioproducts, many of them are genetically intractable and strain improvement is needed, thus constituting a major challenge (Wendt & Pakrasi, 2019). Current bioengineering strategies focus on model cyanobacteria for which genetic systems and—for a few strains—genome-scale metabolic network models exist (Gudmundsson et al., 2017).

Many strains of cyanobacteria are amenable to genetic modification using a variety of different methods (Figure 2). Some cyanobacteria are naturally transformable and readily use exogenously added DNA as a source of genetic information. This method allows the incorporation of DNA into the genome of the recipient strain via homologous recombination and makes engineering of these strains quite straightforward; several model strains of cyanobacteria are naturally transformable with several simple protocols popularizing their use (Heidorn et al., 2011; Wendt & Pakrasi, 2019). This includes the widely used unicellular model strains *Synechocystis* sp. PCC 6803 (Syn6803), *Synechococcus elongatus* PCC 7942 (Syn7942), and *Synechococcus* sp. PCC 7002 (Syn7002). However, a number of other cyanobacteria are also amenable to natural transformation including the filamentous *Nostoc muscorum*, the thermophile *Thermosynechococcus elongatus* BP-1, and the recently isolated fast-growing strain *Synechococcus elongatus* PCC 11801 (Jaiswal et al., 2018; Onai et al., 2004; Trehan & Sinha, 1981).

Conjugation is another widely used method to introduce recombinant DNA into cyanobacterial hosts via *E. coli* (Thiel & Wolk, 1987). This method is sometimes more efficient than natural transformation, works in many strains that are not naturally transformable, and can be used both for genetic constructs designed to be integrated into the chromosome and for constructs carried on replicating plasmids. The presence of endogenous restriction enzymes hinders the transfer and incorporation of recombinant DNA into cyanobacterial genomes. However, recombinant DNA can be protected through methylation

using specific methylases in the parental *E. coli* strain (Tandeau de Marsac & Houmard, 1987). Electroporation to transfer plasmid DNA into the cells is possible for at least some cyanobacteria, and protocols for this are available for several strains (Thiel & Poo, 1989).

Cyanobacteria often harbor multiple copies of their genome per cell, depending on the strain, growth phase, and growth conditions as well as unequal copy numbers of chromosomes and plasmids (Watanabe, 2020). Therefore, it is necessary to select for complete segregation of the chromosome when integration of a genetic construct is desired, for the introduced modification to be stable over time and conditions. One way to overcome this, is the use of self-replicating vectors which can be either synthetic or repurposed natural plasmids (Chen et al., 2016). The use of self-replicating vectors does not come without its own challenges: need for constant selection pressure to maintain selection, limitations on the selection markers that can be used, as well as limited toolbox of available plasmids. Plasmids used as self-replicating vectors tend to be large and, due to the low-copy number in *E. coli*, cumbersome in handling and sub-cloning. Overall, the advantages of genome integration or vector expression are not always clear when a transgene expression experiment is designed, and the best approach depends on the application. Therefore, researchers should be prepared to shift between strategies when troubleshooting or optimizing strains.

Many, but far from all, SB tools and genetic parts developed for use in *E. coli* can be readily adapted for use in cyanobacteria (Figure 3). Cyanobacterial codon usage also matches that of *E. coli* reasonably well, so that genes for expression can be used in both hosts. However, due to differences in transcriptional machinery, genetics, and regulation, tools cannot always be directly transferred from heterotrophs to autotrophs (Stensjö et al., 2018). SB in cyanobacteria has therefore focused on characterizing promoters, ribosome binding sites, and other genetic elements that enhance predictable protein expression. The availability of SB tools for the standardized assembly of genetic constructs is also of critical importance (Figure 3). One such standardized modular cloning (MoClo) system called CyanoGate has recently been developed for Syn6803 and SynUTEX2973 (Vasudevan et al., 2019).

Among the presently available cyanobacterial SB tools we find well characterized promoters, such as the strong light regulated promoters P*psbA2* and P*cpc560* (Englund et al., 2016; Markley et al., 2015; Zhou et al., 2014), and modified versions of the well-studied *E. coli* promoters P*lac* and P*tet* (Ferreira et al., 2018; Huang & Lindblad, 2013). The light-dependent regulation of many genetic parts in photosynthetic microorganisms adds an extra level of complexity when assessing genetic parts behavior: growth conditions, such as light intensity, light quality, light regimes, and cell density at the time of sampling, should be carefully monitored and reported. Inducible promoters with a wide dynamic range have successfully been developed for cyanobacteria based mainly on these foreign and endogenous promoters (Huang & Lindblad, 2013). Among these, the strong L03 promoter and the well repressed L22 promoter have been used in SB approaches (Yao et al., 2016). Ferreira et al. (2018) reported a collection of well-characterized promoters analyzed in Syn6803. However, the cyanobacterial bioengineering community lacks reliable promoters of determined expression strength (ranging from weak to very strong) that work across species (Vasudevan et al., 2019). The lack of well-controlled inducible expression systems is a serious hindrance in developing genetic circuits. However, a few recent studies tackle this issue (Behle et al., 2020; Ferreira et al., 2018; Kelly et al., 2018).

Heterologous constitutive and inducible promoters are used primarily in unicellular model cyanobacteria such as Syn6803, Syn7942, and Syn7002. Efforts have also been made to use synthetic well-defined promoters in filamentous cyanobacteria. A minimal synthetic promoter that gives cell-specific expression was reported from *Nostoc* sp. PCC 7120 (Nostoc7120; Wegelius et al., 2018). Furthermore, for defined gene expression both transcription and translation need to be well controlled. Therefore, both native and rational designed RBS sequences giving a high fold-range of expression levels have been developed for model strains (Englund et al., 2016; Heidorn et al., 2011; Huang et al., 2010; Wang et al., 2018).

Nevertheless, promoter-RBS constructs often lack the predictability needed for controlled protein expression even when using well-characterized promoters and RBS sequences (Mutalik et al., 2013). Secondary structures of the 5′UTR may cause unpredictable expression levels (Mutalik et al., 2013). The use of insulator sequences, such as in the bi-cistronic design (BCD) first described in *E. coli* (Mutalik et al., 2013), orthogonal riboswitches, and fusion constructs have been reported to increase level and predictability of protein expression in cyanobacteria (Englund et al., 2018; Formighieri & Melis, 2015; Taton et al., 2017). Genome editing to achieve marker free knock-outs and knock-ins is routinely performed by homologous recombination (HR). However, time-consuming mutant selection and chromosomal segregation in cyanobacteria is often a hurdle for efficient genetic engineering. Multiplexed engineering, together with multiplexed gene silencing, would be advantageous to speed up development of new strains, and this true potential of CRISPR-based editing for bacterial engineering has not yet been explored in cyanobacteria to the extent observed in other heterotrophs. CRISPR/Cas (*c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeats/CRISPR *a*ssociated protein) based tools for genome editing have lately gained attention (Pattharaprachayakul et al., 2020). In early reports, CRISPR/Cas9 was used, and demonstrated to work in SynUTEX2973, Syn7942 and Syn6803 (Li et al., 2016; Wendt et al., 2016; Xiao et al., 2018). CRISPR/Cas12a (Cpf1) was introduced into the cyanobacteria genetic toolbox more recently due to the reported toxicity of Cas9 in cyanobacteria (Ungerer & Pakrasi, 2016). Cas12a has proven useful for multiple genome editing, in the unicellular model species Syn6803, SynUTEX 2973, Syn7942, in the filamentous *Nostoc*7120, and in the recently characterized fast-growing strain *Syn*11801 (Sengupta et al., 2020; Ungerer et al., 2018; Ungerer & Pakrasi, 2016).

CRISPRi, a technique based on the use of a deactivated Cas9 (dCas9), allows the repression of gene expression of multiple target genes (Gordon et al., 2016; Higo et al., 2017; Yao et al., 2016; Yao *et al*., 2020). Strategies to control the dynamic range and inducibility of target gene expression in cyanobacteria have in part been based on

SB approaches involving a variety of promoters and RBS and resulted in regulation of dCAS9 expression levels (Gordon et al., 2016; Yao et al., 2016). CRISPRi based on dCas12 was reported to be efficient for multiple gene repression in SynUTEX 2973 (Knoot et al., 2019) and in Syn7942 (Choi & Woo, 2020). This technique has also been successfully used in other model cyanobacteria, thus showing its value as a SB tool in metabolic engineering of cyanobacteria (Ungerer & Pakrasi, 2016).

Strains of cyanobacteria have been engineered to produce numerous compounds (Savakis & Helligwerf, 2015). The initial step involves the addition of the necessary genetic capacities, and often a simultaneous deletion of a competing pathway or unwanted product formation. A systematic approach was employed to engineer cells of *Syn*6803 to produce the attractive commodity chemical and gasoline substitute 1-butanol (Liu et al., 2019). By introducing and recasting the 1-butanol biosynthetic pathway, optimizing the 5′-regions of expression units for tuning transcription and translation, rewiring carbon flux and rewriting the photosynthetic central carbon metabolism to enhance the precursor supply, a cumulative 1-butanol titer of 4.8 g l^{-1} with a maximal rate of 302 mg l^{-1} day^{-1} was observed from the engineered *Synechocystis* (Liu et al., 2019). In the best performing strain, eight genes organized in three operons were introduced at three different locations in the *Synechocystis* genome that abolished the capacity to synthetize acetate and polyhydroxybutyrate, and at a neutral site.

2.2 | Chlorophytes (Green algae)

Chlamydomonas reinhardtii is the best-known member of Chlorophyta and the model photosynthetic microalgae (Figure 1). With ever-increasing knowledge, *C. reinhardtii* is now entering the SB era (Crozet et al., 2018). Chlorophyta contains also other important model algae, e.g. *Dunaliella*, *Haematococcus*, *Chlorella*, and *Tetraselmis* species used for production of carotenoids, nutritional or feed products, and emerging members of the Coccomyxaceae and Scenedesmaceae family with ability to produce lipids and being use in wastewater treatments (Liang et al., 2020). There are now over 100 whole-genome sequences available for members of the Chlorophyta ranging in size from 13 to 365 Mb (Blaby-Haas & Merchant, 2019; Hanschen & Starkenburg, 2020). The best representation of genome sequences is available for the species of *Chlamydomonas* (Hanschen & Starkenburg, 2020), which is widely used as a model system to study genetics and physiology (Blaby-Haas & Merchant, 2019). The availability of high-quality genome sequences and genome-scale metabolic network models promotes opportunities to exploit algal metabolism for biotechnological purposes.

Some members of the Chlorophyta are readily transformable. However, the members of Chlorophyta have thick cell walls and many strains need significant method optimization or cannot be transformed at all (Dehghani et al., 2018; Kania et al., 2020; Suttangkakul et al., 2019). Various methods are applicable for transformation of *C. reinhardtii*, including the glass bead method, particle bombardment, *Agrobacterium*-mediated conjugation, and electroporation being the main methods employed (Figure 2). Electroporation is preferred, because of higher transformation efficiency, whereas particle bombardment is useful for transformation of mitochondria or chloroplast genomes (Wang et al., 2019). Similar methods have been used for the transformation of *Chlorella* (Yang et al., 2016), *Coccomyxa* (Kania et al., 2020; Kasai et al., 2018), and for *Scenedesmus/Acutodesmus* species (Dautor et al., 2014; Guo et al., 2013; Muñoz et al., 2018; Suttangkakul et al., 2019). In addition, an *E. coli*-mediated conjugation was reported for transformation of *Acutodesmus obliquus* (Muñoz et al., 2019). Compared with *C. reinhardtii* the transformation efficiencies for the other Chlorophyta algae remain low.

The transformation of the chloroplast genome offers an exciting bioengineering alternative to the challenging transformation of the nucleus (Dyo & Purton, 2018) Methods for the transformation of the chloroplast are established for *C. reinhardtii*, *Haematococcus pluvialis*, and *Dunaliella tertiolecta* (Dyo & Purton, 2018). Plastid transformation has the advantage of inserting genes in a prokaryotic-like genetic environment. It is preferred for high expression of heterologous proteins because random integration in the nuclear genome results in inconsistent protein expression levels. The selection of nuclear transformants can be based, e.g. on arginine deficiency or on resistance to antibiotics. Commonly used antibiotics include paromomycin, hygromycin, spectinomycin, kanamycin, and zeocin. The selection of chloroplast transformants is possible using spectinomycin, or light-sensitive mutants, which is a rapid way to achieve marker-free engineered *C. reinhardtii* strains (Esland et al., 2018).

Genetic engineering of *C. reinhardtii* has developed during the last decades (Crozet et al., 2018). A variety of genetic constructs are now available and recently a toolkit for modular cloning with 119 described parts: promoters, terminators, introns, UTRs, tags, marker and reporter genes was described (Crozet et al., 2018; www.chlamycollection.org/). Several strong native promoters have been used for nuclear transgene expression in *C. reinhardtii*. The most commonly used are promoters regulating the expression of *PSAD* (photosystem I protein D), *RBCS2* (small unit ribulose-1,5-bisphosphate carboxylase/oxygenase) and the chimeric *RBCS2-HSP70A* (heat shock protein 70A) promoter (Fischer & Rochaix, 2001; Schroda et al., 2000). Inducible promoters are also available and include, *NIT1* (nitrate-repressible, ammonium-inducible; Schmollinger et al., 2010) and *CYC6* (copper dependent repression and nickel-dependent induction) promoters (Quinn et al., 2003). Terminators can be derived, e.g. from *RBCS2*, or *PSAD* genes.

The introduction of introns into expression constructs has been shown to increase nuclear transgene expression in *C. reinhardtii* (Baier et al., 2020). The introns from *RBCS2* are the most studied and are recommended for high-level protein expression (Baier *et al*., 2018), although more recently other introns have been reported to enhance expression even further (Baier et al., 2020). A tool for designing intron containing genes also exists "Intronserter" (https://bibiserv.cebitec.uni-bielefeld.de/intronserter; Jaeger et al., 2019). Codon optimization is crucial for high transgene expression, and an algorithm for sequence optimization has been developed (Weiner et al., 2018).

Chloroplast transformation can make full use of the extensive toolbox developed for plant chloroplast transformation. Fluorescent proteins and luciferases are commonly used as reporters and can be targeted to different compartments (Esland et al., 2018). Genome scale metabolic models, transcriptome and other omics data are available, and summarized in Kumar et al. (2020), and a mutant library covering 83% of the protein-encoding genes, is available at the Chlamydomonas Resource Center (https://www.chlamycollection.org/products/clip-strains).

The knowledge gained with *C. reinhardtii*, its genetic parts, or alternatively those used for gene expression in plants, have been applied to engineer other members of the Chlorophyta (Crozet et al., 2018). The availability of genome sequences promotes the generation of species/strain specific endogenous parts, and the number of promoters used in microalgae research is constantly increasing (Kumar et al., 2020). The cauliflower mosaic virus 35S (CaMV35S) promoter used in plants has been the most frequently used promoter in *Chlorella* species (Kumar et al., 2018). *RBCS* and nitrate reductase promoters are also used in *Chlorella* (Yang et al., 2016). Enhancer or enhancer-like elements, introns or retrotransposons have also been studied for improved expression (Yang et al., 2016). The endogenous *RBCS* promoter, and also the first intron of *RBCS* (Kania et al., 2020; Kasai et al., 2018) as well as the promoter of the elongation factor 1 alpha gene have been used to drive gene expression in *Coccomyxa* species (Kasai et al., 2017). A marker recycling system and marker-free engineering have also been reported (Kasai et al., 2017, 2018). In the Scenedesmaceae family, the *RBCS2-HSP70Ap* promoter, and the CaMV35S promoter have been used (Guo et al., 2013; Muñoz et al., 2018, 2019; Suttangkakul et al., 2019).

Genome editing of *C. reinhardtii* has been reported with Zinc-finger nucleases (ZFNs; Greiner et al., 2017), transcription activator-like effector nucleases (TALENs; Gao et al., 2014), different CRISPR/Cas9 approaches (Jiang et al., 2014; Wang et al., 2016; Greiner et al., 2017) and through the Cas12a (Cpf1) nuclease (Ferenczi et al., 2017). RNA interference (RNAi) and microRNAs (miRNA) can be used to alter expression by targeting mRNA (Schroda, 2006; Molnár et al., 2007; Kim & Cerutti, 2009; Figure 3).

The use of CRISPR/Cas9 in deletion of omega-3 fatty acid desaturase (fad3) has recently been reported in *C. vulgaris* (Lin & Ng, 2020). A starch-less mutant of *Coccomyxa* sp. strain Obi was obtained by TALEN mediated method (Takahashi et al., 2018). Yoshimitsu et al. (2018) used CRISPR/Cas9 to edit the genome of the *Coccomyxa* strain KJ by guiding the RNA-Cas9 protein complex to knockout the FTSY gene. Such genome editing has not yet been reported for the members of the Scenedesmaceae family.

2.3 | Eustigmatophytes

The class of Eustigmatophyceae is represented in the SB community by the heterokont species of the *Nannochloropsis* genus, which are unicellular oleaginous microalgae (Figure 1). They represent a rich source for the omega-3 LC-PUFA eicosapentaenoic acid, in addition to high amounts of triacylglycerols, which can be processed into biodiesel (Simionato et al., 2013). This group harbors haploid nuclear genomes that span over 30 chromosomes, with 25–30 Mb in size. Very recently, a pan-genome database has been published, which constitutes a wealthy resource for multi-omics data collected from a variety of explored *Nannochloropsis* species, such as *N. oceanica*, *N. salina*, and *N. gaditana* (Gong et al., 2020). In addition, genome-scale metabolic models (Loira et al., 2017; Shah et al., 2017) for the *Nannochloropsis* algae were reconstructed that assist in identifying metabolic capacities of this interesting organism. Given their high biotechnological potential, a significant effort has been invested in the past years for developing a SB toolbox for *Nannochloropsis*, to be used for fundamental and applied research.

Stable transformation protocols for targeting the nuclear and chloroplast genomes in various *Nannochloropsis* species are reported (Cui et al., 2020; Gan et al., 2018; Figure 2). Transformation of *Nannochloropsis* is primarily conducted using electroporation (Kilian et al., 2011), where either the plasmids are linearized prior to the transformation procedure, or by using PCR fragments (Li et al., 2014). However, particle bombardment and *Agrobacterium*-mediated transformation are also in use (Cha et al., 2011). For strain selection, genes conferring resistance to zeocin, hygromycin B and blasticidin S antibiotics are mostly used as genetic markers (Kilian et al., 2011). In addition, G418 and Nourseothricin were recently found to be effective as well (Poliner et al., 2020), especially when high transgene expression is desired.

A variety of constitutive and inducible endogenous promoters in different strengths have been identified in *Nannochloropsis* species. These mainly include the VCP2, β-tub, HSP70, UEP, EF, rbcL, LDSP, and NR promoters (Radakovits et al., 2012). Recently, two new constitutive promoters (HSP90 and EPPSII) were identified, which outperform the commonly used ones (Ramarajan et al., 2019). Of main interest are the bidirectional promoters (VCP2 and Ribi) that are useful for multigene expression (Moog et al., 2015; Poliner et al., 2018a). Terminators comprise, among others, genes encoding the psbA, rbcL, CaMV35S, nopaline synthase, and LDSP (Vieler et al., 2012; Zienkiewicz et al., 2017). Monitoring of the gene/protein expression is mostly achieved by using reporter genes coding for GFP and YFP, although RFP and CFP fluorescent proteins are also employed in some *Nannochloropsis* species (Zienkiewicz et al., 2017). In addition, the utilization of Luc, chromoproteins and GUS was proven useful for expression evaluation (Poliner et al., 2018a). Luciferases and chromoproteins are particularly advantageous over the fluorescent proteins. Luciferases show higher signal-to-noise ratios and can be simultaneously measured (due to usage of specific substrates). On the other hand, chromoproteins offer simplicity, as there is no need for the application of selective pressure for screening of transformants, nor specialized equipment for the observation of a fluorescent signal. An expansion of the transgenic techniques now aids in the assembly of multiple genes (gene stacking) into one expression cassette (Poliner et al., 2020; Verruto et al., 2018). In contrast to cyanobacteria (Vasudevan et al., 2019) and *C. reinhardtii* (Crozet et al., 2018), a standardized toolkit for the modular assembly of different genetic parts for *Nannochloropsis* is not yet available. However, the development of an open-source *Nannochloropsis* MoClo platform is in progress (https://algalsyntheticbiology.com/open-algae-project/nanno-

moclo/). The current design allows for the use of bidirectional promoters and the expression of multiple transgenes built on gateway vectors (Poliner et al., 2020).

Unlike most other algal species, *Nannochloropsis* is amendable to high-frequency homologous recombination when targeting its nuclear genome (Kilian et al., 2011). Moreover, it has also been documented that *Nannochloropsis* employs a wide selection of Cas9-mediated methods for sequence-specific manipulations. These include vector-based stable expression (Wang et al., 2016), synthetic episomal delivery (Poliner et al., 2018a), pre-assembled Cas9 protein-gRNA ribonucleoproteins (RNPs) and CRISPR/Cpf1 variants (Naduthodi et al., 2019), Cas9-Cre combination (Verruto et al., 2018), and TALENs (Kurita et al., 2020). Different experimental methods for the attenuation of expression of selected genes include an RNAi-based approach (Wei et al., 2017). Apart from site-specific genome modifications, a non-targeted mutagenesis has been carried out by Osorio et al. (2019).

The CRISPR/Cas9 method for the precise genome editing in *Nannochloropsis* was first introduced in 2016, by Wang et al. (2016). In that strategy, a vector-based Cas9 is integrated into the algal genome, leading to the constitutive expression of the Cas9/gRNA complex within the host strain and the progressive accumulation of undesired off-target mutations. In contrast, Cas9-RNP enables the transient expression of the Cas9 molecule, potentially toxic to some *Nannochloropsis* species, by rapid degradation in vivo, and increases the on-target efficiency. Naduthodi et al. (2019) also made use of the Cas12a, which recognizes different PAM motifs, therefore extending the target recognition (e.g. non-coding RNAs or different UTRs). An episomal-based delivery of Cas9 was shown to improve the localization of the protein throughout the cell and thus allowed for a more uniform transgene expression. Poliner et al. (2018b) and Verruto et al. (2018) further provided a significant technological advancement, as they represented an innovative approach for the generation of non-transgenic and marker-free *Nannochloropsis* mutant strains, thus alleviating the regulatory concerns about GMO-based bioproducts.

As noted from the above, species of the *Nannochloropsis* genus are considered promising production hosts for the biofuel and nutraceutical sectors. Powered by omics data and CRISPR/Cas9 system, Ajjawi et al. (2017) recently modulated the transcription factor Zn2Cys6, a homolog of the fungal Zn(II)2Cys6, which regulates TAG biosynthesis in the industrial *N. gaditana* strain. This reverse-genetics approach led to the doubling of lipid production over that of the wild type under nutrient-repleted conditions.

2.4 | Diatoms

Diatoms are important primary producers in the world's oceans and secondary symbionts with complex genomes reflecting their symbiotic history (Armbrust et al., 2004; Mock et al., 2017). Diatoms were selected for genome sequencing because of their ecological importance as primary producers (Armbrust et al., 2004; Bowler et al., 2008) but also because of their ability to form toxic blooms. Diatoms are rich in fatty acids and have potential as protein expression platforms (Kroth, 2007). They have been also engineered to overproduce photosynthetic pigments and other heterologous terpenoids (D'Adamo et al., 2019). While the ecological and environmental importance of diatoms is well established, until recently they were overlooked as biotechnology and SB hosts. This changed in the recent few years, when the first genetic engineering studies were published (Huang & Daboussi, 2017).

Thalassiosira pseudonana and *Phaeodactylum tricornutum* are important model organisms for cell biology and ecophysiology due to their rapid growth and small genome sizes of around 30 Mb (Armbrust et al., 2004; Bowler et al., 2008). There are now 19 whole genome sequences from 15 diatom species publicly available ranging in size from 21 to 98 Mb (Armbrust et al., 2004; Bowler et al., 2008; Galachyants et al., 2015; Lommer et al., 2012; Mock et al., 2017; Tanaka et al., 2015). There is also substantial interest in biotechnological applications because diatoms are excellent candidates for biodiesel fuel production due to their high productivity and lipid content (Tanaka et al., 2015). Genome-scale models have provided insights into the metabolic basis of biomass partitioning in these model diatoms and provide a basis for biotechnological exploitation (Ahmad et al., 2020; Levering et al., 2016). The complexity of diatom genomes with allodiploid genome structure (Tanaka et al., 2015) and highly heterozygous genomes was reported (Mock et al., 2017). The lack of high-quality genome annotations for diatoms hinders the construction of robust genome scale models for metabolism.

The diatom SB studies have taken place using *P. tricornutum* and *T. pseudonana*. Genetic engineering is possible using biolistic transformation, conjugation, and electroporation, while both plasmid and nuclear transformation has been reported (Huang & Daboussi, 2017; Figure 2). The chloroplast transformation of *P. tricornutum* was established in 2014 (Xie et al., 2014). Efficient biolistic transformation was recently reported for *P. tricornutum*, where CRISPR/Cas9 ribonucleoproteins were able to generate double-gene knockouts with 65%–100% efficiency (Serif et al., 2018). Diatoms can maintain heterologous DNA exosomally as well, allowing the use of diatom-specific vectors. Diatoms have a limited set of characterized genetic parts like promoters, terminators and resistance markers (Huang & Daboussi, 2017). The diatom toolbox mainly benefits from parts and techniques developed for other eukaryotic algae. However, heterologous expression of the endogenous *fcp* promoter is commonly used due to high and predictable expression (Apt et al., 1996). Also, a number of constitutive and inducible promoters regulated by the nitrogen status have been explored in *P. tricornutum* (Adler-Agnon et al., 2018). For genome editing precision nucleases such as CRISPR, TALEN, and zinc finger nucleases are established, together with RNAi gene silencing (Daboussi et al., 2014; De Riso et al., 2009; Nymark et al., 2016; Slattery et al., 2020).

2.5 | Critical overview

SB applications as well as the number of research groups working with photosynthetic microorganisms pale in comparison to those

involved in studies with heterotrophic bacteria and yeasts, mammalian, and plant cells (Fabris et al., 2020). However photosynthetic microbes have received more attention in recent years, as they became easier to engineer and use in SB studies. SB applications in this area age less than a decade, and the corresponding bioengineering potential is progressively improving.

Model algal and cyanobacterial species used in SB studies are usually not the ones used in industrial applications. There is a wealth of successful bioengineering achieved using *Synechocystis* and *Chlamydomonas*, while making even simple genetic modifications in *Arthrospira* (*Spirulina*) and *Chlorella* is challenging (Dehghani et al., 2018). The aims of developing SB tools and applications for photosynthetic microorganisms are not exhausted in the generation of industrial strains, but also to enrich our understanding of their physiology and metabolism. Thus, SB will aid in our exploration of the unique physiological features of microalgae, such as efficient carbon concentrating mechanisms and photosystems, and in the generation of metabolic models that can be adopted into true production strains.

Both cyanobacteria and algae possess an almost totally unexplored wealth of secondary metabolic pathways for the production of natural products with commercial interest. However, the biosynthetic pathways for these metabolites differ between the organism groups. These differences in metabolism will be of importance for the choice of the production host. Thus, there is a value in assessing the different groups of organisms for their suitability for different applications. Cyanobacteria are prokaryotic species with rapid (few hours) growth, generally easy to manipulate due to their small genomes and homologous recombination. The unicellular cyanobacteria are excellent vessels to study photosynthesis as a process, and they have achieved remarkable success as metabolic engineering platforms (Liu et al., 2019). The filamentous cyanobacteria can provide invaluable insights on how life became multicellular and how cell types differentiate to perform specific functions, such as ensuring a micro-oxic environment for oxygen sensitive enzymes such as nitrogenases and hydrogenases.

To increase the biotechnological potential of cyanobacteria, there is a need to develop models with faster doubling time, such as SynUTEX 2973 and SynPCC11801. Further genome scale computational tools are needed, including models for prediction of metabolism and flux balance analyses. The genome scale models available today cover less than 30% of any of the annotated cyanobacterial genomes (Broddrick et al., 2016). As a result, a higher coverage is required to allow for increased robustness and predictability. This might be feasible by integrating multiomics data and biochemical data stored in scattered available databases. A true revolution in cyanobacterial SB will come with the full integration of the advanced genome editing tools that was explored almost a decade ago (Jinek et al., 2012). Strategies for marker-less gene modifications will release the dependency of available selection markers, thus enabling complex systems metabolic engineering. The relatively late adoption of

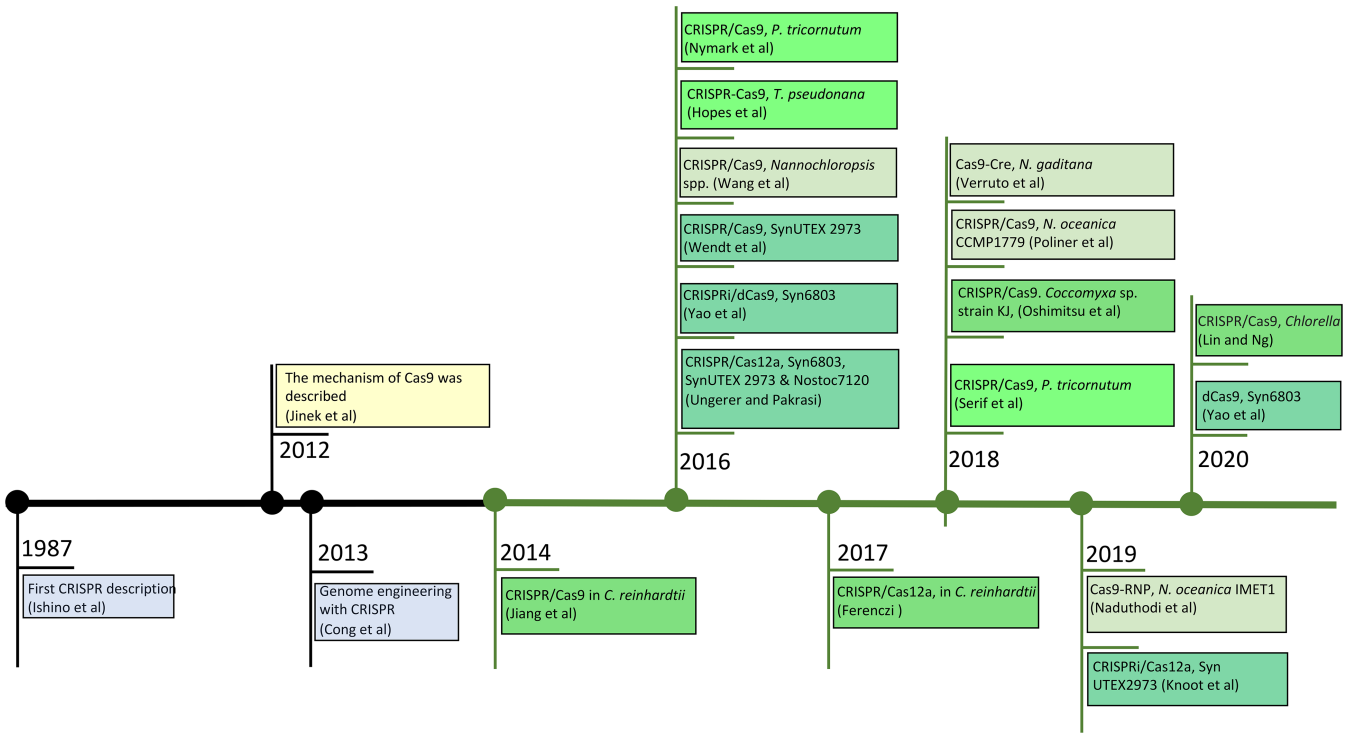

FIGURE 4 Milestones of genome editing by CRISPR strategies from the first discovery of CRISPR arrays in *E. coli* in 1987 up until today. The blue-colored boxes represent the first described CRISPR sequences in *E. coli*, by Ishino et al. (1987), and the first reported CRISPR/Cas-based genetic engineering in eukaryotes, by Cong et al. (2013). The yellow box showcases the study by the groups of J. Doudna and E. Charpentier (Jinek et al., 2012) where they reported the mechanism that directs Cas9 to introduce site-specific double-stranded breaks. Green boxes highlight the use of CRISPR-based techniques for the engineering of photosynthetic microorganisms

CRISPR tools on cyanobacterial platforms (Figure 4) is likely because homologous recombination is a well-established technique that works well and reliably for single modifications. Recent developments in using multiplex CRISPR-based techniques such as CRISPRi for strain development (Yao et al., 2020), and the emergence of a more diverse set of CRISPR tools tested in cyanobacteria should speed up the implementation of CRISPR methodology in the next few years (Pattharaprachayakul et al., 2020).

Chlorophytes, eustigmatophytes and diatoms are among the simplest eukaryotes that perform oxygenic photosynthesis. Many species accumulate metabolites of interest, such as unsaturated fatty acids, proteins and photosynthetic pigments with nutritional and bioindustrial value (Kumar et al., 2020). The popular model species share a lot of tools and resources, and genetic engineering becomes less and less challenging. The need for well characterized promoters for successful synthetic biology applications are shared among all microalgae. Rational engineering and characterization of the most commonly used strong promoters in *C. reinhardtii* will also be beneficial for SB in other microalgae (Einhaus et al., 2021).

Algae have chloroplasts, an extra compartment where genetic engineering for recombinant protein expression can take place (Figure 1). A major obstacle in using the chloroplast as a production chassis is that the product of interest needs to be extracted from the cell and will not be glycosylated. However, transgenes expressed in the nucleus can be targeted for secretion into the growth medium, which simplifies the purification.

To succeed with complex metabolic engineering, including multiple transgenes in microalgae, genetic tools that target biocatalysts or protein products to specific cellular compartments such as chloroplasts and ER would be beneficial. The challenges of working with algae are their complexity and tight genetic and transcriptional regulation that hinder precise genetic manipulation of the nuclear genome. The low and unpredictable transgene expression has been a huge disadvantage for genetic engineering of *C. reinhardtii*. Many species harbor large and complex genomes, with repetitive elements. These difficulties have led researchers to early on adopt advanced SB tools, such as CRISPR (Figure 4). We can see that the development of precision editing tools was very rapid in eukaryotic algae, and we can expect that improved, dedicated methods will open up bioengineering for more species, especially the ones with large commercial interest but recalcitrant to genetic modification.

The recent advancement in robust SB tools for industrial microalgae of the *Nannochloropsis* genus (such as *N. gaditana* and *N. oceanica*) are of great importance. The different techniques for genome editing and metabolic engineering, together with indispensable systems biology and physiological data now open a new venue for the design of strains capable of producing renewable acetyl-CoA-derived valuable biomolecules with extraordinary yields (Ajjawi et al., 2017; Han et al., 2020; Poliner et al., 2018a). Furthermore, it enables the gain of even more attraction of academic and industrial biotechnology sectors for further research and development efforts. Albeit the relatively small genome size in *Nannochloropsis* and the applicability of CRISPR-Cas9-based genome editing tools in several

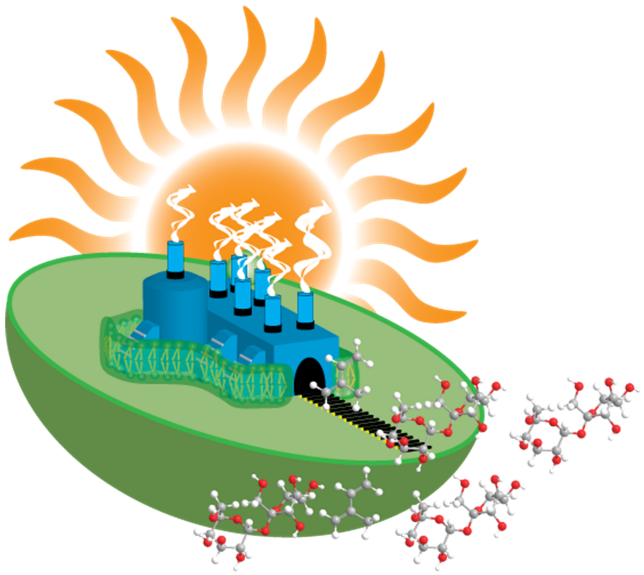

FIGURE 5 A cartoon representing the vision of realization of engineered microalgae as photosynthetic cell factories. Photosynthetic microbes can convert CO_2 into a wide variety of chemicals using water, micronutrients, and sunlight as energy source. The photo-based production ranges from high added value chemicals for food ingredients, pharmaceuticals, cosmetics, to hydrocarbons for high quality fuels. By using synthetic biology, the microalgae can be engineered with new biosynthetic pathways, while their existing cellular "infrastructure" ensures photosynthetic growth, a flow of precursor molecules, and secretion or storage of the biochemicals of interest. The realization of photobiological microbial factories could reduce human emissions of greenhouse gases and thus mitigate climate change. The chemicals produced in this cartoon are isoprene and D-Limonene

Nannochloropsis species (Figure 4), these reports proved the feasibility of DNA deletion of hundreds of base pairs. However, as a eukaryotic microorganism, *Nannochloropsis* still possess regulatory mechanisms that hinder the full exploitation of these photosynthetic microbes for metabolic engineering purposes. The targeted deletion of DNA parts at the chromosomal level has yet not been demonstrated in *Nannochloropsis*. Constructing strains with a reduced genome, yet viable, will allow the investigation of cellular mechanisms that are cryptic in the WT strain. Furthermore, this will facilitate the design of simple cells with genomes tailored to specific functions and environments.

Diatoms are far from biotech-ready hosts. While a lot is known about diatom diversity, their physiology, regulation, and adaptation to different environments is not well understood. Their genome architectures are complex, and they do not have the expanded genetic and SB toolsets that are available for other microalgae. However, *P. tricornutum* and *T. pseudonana* are becoming important model species, and they are backed by an enthusiastic research community. Also, the large extent of multiomics data is an important resource that can expedite the generation of dedicated SB parts and help them establish a niche for specialized applications (Falciatore et al., 2020).

Another set of significant challenges related to eukaryotic algae are related with optimal growth in large scale, a multifactor problem

that is specific for every species and goes beyond the scope of this mini review. It is unclear whether one of the algal groups will dominate the SB landscape or the community will adopt several hosts that serve different purposes.

3 | CONCLUSIONS AND PERSPECTIVES

Biotechnological employment of photosynthetic microorganisms has the potential for truly sustainable, solar driven production of useful chemicals from CO_2 (Figure 5). As described in this review, synthetic biology in photoautotrophic microorganisms is a rapidly developing field, where emerging new tools for genome editing and controlled expression of multiple genes using CRISPR-based technologies is especially promising for speeding up the development of new bioproduction strains and applications using these organisms. Among photosynthetic microbes, cyanobacteria hold the advantage of being easy to engineer, and cyanobacterial model strains are well characterized, in terms of both metabolism and genomics, based on several decades of using them as model organisms for photosynthesis research. While some of the most used cyanobacterial model strains may not be ideal for large scale cultivation, the knowledge about them is now being applied to several newly isolated strains which are selected for their industrially attractive properties, a development that is sure to continue in the next few years. The microalga *C. reinhardtii* is also a widely used model organism for which many SB tools and practices have been developed, while *Nannochloropsis*, *Chlorella* and the cyanobacterium *Arthrospira* (*Spirulina*) are well suited for industrial-scale cultivation, and are already widely used in the marine aquaculture industry.

Continued development of SB tools and techniques will support the implementation of biotechnological applications based on photosynthetic microorganisms. In addition, studies on metabolic flux and adaptation to different growth conditions, as well as the development and refinement of comprehensive metabolic models of new organisms, are building the knowledge base needed for the realization of engineered microalgae as sustainable photosynthetic cell factories. Thus, the next few years are full of promise and excitement about the future of microalgae bioengineering to optimize productivity, and to address additional societal challenges.

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

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