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Biosensors in microalgae: A roadmap for new opportunities in synthetic biology and biotechnology

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ABSTRACT

Biosensors are powerful tools to investigate, phenotype, improve and prototype microbial strains, both in fundamental research and in industrial contexts. Genetic and biotechnological developments now allow the implementation of synthetic biology approaches to novel different classes of microbial hosts, for example photosynthetic microalgae, which offer unique opportunities. To date, biosensors have not yet been implemented in phototrophic eukaryotic microorganisms, leaving great potential for novel biological and technological advancements untapped. Here, starting from selected biosensor technologies that have successfully been implemented in heterotrophic organisms, we project and define a roadmap on how these could be applied to microalgae research. We highlight novel opportunities for the development of new biosensors, identify critical challenges, and finally provide a perspective on the impact of their eventual implementation to tackle research questions and bioengineering strategies. From studying metabolism at the single-cell level to genome-wide screen approaches, and assisted laboratory evolution experiments, biosensors will greatly impact the pace of progress in understanding and engineering microalgal metabolism. We envision how this could further advance the possibilities for unraveling their ecological role, evolutionary history and accelerate their domestication, to further drive them as resource-efficient production hosts.

1. Introduction

The synthesis of bulk and high-value bioproducts from engineered microorganisms often offers cheaper, more efficient, and sustainable alternatives to industrial practices based on fossil or inefficient and unsustainable natural sources. Whereas conventional heterotrophic microbial hosts such as yeasts and bacteria offer a wealth of opportunities nowadays, some photosynthetic microorganisms are emerging as promising autotrophic options, anticipating a whole new landscape of possibilities. Microalgae are aquatic microbes that sustain life on Earth by producing oxygen and converting carbon dioxide (CO_2) into biomass through photosynthesis. They only require sunlight and water to grow and thus hold exceptional potential as renewable and sustainable feedstocks (Benedetti et al., 2018). Eukaryotic microalgae are a highly heterogenous and diverse group of organisms, many groups in this polyphyletic category have evolved through very different paths, and adapted to countless habitats (Gimpel et al., 2015). As eukaryotic organisms, they possess subcellular organelles and can perform complex biological functions. Compared to more established heterotrophic microbes that find large applications in industry such as yeasts and bacteria, algae have not undergone domestication yet and nearly all the strains used in laboratories and industries are natural isolates. As such, their metabolic make-up is still largely uncharted and leaves their huge potential untapped (Fabris et al., 2020a).

To date, several research efforts have provided proof of concepts for the potential of microalgae in being engineered to produce different products of industrial and commercial value (Abdallah et al., 2022; Fabris et al., 2020a, 2020b; Südfeld et al., 2023; Wichmann et al., 2020; Windhagauer et al., 2022). However, the current level of sophistication of metabolic engineering strategies in microalgae is substantially lower than in more established industrial organisms, which emphasizes the need for considerable advancements to develop new biotechnological resources. This is mostly due to limitations in their genetic tractability and available genetic tools, which are rapidly expanding and improving, and by a consequential lower understanding of their biology and biochemistry (Sreenikethanam et al., 2022). Some remarkable discoveries based on functional genetics approaches have outlined a very diversified and novel metabolic landscape but due to their highly

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dynamic metabolism, analysis with analytical chemistry or omics resources remains difficult to perform, as it poses relevant methodological biases and limitations (Alexandrov and Vickers, 2022; Grama et al., 2022).

Knowledge on metabolic traits, pathway regulation and fluxes, and phenotyping of engineered strains requires miniaturization of experimental set-ups, dynamic and non-invasive analyses, and high-throughput systems which now are theoretically feasible with the aid of biosensors. Biosensors are robust biomolecular devices that could overcome these limitations, as they have the capacity of sensing molecules intra and extracellularly, in vivo, and in vitro with high throughput. The advancements in microbial synthetic biology and metabolic engineering alongside the adoption of biosensors have been extremely useful in elucidating metabolic capabilities, traits, and flux regulators (Boada et al., 2020; Zhu et al., 2021), as well as invaluable tools for high-throughput phenotyping (Kaczmarek and Prather, 2021), or fine-tuning or selection of engineered strains (Liu et al., 2015; Teng et al., 2022). While these synthetic biology components have found large applications in model and established heterotrophic microorganisms, they have never been implemented in microalgal research. Similar to other genetic and molecular resources, advancements in algae often appear as progressing at a slower pace compared to the above mentioned established models. This is mainly imputable to the "younger" age of this field, and to the much smaller – yet growing, research communities and industrial sectors involved, compared to those dedicated for example to *Saccharomyces cerevisiae* and *Escherichia coli*. Moreover, some factors intrinsic to the biological and genetic features of microalgae have been posing particular challenges, for example the generally slower growth rate and the diverse composition and thickness of their cell walls, which often make their transformation challenging (Poveda-Huertes et al., 2023). Other challenges that the research community has been facing in are the limited availability of characterized dynamic regulatory components and limited knowledge on endogenous silencing, DNA integration and repair systems, and novel biological, genetic or biochemical traits. Considering this, while the availability of readily usable tools in algae synthetic biology may seem limited now, the development of new resources and technologies for microalgae is progressing relatively fast and benefiting from the overall advancements of synthetic biology in conventional microbial hosts (Section 3).

Here we discuss the suitability, feasibility, opportunities, and challenges for the development and implementation of some specific types of biosensors in eukaryotic microalgae. We project that they will enable high-throughput, refined approaches both to the understanding of the fundamental biology, to the prototyping of engineering strains and to drastically accelerate the iteration of *Design-Build-Test-Learn* cycles. We envision that these innovations will overcome current limitations and ultimately favor and accelerate the domestication of microalgae, opening avenues for the full industrial exploitation of their biosynthetic potential.

2. Biosensors as biomolecular devices for microbial engineering

Biosensors span a variety of modes of functioning and applications. Here, we specifically address protein-based and genetically encoded, transcription factor-based biosensors designed to detect specific metabolites. Biosensors of this type interact with target molecules coupled to a regulator or transducer component which converts the interaction with a small target molecule into a measurable signal or biological response that is proportional or dependent to the sensed metabolite (Dupont et al., 2018). Most biosensors are generally designed to operate intracellularly or on the cell surface and immediate surroundings, and to generate collective data at the single cell-level (Chen and Yang, 2022). Such biological devices enable the detection of metabolites non-invasively and allow rapid and multiplexed phenotypic evaluation of wild-type or mutant strains over traditional analytical techniques, which generally require full-scale experiments (Alexandrov and Vickers, 2022; Shi et al., 2018; Yu et al., 2023). As the definition of biosensors is broad, and the design and functioning of biosensors have been described in depth by recent reviews (Alexandrov and Vickers, 2022; Yu et al., 2023), here we specifically address applications of biosensors of two main groups that we envision could be readily implemented in microalgal systems: those which response is mediated through the expression of an encoded genetic function (reporter or selection) and those which directly operate at the protein level.

Transcription factor (TF) based biosensors involve a transcriptional regulator (often a TF) and an genetically encoded effector, which is transcriptionally regulated by the TF through a specific promoter region. The TF component is expressed independently and exists in its inactive or repressed form in the cell. Once specifically bound to the target ligand, the TF activates or de-represses the transcription of a reporter gene or a genetic circuit effector that modulates cellular functions (i.e. selectable markers) (Alexandrov and Vickers, 2022; Ellis and Wolfgang, 2012) (Fig. 1 a, b). The TF component possesses a DNA binding domain and a ligand binding domain (Ulrich et al., 2005). As the onset of the signal is mediated by gene expression, the response of these biosensors is generally slower than in protein-based systems (Alexandrov and Vickers, 2022). TF-based biosensors have been employed in heterotrophic biosystems to determine changes in the concentration of target metabolites (Skjoedt et al., 2016), define optimal growth parameter, detect metabolic heterogeneity, and metabolic fluxes in populations (Monteiro et al., 2019). Biosensor-based high-throughput screening (Williams et al., 2017), adaptive evolution (Snoek et al., 2020; Williams et al., 2017), dynamic regulation of metabolic flux (Boada et al., 2020), optimizing heterologous pathways via the implementation of dynamic control circuits (Xu et al., 2014), and monitoring of single-cell productivity has also been demonstrated (Binder et al., 2012).

The second type are genetically encoded protein-based biosensors which are expressed in the cell, on its surface, or in its surroundings and are activated upon direct binding of effector molecules (Fig. 1 c, d). Upon that, they regulate an actuator part (e.g., fluorescent reporters, regulatory switches, or selection markers) by conformational changes, dimerization, or selective binding, which acts as a detectable proxy for the presence of the target compound or condition (Leonard and Whitehead, 2022). The output is either a phenotype or a signal that is ideally measurable with laboratory instruments at low levels (Turner, 2013). Protein degradation tags are often attached to reporter proteins to substantially reduce the half-lives, as these fluorescent reporter proteins have greater half-lives, which often affects real-time measurements (Houser et al., 2012). Alternatively, a bioluminescent luciferase system with a short half-life has been developed to characterize the circadian behavior of promoters and transcription factors (Sagi et al., 2003).

A repertoire of perceptible metabolites and enzymes and the characterization of transcription factors are critical to designing a biosensor. The limiting factors even for most advanced biosensor devices are affinity, selectivity, sensitivity, dynamic working range, detection range, and response time which need to be addressed (Alexandrov and Vickers, 2022). It has been demonstrated that computational design workflows can be implemented in developing new or improving existing affinities of biosensor proteins and small molecules (Glasgow et al., 2019).

3. Suitability of microalgae for biosensor-based research and applications

The requirements for the implementation of biosensors in a microbial organism include the genetic tractability of the hosts, controlled and consistent transgene expression, the availability of sufficiently characterized and diversified genetic parts (promoters and terminators, reporter genes and genetic pathways/cascades), as well as rapid and efficient transformation procedures. Interest, applications and resources in algae molecular and synthetic biology have risen considerably in the past few years (Fabris et al., 2020a). Technologies and advancement in

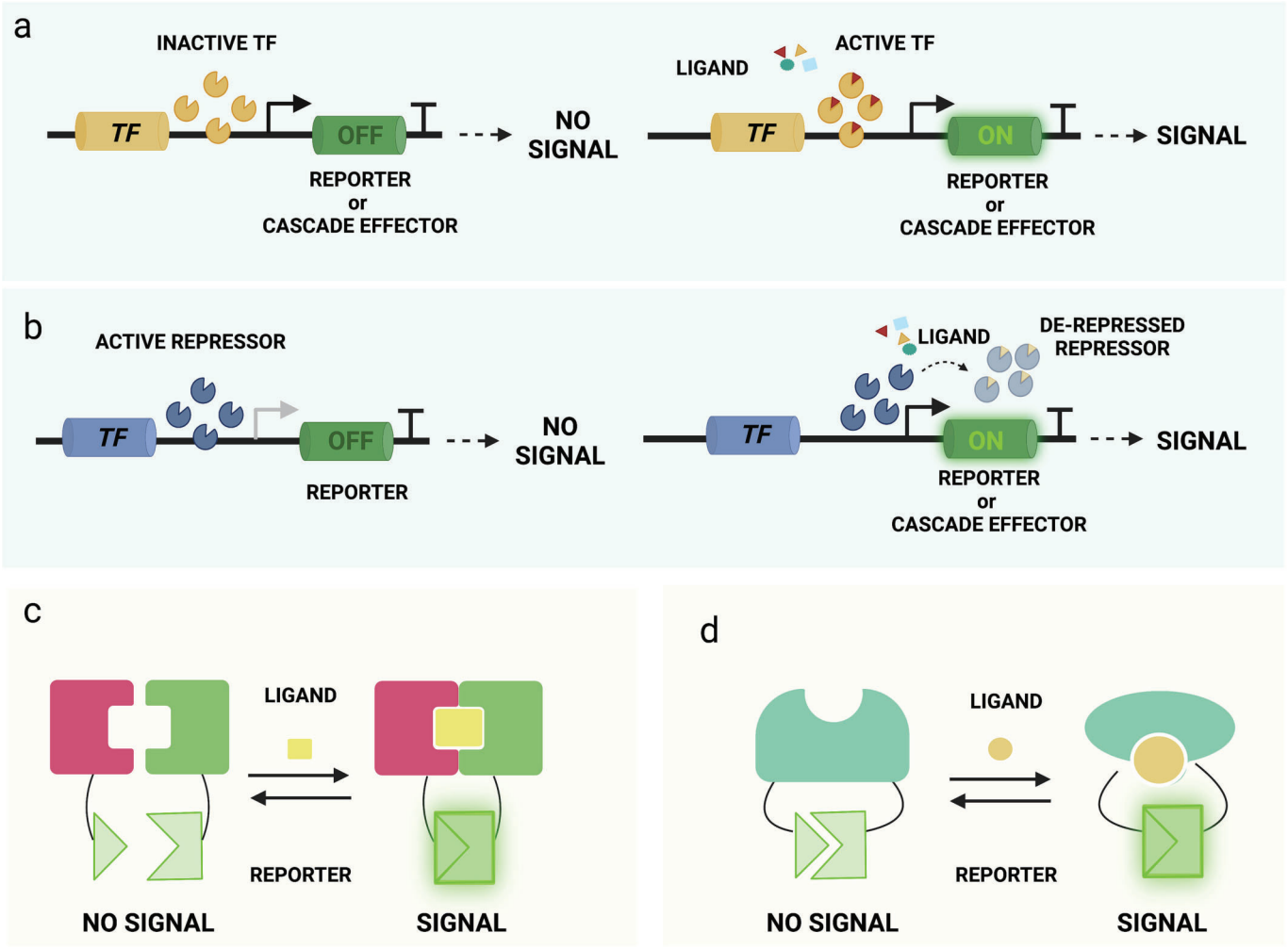

Fig. 1. A schematic representation of biosensors suitable for microalgae. (a-b) TF-based biosensors: in the absence (left) or in presence (right) of target metabolites; (c-d) protein-based biosensors: split reporter system where two parts of reporter are separated but linked to ligand-responsive proteins, which bind in presence of the target ligand (left), or undergo conformational changes (d) bringing the split reporter portions in close proximity to enable its activity.

algae genetics, genetic engineering, and synthetic biology have primed from model species, representative of larger groups with clear industrial potential, such as *Chlamydomonas reinhardtii* (Chlorophyta), *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Bacillariophyta), and *Nannochloropsis* spp. (Eustigmatophyta). However, non-model species have seen substantial and encouraging developments, which place them as next-generation industrial algal species (Kumar et al., 2020).

The engineering of biological functions such as biosensors requires standardized resources and tools. This is key to generate and prototype biosensors *in alga*, which involve the assembly and expression of entire libraries of construct variants. Recently developed standardized strategies based on Golden Gate Modular cloning allow building of modular complex multigenic DNA from basic genetic elements (Crozet et al., 2018; Pollak et al., 2020), which enable gene-stacking designs. The functional characterization and development of genetic parts is steadily increasing and will not be reviewed here, as it has been covered elsewhere (Fabris et al., 2020a; Vavitsas et al., 2019).

Besides the capacity of expressing multiple genes, it is imperative that the biosensor components are expressed in a consistent and controlled manner in the cell. In model yeasts and bacteria, these can either be inserted in specific loci in the genome or maintained on plasmids. In microalgae, with some exceptions, most of the genetic transformation approaches are currently based on random integration of the transgenes in the nuclear chromosomes, which has clear incompatibility with the fine and consistent expression levels required by biosensor components. Indeed, these are characterized by uncontrolled genetic rearrangements and endogenous silencing mechanisms and position effects (George et al., 2020; Plucinak et al., 2015). A few stramenopiles and rhodophyta species, can maintain large extrachromosomal expression vectors containing centromeric sequences (Karas et al., 2015), which possibly overcome these issues (George et al., 2020; Li and Bock, 2018; Poliner et al., 2020). These can be delivered by bacterial conjugation to the algal cells, allowing the transfer of large DNA constructs that could host entire genetic circuits (Karas et al., 2015; Slattery et al., 2018). However, it is still unclear how artificial extrachromosomal DNA constructs are maintained in algae nuclei and their stability is often regarded as a concern (Diamond et al., 2023), with contrasting observations (Diamond et al., 2023; George et al., 2020). Episomal expression may allow to avoid interferences in transgene expression caused by position effects and chromosomal re-arrangements, and potentially offer a more reproducible strategy for the implementation of biosensors. However, this is affected by higher clonal heterogeneity of phenotypes, possibly due to episome segregation dynamics or endogenous silencing mechanisms, which might affect the resolution of biosensor-mediated measurements (Diamond et al., 2023; George et al., 2020; Karas et al., 2015).

Biosensor-based experiments in combination with metabolic engineering approaches often require several heterologous constructs to be co-expressed, implying several rounds of super-transformation, thus several selection markers are needed. Additionally, biosensors can be

designed to orchestrate genetic circuits linked to the modulated expression of selection markers or to be coupled with titrated concentration of antibiotics in the medium to drive and enrich populations with superior production phenotype, for example in assisted laboratory evolution experiments or large-scale screenings. Selectable markers in microalgal research are conventional antibiotics such as hygromycin B in *C. reinhardtii* (Berthold et al., 2002), zeocin in *P. tricornutum* (Apt et al., 1996) nourseothricin in *P. tricornutum* and *C. reinhardtii* (D'Adamo et al., 2019; Yang et al., 2019), and blasticidin s-deaminase in *C. reinhardtii* and *P. tricornutum* (Buck et al., 2018; de Carpentier et al., 2020) and their respective resistance genes, and auxotrophic markers. Recently, an endogenous selectable marker conferring resistance to norflurazon was developed for *P. tricornutum* (Taparia et al., 2019). In another instance, the disruption of the endogenous gene encoding a uridine monophosphate synthase (UMPS) confers uracil auxotrophy and allows counter selection on 5-fluoroorotic acid (5-FOA) in *P. tricornutum*, which is increasingly used as auxotrophic selectable marker for transgene expression and DNA-free genome editing (Pampuch et al., 2022; Sakaguchi et al., 2011; Serif et al., 2018). However, the *PtUMPS* locus can accumulate spontaneous or transposon-induced mutations (Abbriano et al., 2023) and might not be an ideal marker for biosensor-based studies, underscoring the requirement for additional auxotrophic markers.

4. Microalgae as source and inspiration of novel biosensor components

In the wild, phytoplankton thrive in an extremely complex network of chemical signals, which drive many, if not all interactions within cells and populations (Seymour et al., 2017). This, yet elusive chemical language drives massive events such as blooms as well as chemical warfare between competing species and consequently large die-offs. Molecules and mechanisms triggering these phenomena are largely unknown, however in the past years a lot of progress has been made and several signaling molecules have been identified, particularly in diatoms (Amin et al., 2015; Gallo et al., 2017; Gillard et al., 2013; Shibl et al., 2020). Chemical cues are sensed in small concentrations and possibly trigger signaling cascades that induce transcriptional reprogramming in the cells, which reflect on growth and metabolism (Amin et al., 2015; Gallo et al., 2017; Gillard et al., 2013; Shibl et al., 2020). Identifying and decoding these signal transduction pathways, may provide elements and parts to re-design them to sense different molecules and activate artificial genetic elements, for example linked to the expression of reporter genes or selectable markers (Miettinen et al., 2022). These types of biosensors could greatly enhance the output of biosensor-assisted laboratory evolution or large-scale screening of mutants, as outlined in the following sections. Finally, mapping the variety of molecules and secondary metabolites in phytoplankton populations, will drive the discovery of novel ligand-protein interactions and expand the repertoire of ligand-receptor couples. This will provide the base ground for the design and development of novel biosensors, not only for microalgae, but also for other microbial platforms.

To design new TF-based biosensors, it is necessary to possess sufficient knowledge on the interactions and specificity of endogenous TFs and regulatory DNA sequences, which in microalgae is still in early stages. Systematic approaches have been established to mine information on regulatory elements and their corresponding effectors, a basic requirement for modulating biosensors at the genome-scale level. For example, 16 putative inducible systems from a pool of 400 transcription regulators (TR) were identified through a genome-wide approach using BRENDA in a soil-dwelling bacterium, *Cupriavidus necator* H16. The system was experimentally validated to be functional not only in *C. necator* but the authors further demonstrated the utility of this transcription factor-based-inducible biosensor system by employing the transcription regulator-promoter pairs in the model bacteria *E. coli* and *Pseudomonas putida* (Hanko et al., 2020). This potential of the genome-scale approach in bacteria for developing biosensors is yet to be harnessed in microalgae. Advancements in synthetic biology approaches in photosynthetic microalgae are underway to unleash their full potential as exceptional microbial cell factories (Wang et al., 2020a). Putative regulatory elements have been identified by experimental and computational methods on a genome-wide scale in many species of microalgae such as *C. reinhardtii* (Ding et al., 2012) and *Nannochloropsis* spp. (Hu et al., 2015) which could be the starting point to modulate and validate novel biosensors.

5. Opportunities for biosensor-based research and engineering in microalgae

To the best of our knowledge, biosensors have never been implemented in microalgae research, but recent advancements have underlined the cruciality to the understanding of metabolic pathways and to construct novel biological systems via synthetic biology. Biosensors have become essential tools to accelerate any metabolic engineering research. In the following sections we propose areas and applications in which biosensors could be implemented in microalgae research, to deepen and extend its reach both in fundamental and applied contexts (Fig. 2).

5.1. Understanding the dynamic metabolism of algae

Cellular metabolism is a highly intricate and dynamic network, which can undergo drastic changes in minutes or even seconds. This is particularly relevant in organisms that evolved in highly variable environmental conditions, such as algae and phytoplankton. The overall understanding of their metabolism consists of partial and 'static' views of metabolic pathways and enzymes. Fast regulation and dynamic metabolic shifts are at the basis of the most relevant ecological phenomena, such as blooms, die-offs, nutrient depletion, and competition, as well as in industrial bioprocessing settings, when different heterologous pathways are induced and when algae are cultivated in different and intensive modes. While several high-resolution works that combined transcriptomics, proteomics, and metabolomics have unveiled some of these dynamics, these approaches always suffered from the necessary limitation of sampling, metabolite extraction, and measurements. Analytical chemistry-based methods have limited temporal and spatial investigative resolution. Biosensors allow to track and monitor the unrolling of metabolic phenomena *in vivo*, in real time (Alexandrov and Vickers, 2022), and have increasingly become versatile research tools in many laboratories, applied to different organisms. For example, fluorescent biosensors allowed to track nitrate dynamics in plants at the single-cell level (Chen et al., 2022), and to monitor redox dynamics in mammalian cancer cell lines (Zhao et al., 2016). A redox sensor to investigate the ROS dynamics at the single-cell level was established through the expression of a redox-sensitive GFP (roGFP) in different subcellular compartments of the diatom *P. tricornutum*. Cell-to-cell phenotypic variability was recorded when the diatom population with roGFP targeted to the chloroplast was treated with H_2O_2 and high light. The specific stress cues formed two distinct subpopulations, which accumulated ROS above a threshold and eventually induced cell death and the other resilient cells which survived, demonstrating the link between chloroplast redox state and regulation of the cell fate (Mizrachi et al., 2019). Other promising examples to image and track metabolites in the several algae species using non-genetic biosensors are graphene oxide (GO) nanoparticles used to carry ssDNA aptamers to detect β-carotene and adenosine triphosphate (ATP) in *Euglena gracilis* and a cell-wall deficient strain of *C. reinhardtii* (Jin et al., 2021; Kim et al., 2022). These biosensors, however, were not genetically encoded and required to be delivered at each use. The availability of genetically encoded biosensors for intracellular metabolites will add an exceptional new array of resources to investigate metabolic dynamics, allowing to design miniaturized, rapid, and continuous experiments. In microalgae, these

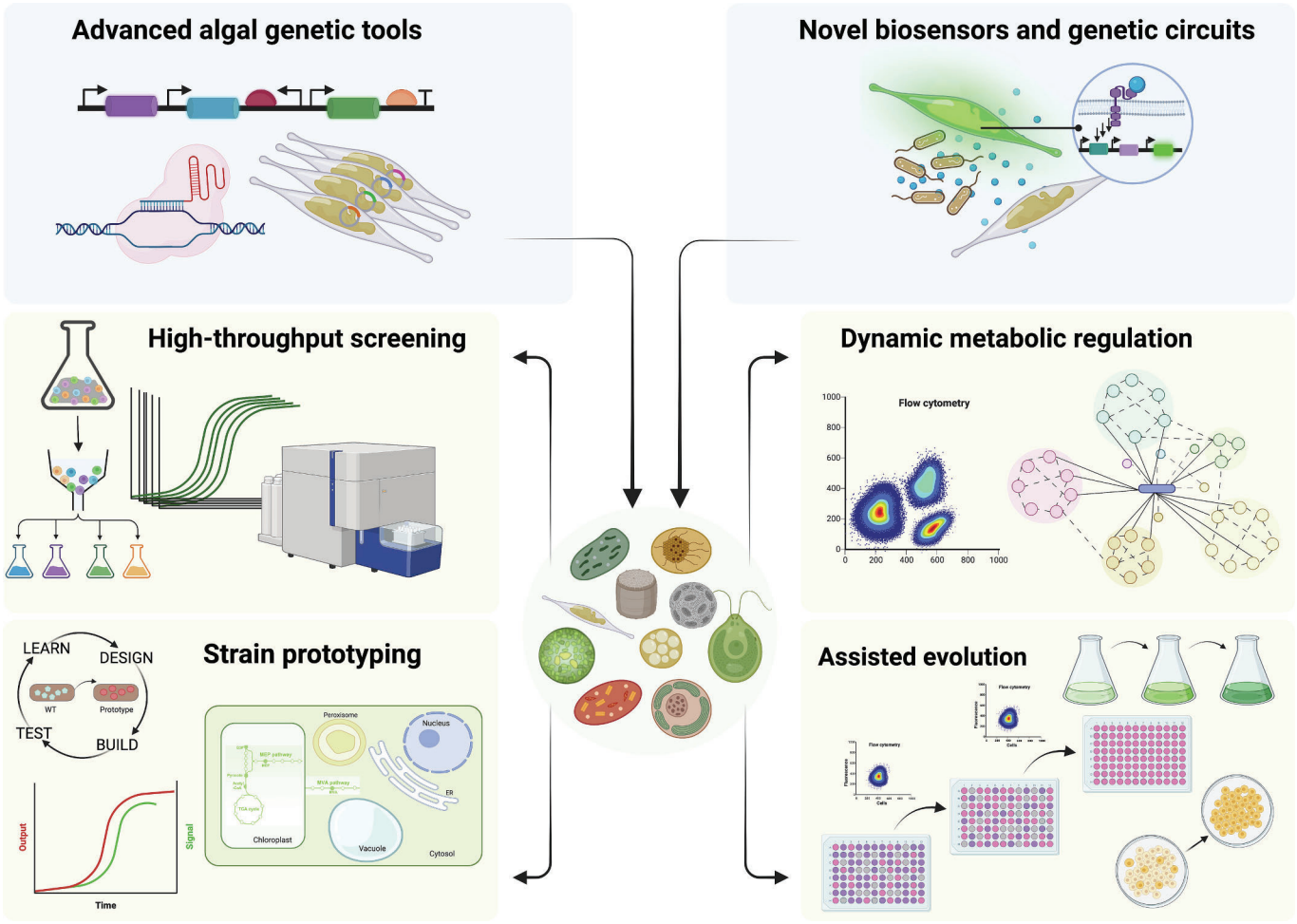

Fig. 2. Visual summary of new opportunities and applications of biosensors in microalgae, spanning from understanding fundamental biochemical traits, to metabolic engineering and synthetic biology.

will enable the non-destructive tracking and visualization of nutrient uptake, metabolite trafficking, and accumulation in different growth stages or stress conditions, and in different phenotypes, in real time at the single-cell level.

5.2. *High-throughput screening and selection*

Installation and optimization of endogenous and heterologous metabolic pathways and other production traits is a process that in microalgae, is in its infancy. Even if such applications have not been demonstrated yet, we envision that in the next few years many landmark demonstrations will consolidate these approaches in microalgae, benefitting from the affordability of DNA synthesis, semi-automated construction, and expression and modulation of multi-gene constructs. In this scenario, the complexity of experimental design will increase exponentially, requiring rapid and miniaturized screening and phenotyping of many genetic configurations, in different environmental conditions. Currently, the application of high-throughput approaches in microalgae are limited to a few traits such as pigment fluorescence or the expression of enzymes or proteins fused to fluorescent proteins. The implementation of biosensors will allow to dynamically screen for the presence of specific metabolites or for modulated traits in response to environmental or cellular stimuli. This will enable not only to screen both engineered or mutant strain libraries, but also entire genetic libraries for gene discovery purposes. Hence, there is a great interest in developing genetically encoded biosensors to enable high-throughput screening and selection. Several biosensor screen modalities in *E. coli, S. cerevisiae, Cornyebacterium glutamicum,* and others have been developed to work in high-throughput fashion with capacity for library sizes ranging from 10^1 to 10^6, for well-plates (Ho et al., 2018; Qiu et al., 2020), agar plates (Qian et al., 2019), fluorescence-activated cell sorting (FACS) (Raghavan et al., 2019; Wang et al., 2020b), droplet-based screening (Saleski et al., 2023) and selection-based methods (Snoek et al., 2018). A recent example is a L-cysteine-responsive transcriptional factor based (CcdR) biosensor developed for the engineering and screening of L-cysteine overproducers in *E.coli.* The L-cysteine-responsive TF, CcdR (cysteine-inducible cysteine desulfhydrase) was fused with GFP as the reporter gene, and the resulting biosensor construct was employed as a high-throughput screening platform to screen an extensive random mutagenesis library to narrow down the strains from 1000 colonies to 10 strains with considerably higher cysteine production compared to wild type parents, demonstrating the efficiency of biosensor based high-throughput screening compared to conventional approaches (Gao et al., 2022).

Genetically encoded biosensors can be employed in microalgae to accelerate metabolic engineering approaches in algal phenotyping, to identify natural, mutant, or engineered strains that are associated with higher accumulation of valuable compounds, or key substrates. Many high-throughput screening procedures have been developed for several model microalgae species, clearly demonstrating that miniaturized high-throughput approaches are feasible and could be employed in the context of biosensor based high-throughput screening and selection. For example, *C. reinhardtii* colonies engineered to express a terpene synthase fused with reporters, could be directly screened on agar-plates through

combined high-throughput fluorescence and luciferase analysis to identify high producers of patchoulol (Abdallah et al., 2022). Diatoms, and in particular *P. tricornutum,* are particularly suitable for phenotypic assays based on high-throughput flow cytometry. Semi-automated flow cytometry workflows have been employed to rapidly screen pigment content and composition (Macdonald Miller et al., 2021) and to screen and profile large libraries of transformants of different natures, enabling the isolation of particularly high-performing strains (George et al., 2020). The approach of high-throughput quantitative phenotypic assay (QPA) to evaluate growth rate, cell size, granularity, chlorophyll *a*, neutral lipid content, silicification, reactive oxygen species accumulation, and photophysiology parameters was demonstrated in *Thalassiosira* spp. where these traits were quantified simultaneously in one miniaturized experimental workflow in multi-well plates. The trait data could be collected within a week of inoculation (Argyle et al., 2021). This assay provides a valid example of the feasibility of this experimental framework for designing high-throughput multiplexed experiments involving microalgae such as diatoms.

The potential of these approaches in microalgae can be anticipated from the large number of successful examples of the use of biosensors for high-throughput screening in various microbial chasses. For example, a genetic biosensor circuit in *E. coli* was developed for screening a focused mutant library based on phenol-detecting transcription factors coupled with cell sorting using flow cytometry. It proved to be an efficient high-throughput system for hypersensitive genetic screening to create a new active site and detect various enzyme activities, with a rate of up to 10^6 clones per day (Kwon et al., 2018). A whole-cell lactulose biosensor was successfully utilized for high-throughput screening of the cellobiose 2-epimerase mutants for lactulose hyper-producers (Wu et al., 2017). In addition, a ligand-responsive transcription-factor biosensor was developed in *S. cerevisiae* to detect varying levels of para-hydroxybenzoic acid (PHBA) production. The biosensor enabled FACS isolation of producer cells in the ratio of 1:10000 mixed with non-producers (Williams et al., 2017). Droplet-based microfluidics can be used to screen libraries in ultra-high-throughput fashion as droplets can encapsulate libraries of interest and genetically identical microcolonies. The combined efforts of a genetically encoded biosensor and fluorescence-activated droplet-based sorting (FADS) to screen 3-dehydroshikimic acid (3-DHS) strain library has demonstrated that this approach offers a higher enrichment rate compared to the FACS-based sorting and droplet microfluidics also benefit with the control of low volumes and sorting throughput of millions of droplets in a short amount of time (Baret et al., 2009; Kaczmarek and Prather, 2021; Siedler et al., 2017; Tu et al., 2020). Combinatorial or multivariate optimization-based engineering approaches generates complex libraries with countless constructs and huge genetic diversity, when coupled with the droplet-based microfluidic biosensors can prove to be an attractive and fast way to screen huge libraries up to 10^8 clones per day furthering the efforts of microbial engineering (Naseri and Koffas, 2020; Siedler et al., 2017). Droplet-based microfluidic technologies were successfully demonstrated in two distinct species of microalgae, *P. tricornutum* and *N. gaditana,* where transconjugants microalgal cells were enclosed in discrete droplets and were effectively screened based on several parameters based on fluorescence, such as chlorophyll autofluorescence and eGFP signal. This allowed effective screening and sorting workflows with efficiencies two-times higher than traditional screening protocols (Yu et al., 2021), laying the ground-work for fluorescent-based biosensor microfluidic assays.

5.3. Engineered strain prototyping

Strain engineering and prototyping require an iterative optimization pipeline of *Design-Build-Test-Learn* (DBTL) cycles (Robinson et al., 2020). This cycle can be substantially accelerated with a rapid readout of strain performances avoiding full-scale experiments and laborious analyses. Biosensors revolutionize engineering efforts as their application can shorten the prototype screening time. The test phase of the design-build-test-learn cycles is a critical bottleneck in the development and improvement of novel production strains, which is time-consuming and costly.

This could be achieved by employing cell-free biosensors as they enable the design-build-test experimental cycle to be completed more quickly and conveniently removing the barrier of target permeability of cells and avails diversified sensing and signal output elements (Pandi et al., 2019). Cell-free biosensors are based on cell-free expression systems which harness the transcription and translation (TX-TL) machinery of living cells. The *in vitro* TX-TL reaction constitutes of cellular synthetic machinery and cofactors with DNA-encoded genetic information. The working principle of cell-free biosensors is to select the suitable target recognition elements such as nucleic acids, aptamers, transcription factors, and reporter genes (Lee and Kim, 2019; Wang and Lu, 2022). A landmark example consists of computationally engineered sensor proteins composed by an ankyrin repeat (AR) protein and a maltose binding protein (MBP) fused to a split reporter system, in which the input was computationally designed for specific binding of farnesyl pyrophosphate (FPP), an important isoprenoid precursor. The input-output response of the cell-free biosensor was demonstrated by utilizing the *in vitro* TX-TL machinery encoded with sensor proteins with a defined addition of FPP, resulting in the dimerization of sensor proteins, validating the functionality of cell-free biosensors (Glasgow et al., 2019). Cell-free biosensors have also gained extensive attention and rapid development as an *in vitro* tool to characterize regulatory elements in non-model organisms such as *Bacillus megaterium* which is particularly difficult to engineer (Jiang et al., 2018; Moore et al., 2016). Similarly, the potential of many non-model microalgae species is often limited due to the lack of genome sequences, high-throughput screening approaches, and genetic tools for the expression of multi-gene constructs. In these cases, extracellular, cell-free biosensors could provide valuable opportunities to develop and prototype strains on model as well as non-model microalgal species in a high-throughput fashion.

Industrial scale microalgal biomass and bioproducts production require the development of robust and highly efficient engineered strains with desirable metabolic and physiological capabilities, which can reduce the upstream processing costs. A high-throughput rational strain workflow was developed for *C. glutamicum* by automated conjugation accompanied by a software tool to record the transformants. To validate the applicability of the workflow, different versions of amino acid biosensor libraries based on a transcriptional regulator *Lrp*, were constructed, and characterized. Herein, the biosensor design coupled with high-throughput screening was employed to screen and develop novel biosensor strains with improved dynamic range (Tenhaef et al., 2021) which will potentially minimize the laborious engineering of strains and hence decelerating the DBTL cycles. The approach has been proven not to be limited to a target species but can be utilized for any microbe responsive to horizontal gene transfer through conjugation, such as different species of stramenopiles, red algae, and other microalgal species, to prototype strains (Karas et al., 2015; Li and Bock, 2018; Poliner et al., 2020; Slattery et al., 2018).

In another approach, *in vitro* rapid prototyping of a synthetic pathway was demonstrated by a single reaction recombinase-based toolkit "SCRaMbLE-in" to pre-assemble genes with regulatory elements into targeted pathways. One key gene with the desired integration position in the pathway is engineered with a single recombinase recognition site. A combinatorial library is generated by the integration of recombinase into the target recombination site, which was transformed into yeast for downstream screening and analysis (Liu et al., 2018).

5.4. Adaptive laboratory evolution

Adaptive Laboratory Evolution (ALE) is a powerful tool to derive and select new superior phenotypes with specific traits, with great potential in the context of strain improvement for bioproduction. An artificial

selective pressure is put on a cell culture and beneficial phenotypes are selected either continuously or throughout consecutive selection cycles. Biosensors greatly empower these approaches, either by coupling the presence of a certain metabolite to the expression of a resistance gene, or via fluorescent biosensors that facilitate FACS-mediated selection in consecutive selection cycles. Even though biosensor-assisted ALE experiments have been frequently implemented in the past to select for either superior production strains or screen for novel genetic targets for pathway engineering in heterotrophic organisms (Williams et al., 2016), genetically encoded biosensors have not yet been implemented in microalgae for ALE purposes.

Biosensors mainly have been used to enhance the production titers of different metabolites in established hosts such as *E. coli*, *S. cerevisiae*, or *C. glutamicum*, by the implementation of genetic circuits, which are regulated by metabolite concentrations. Notably, amino acid biosensors often have been implemented for biosensor-assisted ALE experiments, to increase the production titers of e.g. tyrosine (Chou and Keasling, 2013), L-valine (Han et al., 2020; Mahr et al., 2015), and tryptophan (Gwon et al., 2021). Biosensor-assisted ALE experiments also have been implemented to enhance tyrosine and isopentenyl diphosphate (IPP) production in *E. coli* (Chou and Keasling, 2013). IPP is a pathway precursor to the synthesis of high-value terpenoids, and as photosynthetic organisms naturally are suited for the production of these metabolite families, an IPP biosensor would be attractive for the implementation in microalgae ALE experiments. Artificial TFs have never been implemented in microalgae, thus this could be a first crucial step to facilitate such experimental approaches.

Microalgae are often natural and efficient producers of lipids, yielding especially high amounts of polyunsaturated fatty acids (Fernandes and Cordeiro, 2021). ALE experiments to boost lipid production have been implemented in the microalgae *C. reinhardtii* (Velmurugan et al., 2014) and *Micractinium* sp. (Deka et al., 2020), by employing lipid-specific fluorescent dyes, flow cytometry, and fluorescence-activated cell sorting (FACS). Remarkably, in the study of Velmurugan et al., lipid production could be increased by up to 175%. Although neither example involved biosensors of the type discussed so far, these demonstrate the feasibility of high-throughput, fluorescent-based evolution experiments operating at the single-cell level, and anticipate the high impact that biosensor-assisted ALE experiments could yield. The implementation of genetically encoded biosensors, with their broader and superior substrate specificity, will enable an even more precisely directed evolution to the increased yield of high-value fatty acids but also offer the opportunity of extending the reach of these powerful approaches to a much wider range of compounds.

Even though several ALE experiments have been successfully performed on evolving microalgae to improve the production of carotenoids (Fu et al., 2013), lipids (Deka et al., 2020), or the ability to biodegrade phenol (Wang et al., 2016), the selection for high producers of specific metabolites remains difficult. In their ability to change a difficultly selectable phenotype into an easily selectable one, biosensors pose the perfect tool for ALE experiments.

6. Outlook of biosensors in microalgae

Designer microbial factories have been increasingly employed for bio-based production of useful chemicals and materials through metabolic engineering and synthetic biology. Integration of biosensors into these advances will revolutionize the engineering of microbial cell factories (Fig. 2). The engineering and implementation of biosensors requires the information on protein or a regulatory system, but for certain applications it also needs a detailed understanding of the transcriptional regulation network and the protein expression system of the host, as well as the availability of sufficient number and variety of genetic parts to assemble a transcriptional response system (Xu et al., 2014). The application of biosensors in well-established hosts such as yeast and bacteria is itself in the developmental stage. Thus, the design of biosensors in microalgae is even more challenging as some groups are phylogenetically distantly related to conventional microbial and plant model systems, and their metabolic pathways and regulation work in substantially different ways.

When implemented, the optimization and practical application of biosensors in microalgal species will initially be limited to a handful of model species due to the unavailability of sufficiently accurate genetic tools. To date, only a few species allow sufficiently precise, controllable, and consistent transgene expression and targeted gene integration is still in its early stages in microalgae with considerable results achieved mostly in *Nannochloropsis spp* (Naduthodi et al., 2019). Also of particular interest, are the efforts directed towards precisely targeting cassettes in genomic safe harbors to ensure reliable and consistent expression. While some of them have tentatively been identified in *P. tricornutum and N. oceanica* (Defrel et al., 2021; George et al., 2020; Südfeld et al., 2022) they require thorough validation. Moreover, CRISPR-based methods for targeted insertion are still lagging in model species like *C. reinhardtii* and diatoms, although some promising advances in this regard have been made in *N. gaditana* (Naduthodi et al., 2019) and *T. pseudonana* (Belshaw et al., 2022; Nam et al., 2022). Even though this can slow the progress in generating efficient production strains in model species that could reach yields and cost-effectiveness for industrial feasibility, the diversity of algae in terms of biological and metabolic capabilities can offer novel engineering opportunities, as well as new functions to be exploited in non-model species with the advancements in biosensor-directed metabolic engineering (Fabris et al., 2020a).

The optimization of biosensor technology and their subsequent implementation in microalgae will enable more streamlined, rapid, and efficient strategies to obtain superior algae production strains (Fig. 2). Further, biosensors will synergize with single-cell analysis methods, accelerating the pace of research on algae biology and genetics, as well as expanding their applications. As such, biosensors show great potential for the future of microalgae engineering to produce superior microalgal strains.

7. Conclusions

Some of the current limitations in understanding and manipulating the metabolism of algae can be overcome by combining the efforts of metabolic engineering and synthetic biology with the aid of biosensors. Development of microalgal biosensors will offer increased experimental capacity and rapidity, including the phenotyping of genotypes with complex genetic designs, rapid strain evaluation, high-throughput screening, and adaptive evolution that can accelerate the design-build test-learn cycle to obtain improved metabolic configurations for synthetic biology applications, besides providing opportunities for the discovery and development of novel biosensors. We anticipate that the development of microalgal biosensors can also shed light on the interactions between phytoplankton, the primary producers, and other living organisms of the oceans and aid in better understanding of the climate and carbon cycle.

CRediT authorship contribution statement

Payal Patwari: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Florian Pruckner:** Investigation, Writing – review & editing. **Michele Fabris:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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