Trends in Biotechnology

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Review

Cyanobacterial biofilms: from natural systems to applications

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Cyanobacteria are the ancestors of oxygenic photosynthesis. Fueled by light and water, their ability to reduce CO_2 to sugar holds potential for carbon-neutral production processes. Due to challenges connected to cultivation and engineering issues, cyanobiotechnology has yet to be able to establish itself broadly in industry. In recent years, applying cyanobacterial biofilms as whole-cell biocatalysts instead of suspension cultures has emerged as a novel concept to counteract low cell densities and low reaction stability, critical challenges in cyanobacterial applications. This review explores the potential of cyanobacterial biofilms for biotechnology and bioremediation. It briefly introduces cyanobacteria as primary producers in natural structured microbial communities; describes various applications in biotechnology and bioremediation; and discusses innovations, challenges, and future trends in this exciting research field.

Highlights

As solar cell factories, cyanobacteria generate value-added compounds directly from CO_2 and sunlight, opening the field of photobiotechnology for carbon-neutral or carbon-negative production scenarios.

Cyanobacterial biofilms are superior to suspended cell cultures in robustness, turnover numbers, and cell densities.

Cyanobacterial biofilms are applied in diverse fields, from agriculture to wastewater treatment and bioremediation to the production of value-added compounds and biofuels.

Cyanobacteria: the game changers?

Cyanobacteria are the organisms responsible for the Great Oxygen Event 2.4 billion years ago. They are the inventors of oxygenic photosynthesis, a process that led to fundamental changes in the atmosphere of Earth and thereby ultimately influenced the evolution of life [1]. Cyanobacteria can transform light energy into chemical energy, depending on water as an electron source, releasing oxygen during the process. This energy is invested in reducing carbon dioxide to sugar in the Calvin Benson Bassham (CBB) cycle, which is the basis for all heterotrophic life. Driven by the prospect of realizing **carbon-neutral** (see Glossary) or even **carbon-negative bioprocesses**, a plethora of proof-of-concept studies in recent years have demonstrated the feasibility of coupling various fermentative pathways to the CBB and thereby generating **value-added compounds** directly from carbon dioxide and water with energy supplied by light [2–6].

Furthermore, the ability of many cyanobacterial species to fix atmospheric nitrogen gas was found to be highly interesting in the context of natural fertilizers for agriculture [7,8]. However, these studies have yet to be translated into broad commercial applications. Due to **light limitation**, cyanobacteria do not reach very high cell densities when grown in suspended cell cultures [9]. They are also sensitive to chemical compounds such as alcohols and organic solvents [10] and grow more slowly than other traditional biocatalytic workhorses such as *Escherichia coli* [11]. However, when growing in biofilms, these organisms profit from extraordinary robustness and high cell numbers [12], which is why this growth format is increasingly being discussed as a potent alternative to planktonic cultures in application-oriented research. This review aims to link the features of cyanobacteria growing in naturally occurring **microbial mats** to fundamental biofilm characteristics and their application. Hence, it provides a short overview of microbial mats and naturally occurring biofilms, emphasizing the unique function of cyanobacteria within these structured microbial communities. It briefly explores cyanobacteria as whole-cell **biocatalysts** and discusses the benefits and challenges of operating these microbial workhorses. Finally, this review highlights applications of cyanobacterial biofilms, including axenic as well as mixed

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cultures; discusses key innovations and various application fields from **white biotechnology** to **bioremediation** and agriculture; and deduces future trends in this exciting research field.

Cyanobacteria are essential primary producers of carbon compounds in structured microbial communities

The ability to build organic carbon molecules from solely inorganic resources makes cyanobacteria important primary producers [13], especially in marine environments [1,14,15]. Many species in this highly diverse group of organisms can also fix atmospheric nitrogen, adding to their importance for the global element cycles [16]. Cyanobacteria evolved into various morphologies with both unicellular and different filamentous forms. As diverse as their morphotypes are the ecological niches to which cyanobacteria have successfully adapted. These reach from various terrestrial ecosystems in all climate zones to aquatic systems such as fresh, salt, and brackish water. They are abundant in moderate and extreme environments (Table 1). Consequently, they have developed numerous traits to prosper in these diverse conditions.

In nature, microorganisms grow in two formats, either freely suspended or attached to a surface. In the huge open water bodies in oceans, lakes, and rivers, billions of cells live as planktonic microbes. On the other end, there are sediments, soils, and all habitats located at the phase boundaries, mostly solid to liquid, but also liquid or solid to gas–air interface. In these habitats, microorganisms are not growing freely suspended and homogeneously distributed but attach to these phase boundaries, often forming 3D structures with remarkably high cell densities. A fascinating lifestyle of some cyanobacteria is as partners in microbial mats. The oldest structures of this kind, stromatolites, date back about 3.5 billion years and thus represent one of the oldest ecosystems we know [38]. In a microbial mat, various microorganisms are embedded in a matrix of self-produced **extracellular polymeric substances (EPSs)**, which are composed mainly of polysaccharides [39]. It serves as a protection shield and a pantry when times get rough, is responsible for the architecture and stability of these 3D structures, and facilitates cell–cell interactions and horizontal gene transfer. In these 3D structures, biochemical gradients governed by microbes' metabolic activities are forming, which define the specific microniche for the different organisms living in such a mat (Figure 1).

Prokaryotes make up the most abundant group of mat inhabitants, including oxygenic phototrophs, anoxygenic phototrophs, purple bacteria, green sulfur bacteria, aerobic heterotrophs, anaerobic bacteria, sulfur-oxidizing bacteria, sulfur-reducing bacteria (SRB), and methanogenic archaea (Figure 1) [17,34,40]. Oxygenic photosynthesis conducted by cyanobacteria is the basic process required for this trophic network to prosper [41,42]. Inorganic carbon is reduced to organic carbon [$(CH_2O)_n$] during daytime and used to form biomass. In dark periods, cyanobacteria respire stored carbon and consume oxygen. Consequently, anoxic conditions are established in which cyanobacteria consume their carbon stock by fermenting, causing the formation of alcohols and organic acids. In addition to the active secretion of metabolites or cell components released due to cell lysis, these compounds serve as nutrients for other inhabitants of the mat, such as methanotrophs or sulfate-reducing bacteria, which often live in close syntropy with other microorganisms. The cycling of carbon and other nutrients through the microbial components of such a community is termed the '**microbial loop**'. It is a model for a perfectly coordinated **self-sustaining microbial ecosystem**.

Biofilms resemble microbial mats, comprising a highly structured, self-organized 3D community of cells and EPS with chemical gradients developing within. These gradients strongly influence the given microbial niche and guide the physiological state of the biofilm inhabitants. Biofilms are usually much thinner and less diverse than microbial mats, but apart from that, the same constraints apply. Biofilm-forming cells do not necessarily need to attach to a static surface. Cells

Glossary

Biocatalyst: refers to a cell or an isolated enzyme that is applied as a catalyst to convert a given substrate to the respective product.
(Bio-)flocculation: occurs when living cells secrete extracellular polymeric substances, commonly known as EPS. Upon EPS secretion, cells form clusters and show physiological traits, as in biofilms.
Biofuels: fuels produced on the basis of biomass or by biocatalysts from renewable resources.
Biophotovoltaics (BPV): novel technology that applies photosynthetic microorganisms to capture solar energy and generate electrical current.
Bioremediation: process for the biological elimination of pollutants. In principle, these processes aim to break down existing contaminants as completely as possible by metabolizing them through microorganisms. However, processes aiming at the removal of contaminants, that is, by adsorption to a biological matrix, are also defined as bioremediation.
Carbon-negative production: carbon emissions during production and dispersal are less than the carbon bound in the product.
Carbon-neutral production: carbon emissions during production and dispersal equal the carbon bound in the product.
Extracellular polymeric substances (EPSs): refers to organic substances actively secreted by microbial cells forming biofilms. EPS mainly contains polysaccharides, proteins, lipids, and extracellular DNA.
Hydraulic retention time: the average time that liquid and soluble compounds stay in a reactor or tank. It is calculated by dividing the reactor's volume by the influent flow rate.
Light limitation: occurs when the available light falls below a threshold level at which photosynthesis is affected.
Lithic surfaces: refers to rock structures serving as an attachment surface for the microbial mat. It comprises endolithic (inside the rock via pores or cracks) and epilithic (on the rock surface) environments.
Microbial loop: describes a material cycle in the food web of marine organisms, in which dissolved organic carbon compounds are taken up by bacteria and passed on along the classic phytoplankton-zooplankton-nekton food chain.

often stick to each other, forming flocs or cell aggregates, also classified as biofilm (Figure 2) [43]. Most known microorganisms can grow biofilms, making them a ubiquitously distributed life form on our planet. The initial triggers leading to biofilm formation are strain-specific. Very often, cell motility is an essential factor in bringing the cells into proximity of the attachment surface, but there are also nonmotile organisms nonetheless capable of biofilm formation. Initial contact with the attachment surface is followed by cell adhesion. At this stage, the attachment is reversible and governed by fundamental physical interactions such as Coulomb and/or van der Waals forces. However, when the environmental factors fit, the cell becomes irreversibly anchored through chemical bonding mediated by adhesins. In this stage, multiple cellular processes are affected, and hundreds of genes become differently regulated, leading to a sessile phenotype that is actively secreting EPS and thereby starts to build micro- and later macrostructures on the surface and ensuring biofilm stability (Figure 2) [44,45]. A mature biofilm behaves, physically speaking, like a viscoelastic fluid in a semisteady state, where biofilm dispersal and regrowth are in equilibrium. Biofilm-forming microbes are extraordinarily robust and difficult to combat at this stage, leading to many problems, especially in the medical field. However, for biotechnological applications, this could be turned into an advantage. In the following sections, the potential of cyanobacteria as a workhorse in biotechnology is explained, and then the benefits and use of cyanobacterial biofilms are discussed in detail.

Cyanobacteria as solar cell factories

Apart from their essential role in ecology, cyanobacteria are explored as potential **microbial solar cell factories** to generate value-added compounds from CO_2 through tailored metabolic pathways. Current commercial production based on cyanobacteria is mostly limited to products obtained from biomass, such as pigments or the biomass itself used as food ingredients, as in the case of phycocyanin from *Arthrospira* [46,47]. However, many cyanobacteria are amenable to genetic modification via natural transformation for some strains or via conjugation for strains that are not naturally transformable. Tools such as vectors and parts for genetic engineering are available and can be applied to several well-characterized model strains. They also often work for newly isolated strains [48,49]. Through genetic engineering, native features of the cells may be deleted, new functions introduced, and metabolic flux rerouted and directed toward the preferred product. Cyanobacteria have been engineered to make a variety of products ranging from hydrogen gas to sugars, alcohols, alka(e)nes, and more complex secondary metabolites [2–6]. Furthermore, the plantlike intracellular environment of cyanobacteria, containing thylakoid membranes and reducing power supplied by photosynthesis, allows the expression of plant enzymes in a functionally beneficial context. Their ability to provide oxygen from photosynthesis and their bias toward NADPH over NADH makes them suitable for certain production pathways, including biotransformation processes [50,51]. These characteristics open up possibilities for future development of cyanobacteria as interesting platforms for chemical products, including **biofuels** and bioplastics, as well as pharmaceuticals and polymers.

Challenges and shortcomings

Although rapid development in engineering cyanobacteria into green cell factories has occurred during the past decade, insufficient space–time yields remain a major bottleneck on the way to energetically, environmentally, and economically sustainable cyanobacterial production systems [52]. However, one needs to be aware that knowledge of cyanobacterial metabolism is still far from the level of established workhorses such as *E. coli or Saccharomyces* sp. We are just beginning to understand the complexity of light-regulated metabolism. Metabolic models of cyanobacteria are being developed for several model strains, and, with more and more large-scale input data (e.g., omics), regulatory networks, light impact, and so forth, available, the use of models and metabolic systems biology to help guide future engineering efforts will increase [53,54].

Microbial mat(s): refers to a stationary microbial community comprising many different microbial species developing at phase boundaries (e.g., liquid to gas or liquid to solid). Cyanobacteria serve as primary producers and supply the mat with mainly organic carbon compounds synthesized from CO_2.
Microbial solar cell factories: engineered microorganisms harboring biosynthetic pathways streamlined to produce chemicals of interest using light as an energy source.
Phyllosphere: refers to a plant's total aboveground surface when considered a habitat for microbes.
Rhizosphere: refers to the total belowground surface of a plant when considered as a habitat for microbes.
Self-sustaining microbial ecosystem: refers to an ecosystem that produces all the resources needed to sustain all organisms indefinitely. There is no need for external inputs or maintenance from humans or other species.
Turnover number: describes the number of conversions that a particular catalyst can accelerate per time and catalytically active center. The turnover rate is a measure of a catalyst's efficiency.
Value-added compounds: summarizes industrially relevant chemicals with a higher value than the feedstock they are derived from.
White biotechnology: refers to the area of biotechnology focusing on using biological systems (isolated enzymes or cells) to produce chemicals relevant for the chemical industry.

Table 1. Cyanobacterial biofilm habitats, species, and application examples[a]

		Natural systems		Technical systems			
Environment		Phototrophs in natural biofilms	Refs	Example organism(s)	Applied field	Growth mode	Refs
Aquatic	Fresh water	*Aphanothece, Calothrix, Chakia, Chroococcus, Dichothrix, Entophysalis, Ewamiania, Gloeothece, Leptolyngbya, Synechocystis, Phormidium, Pseudoanabaena, Rivularia, Schizothrix, Scytonema, Synechocystis, Synechococcus*	[17–21]	*Leptolyngbya* sp. *Chroococcus*-like	Wastewater treatment	Mixotrophic biofilms	[22]
	Marine water	*Aphanothece, Anabaena, Calothrix, Chlorogloeopsis, Chroococcus, Fischerella, Gloeotrichia, Halothece, Hydrocoleum, Leptolyngbya, Lyngbya, Microcoleus, Nodosilinea, Nodularia, Oscillatoria, Phormidium, Pleurocapsa, Schizothrix, Scytonema, Stigonema, Symploca, Synechocystis, Synechococcus, Trichocoleus, Trichodesmium*	[17,23–25]	*Synechococcus* sp.	Biophotovoltaics	Axenic biofilms	[26]
Terrestrial	**Lithic surfaces**	*Hassallia, Tolypothrix, Scytonema, Lyngbya, Calothrix, Aulosira, Nostoc, Camptylonema, Dichothrix, Chlorogloeopsis, Westiellopsis, Leptolyngbya, Phormidium, Rexia, Symphyonemopsis, Scytonema, Haloleptolyngbya, Anabaena, Gloeobacter, Thermosynechococcus, Pseudanabaena*	[27]	*Nostoc punctiforme Tolypothrix* sp.	Hydrogen	Mixotrophic biofilms	[2,12]
	Soil/rhizosphere/**phyllosphere**	*Anabaena, Calothrix, Gloeotrichia, Nostoc, Trichormus*	[19,28]	*Nostoc muscorum*	Heavy metal removal for application in the bioremediation area	Axenic biofilms	[29]
Extreme conditions	Ice glaciers	*Chroococcales, Dichothrix, Leptolyngbya, Nostoc Tolypothrix*-like, *Tychonema*-like,	[30]	*Leptolyngbya* sp.	EPS[b] with antioxidant activity and high water-holding capacity for applications in the food and pharmaceutical sectors	Axenic suspended culture	[31]
	Hot lakes	*Thermosynechococcus, Oscillatoria, Fischerella, Geitlerinema, Gloeomargarita, Halothece, Leptolyngbya, Synechococcus-like*	[32–34]				

Table 1. (continued)

		Natural systems		Technical systems			
Environment		Phototrophs in natural biofilms	Refs	Example organism(s)	Applied field	Growth mode	Refs
	Deserts (including lithic surfaces in deserts)	*Albertania, Chroococcidiopsis*, *Gloeocapsopsis*, *Phormidium*, *Microcoleus*, *Nostoc*, *Leptolyngbya, Scytonema, Calothrix, Oscillatoriales, Fischerella*, *Trichocoleus, Trichormus, Nodosilinea, Pseudoacaryochloris, Pseudophormidium, Schizothrix, Tolypothrix*	[35,36]	*Chroococcidiopsis* sp.	Biocrude oil production	Self-settling suspended culture	[37]

[a]In the middle column, the most abundant cyanobacterial species living in the respective habitat are listed. The right column highlights organisms from this selection that have already been applied in technical systems.
[b]Main EPS components: mannose (35%), arabinose (24%), glucose (15%), rhamnose (2%), and one uronic acid (8%). For more details, please refer to [31].

There are two main challenges: (i) product yield per cell concerning carbon and energy flow needs to become significantly enhanced, with, in the ideal case, the cells acting essentially as biocatalysts, capturing the energy and carbon dioxide via photosynthesis and CBB cycle and funneling these resources primarily into product formation; and (ii) cultivation systems for phototrophic organisms need to be developed to relieve the problem of insufficient cell densities due to light limitation. Both of these challenges may be addressed by cultivating the cells in a state where growth is limited, but resources in the form of light and carbon are abundant, using immobilized or biofilm-forming cells in reactors specifically designed for this purpose (Box 1), which is discussed in the next section.

Cyanobacterial biofilms in biotechnological applications

Microbes growing in biofilms exhibit extraordinary robustness to a wide spectrum of environmental stress factors. The reasons for this are manifold, with the protective EPS layer playing an important part. Furthermore, biofilms are composed of living cells that constantly adapt to environmental changes and regenerate themselves (Figure 3).

Due to their inherent stability and robustness, biofilms have an endless **turnover number** in principle. They are suitable for continuous processes and long-term application targets such as bioremediation or agriculture and with an expanding array of tools for assessing these structures, designing biofilms tailored to specific applications is becoming increasingly easier (Box 2). In the following section, current examples of cyanobacterial biofilm applications are reviewed.

As outlined earlier, cyanobacteria are promising candidates to be used as cell factories for producing high-value chemicals, biofuels, and bioplastics in **carbon-neutral production** processes. Still, they also get increasing attention for their role in wastewater treatment, bioremediation, and agriculture (Figure 2). These approaches often use mixed consortia biofilms comprising cyanobacteria coupled with chemo-organo-heterotrophic partners. Which of the partners dominates the biofilm depends on the cultivation conditions. The cyanobacteria will dominate the biofilm when only an inorganic carbon source is available. Cyanobacteria supply the culture with oxygen, often the limiting factor in biofilm systems composed of aerobic respiration strains. In cyanobacterial biofilm systems, oxygen can accumulate in the EPS to supersaturation levels, which might even become toxic [55]. However, in systems where oxygen-consuming reactions are essential, this is an elegant

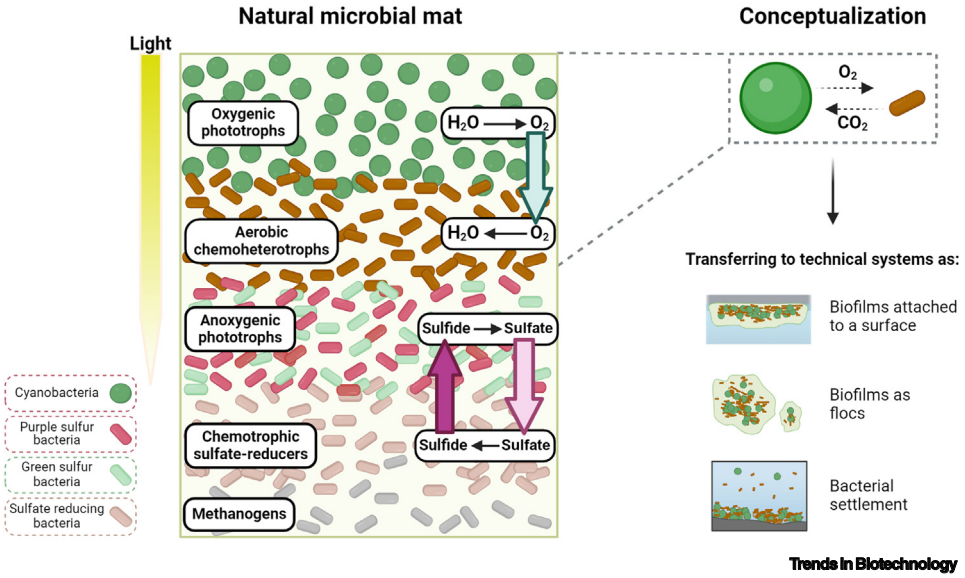

Figure 1. An illustration of the complex system behind natural microbial mats (left) and examples of possible process concepts derived from them (right). The main drivers that control the development of the individual microbial layers are light and oxygen, which penetrate the mat from the upper layer and become increasingly limited in the deeper areas. Organic carbon compounds are built up from CO_2 by the photoautotrophic members of the community and released mainly due to fermentation or cell lysis. Cyanobacteria may be applied as surface-attached film, or flocs, supplying oxygen, carbon, and other nutrients for the aerobic, heterotroph growing partners. Furthermore, induced settlement of the microbes is used for cell harvest in some cases [37].

way of supplying sufficient oxygen to all biofilm layers [56,57] (see the section 'Cyanobiofilms in wastewater treatment and bioremediation' later). Furthermore, examples show that the chemo-organo-heterotrophic partner is necessary for biofilm establishment and stability, as was recently demonstrated for the conversion of cyclohexane to cyclohexanol [58] (see next section).

Cyanobacterial biofilms in biotechnology

When applying cyanobacterial biofilms for production purposes, often defined mixed cultures are used. Cyanobacteria are coupled to biocatalytic workhorses such as *Pseudomonas sp.* in a syntropic community. This approach was successfully applied for the biotransformation of cyclohexane to cyclohexanol. The biofilm culture was much more robust than free-living cells in the presence of the organic solvent cyclohexane and showed a stable conversion activity for over 1 month [58]. Furthermore, it was demonstrated that biofilm formation by *Synechocystis* PCC 6803 [58] and other cyanobacteria [12] was strongly influenced by chemo-organotroph *Pseudomonas* sp. In the presence of this organism, various cyanobacteria showed a much better surface coverage and higher stability [12]. Biofilms are extraordinarily well suited for gaseous products because the constant gas product stream can be easily collected and is highly pure. Ethylene was produced using artificial *Synechocystis sp.* PCC 6803 biofilms carrying a recombinant ethylene-forming enzyme from *Pseudomonas syringae* over 38 days with a yield of 822 ml m^{-2} and a 3.5-fold higher ethylene conversion efficiency than cell suspensions [59]. In addition to carbon-based fuels, some cyanobacteria can produce molecular hydrogen, an energy-dense fuel and important industrial gas [60]. Cyanobacteria can produce H_2 either via biophotolysis, using the activity of a hydrogenase coupled to the photosynthetic electron transport chain [61], or as a side product of nitrogen fixation. Recently, long-term continuous hydrogen production over more than 10 weeks was demonstrated in biofilms containing nitrogen-fixing *Nostoc punctiforme* [2] or *Tolypothrix* spp. [12] (Table 1).

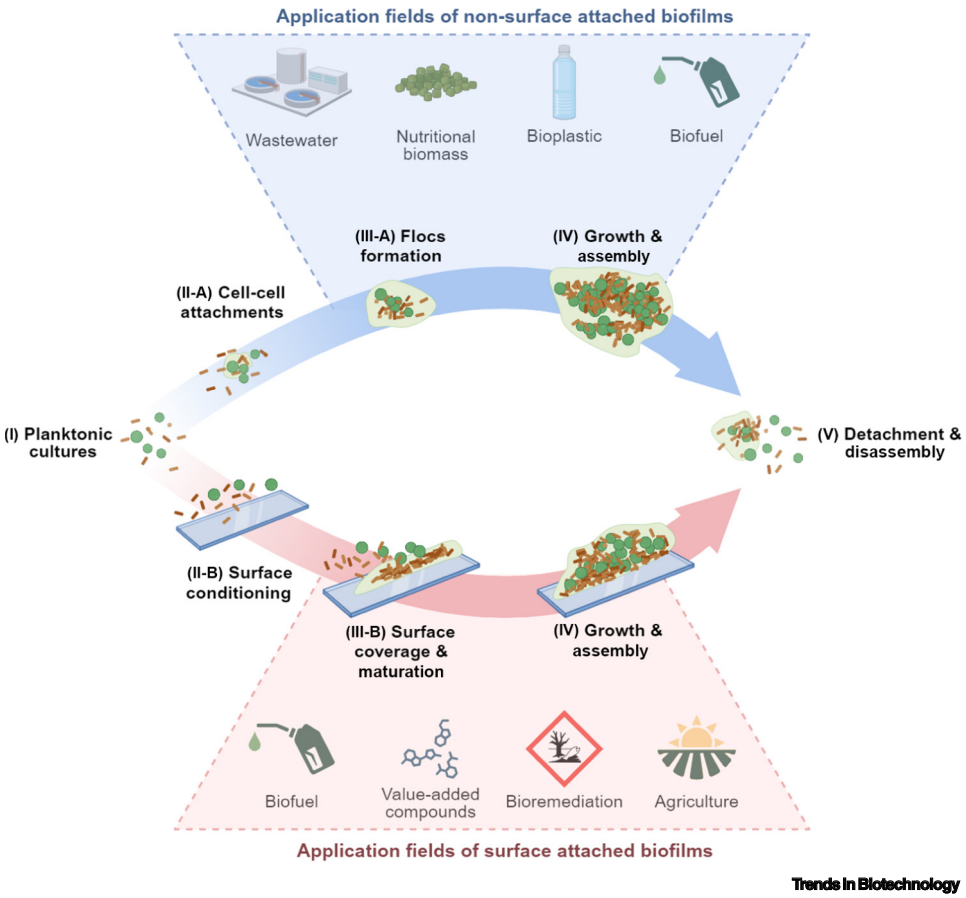

Figure 2. Steps involved in surface- and non-surface attached biofilm formation and possible application fields. Biofilm formation can be divided into five stages. (I) Planktonic cells reside at the beginning of the process, followed by cell-to-cell attachment and the beginning of extracellular polymeric substance (EPS) production (II-A) that leads to flocs formation (III-A) in the case of non-surface attached biofilms. In the case of surface-attached biofilms, surface conditioning occurs because of the interaction of the surface with the surrounding liquid before cells attach reversibly to the respective surface (II-B). Cell attachment becomes irreversible in further development, EPS is produced, and the biofilm develops microcolonies (III-B). These continue growing to a mature 3D structure, which does not increase in thickness at one point anymore. Freshly growing biofilm and biomass leaving the community are balanced (IV). Because of specific triggers, such as nutrient limitation, biofilms can disintegrate at one point (V).

Besides using the cyanobacterial cell as a biocatalyst, the EPS produced by cyanobacterial biofilms is becoming increasingly attractive as a key product. EPSs have water-holding and oil-holding capacity and are known to adsorb heavy metals (see section on bioremediation below). Various cyanobacteria produce an EPS rich in sulfated polysaccharides, previously only reported for animals and higher algae. Depending on the organism of origin, they are known under names such as 'synechan' (for *Synechocystis* sp.) [48], 'spirulan' (for *Arthrospira platensis*) [62], or 'cyanoflan' (for *Cyanothece* sp.) [63]. Sulfated polysaccharides are known for health-beneficial nutraceutical effects such as antioxidant, antiallergic, anti-HIV, anticancer, and anticoagulant activities, making them potential functional nutraceuticals. Thermophilic strains are exciting as their EPS possesses thermostable ingredients such as those recently shown for *Leptolyngbya* sp. IkmLPT16 (Table 1) [31]. First reports on modulating EPS composition and, thereby, EPS function by either modulating the cultivation conditions [64] or genetic engineering [65] are emerging.

Box 1. Biofilm photobioreactors

The growth of phototrophic organisms in technical systems is mainly limited by light availability. In suspended cultures, cell densities usually do not exceed 8 $g_{cell\ dry\ weight\ (CDW)}$/l, which is approximately ten times less than what chemotrophic growing cultures reach. Growing phototrophic organisms as biofilms may provide a solution to this shortcoming as in this format, cell densities are significantly increased (up to 60 g_{CDW}/l have been reported) [12]. While there is a variety of photobioreactors (PBRs) for suspended cultures, only a few systems for growing phototrophic biofilms are available, mostly as adaptations of PBRs. A major constraint is the available surface area the organisms need for attachment, which, in the case of phototrophic organisms, needs to be accessible for light. The large biomass content of the biofilm allows less energy-intensive operation and biomass harvest. Biofilm PBRs are usually operated in a (semi-)continuous mode. Thus, media recycling loops must be integrated to reduce medium consumption [85].

Cyanobacterial biofilms are cultivated using continuous and periodic PBRs, including drip-flow, flat panel, and tubular reactors, as well as systems containing a porous attachment surface allowing a 3D seeding process such as in twin-layer solid-state reactors (Figure I). Drip-flow reactors (Figure IA) incorporate flat coupons as attachment surfaces and supply the medium in a drip-and-flow fashion [86]. Cultured biofilms are typically dense and resemble naturally occurring biofilms, where fluid flows along the solid phase–air interphase. Tubular PBRs (Figure IB) are composed of rows of translucent tubes connected by manifolds. Inspired by tubular PBRs, a capillary biofilm reactor offers a superior surface-to-volume ratio of over 1000 m^{-1} [58]. The low tube diameter reduces the light path and maximal light supply. Emersed PBR (Figure IC) offers better light availability due to glass rods transferring the exterior light directly into the system [87]. Newly developed low-weight multiskin sheet emersed PBR can reduce water consumption due to aerosol use. Flat panel PBRs (Figure ID) are transparent tanks using airflow to mix the contents inside the reactor. Light supply is achieved using bilayer, vertical flat panels with a large surface area and small diameter, preventing self-shading [88]. Finally, twin-layer solid-state PBRs (Figure IE) have two types of layers: a source layer on one side with culture media being transported by gravitational force and a substrate layer serving as an attachment surface for the organisms on both sides of the source layer. The resulting open system makes it possible to efficiently extract adherent phototrophs from their growth medium [89].

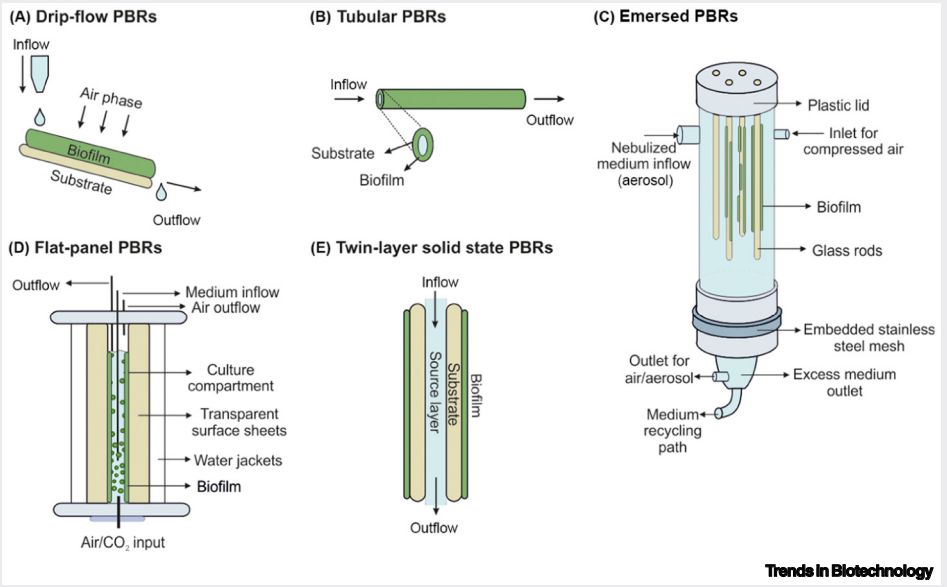

Figure I. Schematic representation of different photobioreactors (PBRs). (A) Drip-flow PBRs, (B) tubular PBRs, (C) emersed PBRs, (D) flat panel PBRs, and (E) twin-layer solid-state PBRs.

In biofuels or biomass production, biofilm formation relieves one of the major disadvantages in this area: the high costs connected to growth, harvest, and the massive amount of necessary fresh water. **Bioflocculation** or strains directly growing as floc, floating mats, or biofilms like filamentous cyanobacteria such as *Chroococcidiopsis* sp., *Tolypothrix* sp., *Phormidium autumnale*, *Phormidium murrayi*, and *Planktothrix* sp. are of interest as they facilitate cell harvest and reduce water consumption (Table 1) [12,37,66].

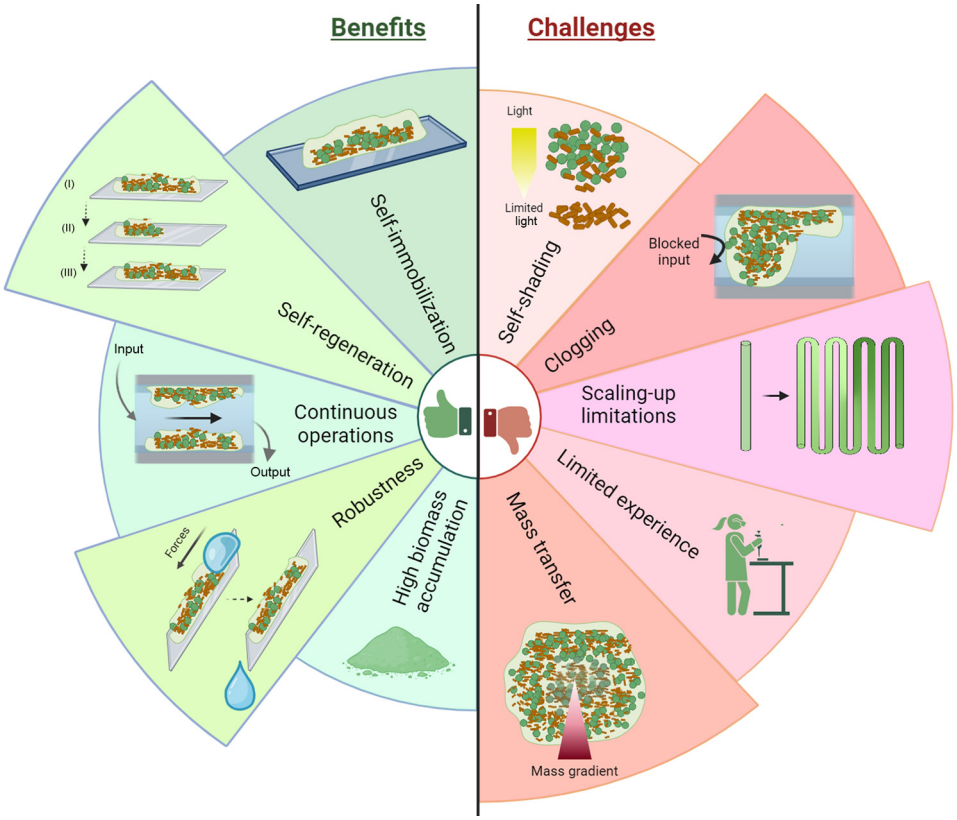

Figure 3. Key benefits and pending challenges for establishing biofilms as a production concept in biotechnology. Apart from numerous benefits connected to cell robustness, constant regeneration and adaptation, and high biomass, several challenges must be addressed before the biofilm process concept can be implemented broadly in the industry. Self-shading leading to light limitation and clogging are potential issues in biofilm cultivation, depending on the reactor system. One of the major bottlenecks is missing reactor technology. Current developments are based on established bioreactors mainly used to cultivate suspended cell cultures and chemoheterotrophic organisms. New developments allowing large-scale cultivation of cyanobacterial biofilms are urgently needed.

Cyanobacterial biofilms in electrobiotechnology

In the young field of **biophotovoltaics (BPV)**, the electrons liberated in the water-splitting reaction of cyanobacteria are transferred to an anode of an electrochemical cell either directly or indirectly via mediator molecules. Direct electron transfer can be achieved if organisms grow as biofilms directly on the anode, benefiting from the close cell-to-anode contact [67]. From the anode, electrons are transferred to the cathode, where they are either used to reduce protons and generate a pure hydrogen product stream or fuel other reduction reactions [67]. Such processes can also run in the dark, yielding dark currents, as cyanobacteria feed on storage compounds such as glycogen produced during the daytime [68]. In a recent study using marine phototrophic strains growing in biofilms on anodes, it was reported that the chlorophyll-*a* (chl-*a*) content correlated with the power density. The cyanobacterium *Synechococcus* UMACC 371 reached a power density of 0.065 ± 0.015 mW m^{-2} with a chl-*a* content of 0.065 mW m^{-2} (Table 1) [26]. Using marine strains enables higher salt concentrations with increased conductivity, leading to high current densities [69]. Another exciting development is supercapacitive microliter-scale photosynthetic microbial fuel cells to attain stable high power and current density (38 μW cm^{-2} and 120 μA cm^{-2}). The proposed technique is

based on a 3D double-functional bioanode concurrently exhibiting bioelectrocatalytic energy harvesting and charge storing [70].

Using a mixed-species biofilm comprising microalgae and cyanobacteria, a maximum photocurrent of up to 20 μA cm^{-3} was reported [71]. In the respective study, mediators such as *p*-benzoquinone and hexacyanoferrate(III), as well as enzymes for the neutralization of reactive oxygen species (ROS), have been added for stable current production. The power output can be optimized by including other additives such as NH_4Cl, which increases the power density produced in a BPV system with *Synechocystis* sp. PCC 6803 by 40.5-fold to 148.27 mW m^{-2}[72]. Other options are elucidating different electrode designs such as structured indium-tin-oxide electrodes with nano- or micropores supplying a high surface area for biofilm formation of *N. punctiforme* or *Synechocystis* sp. PCC 6803. The peak photocurrent was increased approximately by a factor of 300 compared with nonporous electrodes [73]. Additionally, the coating of electrodes with conductive materials is used for the expansion of BPV systems beyond power generation toward the detection of atrazine-class pesticides [74]. Further applications can include the cathode reaction to form hydrogen gas [75].

Cyanobiofilms in wastewater treatment and bioremediation

Biofilms are an established technology in wastewater treatment and bioremediation. Biofilms composed mainly of chemoheterotrophic organisms are grown on supportive structures such as Raschig rings and are applied to reduce the organic carbon load in wastewater. In the 1960s, several studies started to investigate the effect of combining heterotrophic growing bacteria with photoautotrophic microalgae in so-called high-rate algal ponds to solve the problem of oxygen limitation. By concomitantly cultivating microalgae and heterotrophs, the phototrophic organisms provide oxygen for the respiring part of the microbial community. So-called microalgal-bacterial aggregates (MABAs; also known as bioflocs, granular activated algae, photogranules, or bioflocculent algal-bacterial biomass) are now a 'hot topic' in wastewater treatment and are mainly applied for wastewater with a high carbon load. MABAs are aggregates or flocs of chemotrophic and phototrophic microbes, mainly cyanobacteria [56,57]. A *Leptolyngbya* sp. dominated biofilm consortium was applied for the post-treatment of activated sludge processed brewery wastewater and was shown to be better at chemical oxygen demand (COD) reduction than suspended cultures (Table 1) [22,57]. Recently, photogranules have been applied to wastewater with a low carbon load and low **hydraulic retention time** and very effectively reduced the carbon, phosphorous, and nitrogen load below the limits set by the European Water Framework Directive [76]. Apart from reducing the nutrient load in wastewater, biofilms (or MABAs) also exhibit excellent heavy metal adsorption capabilities, mainly due to their EPS [77]. Biosorption is discussed as a promising strategy for metal removal, recovery, and subsequent reuse of heavy metal pollutants. Especially bivalent metals are well absorbed. A recent study examined marine cyanobacteria for continuous EPS production and heavy metal removal. All tested organisms showed very high affinity toward Cu, but also Ni and Zn were removed in ample amounts [78]. A similar observation was reported for EPS from the thermophilic cyanobacterium *Gloeocapsa gelatinosa* [79] and *Nostoc muscorum* biofilms, removing Cd(II) from aqueous solutions (Table 1) [29]. EPS produced by *Synechocystis* PCC 683 biofilms was reported to adsorb and transform arsenic pollutants [80], whereas a biocrust of *Leptolyngbya sp.* XZMQ was used for arsenic removal directly from farmland soil [81].

Cyanobacterial biofilms in agriculture

In agriculture, cyanobacterial applications focus on the **rhizosphere**. Cyanobacterial biofilms are discussed as natural fertilizers due to the ability of many species to fix atmospheric nitrogen. Furthermore, their extracellular metabolites play an important role as chemoattractant, promoting

plant–microbe interactions, adding to their importance as inoculants in agriculture [82]. Hydroponic plant cultivation systems have been established, where plants are fixed on medium surfaces in special containers while the roots are growing into the liquid phase. The roots serve as a biofilm attachment surface for nitrogen-fixing cyanobacteria, and thereby close interaction of N-supplier (biofilm) and N-consumer (plant) is ensured [7].

Additionally, the EPS excreted by cyanobiofilms stabilizes the soil surface, enhances the water-binding capacity of soil [83], and provides a habitat rich in nutrients for various heterotrophic organisms and a variety of agronomically relevant bacteria. These include *Pseudomonas*, *Azotobacter*, *Rhizobium*, and fungi (e.g., *Trichoderma*), which are identified as organisms living near or in the cyanobacterial biofilm. Several studies have demonstrated the beneficial impact of such biofilms in terms of growth in legumes, cash crops, vegetables, and cereals. Upon inoculation, soil parameters such as N, P, S, and C content and other growth-regulating substances, such as amino acids and vitamins, are increased, leading to faster plant growth, enhanced grain weight, and significantly reduced artificial fertilizer [8,84].

Concluding remarks and future directions

In the areas of bioremediation, wastewater treatment, and agriculture, cyanobacterial biofilms are in the process of becoming established technologies. However, for productive biotechnology, this is different as some major challenges are persisting. Although cyanobacteria hold great promise for carbon-neutral or even **carbon-negative production** systems, they have only played a small role in biotechnology so far. Medium- to large-scale applications are limited to biomass production or high-priced specialties such as pigments. Space–time yields need to be significantly improved to raise cyanobacterial production systems to the next level and make them energetically and economically viable. In an ideal system, the photoautotrophic microorganism should function as a solar cell factory, transforming light energy, carbon dioxide, and water into valuable raw materials for chemical production while reducing its investment into biomass to a minimum once it is established (zero-growth, maximal productivity). However, a profound understanding of the cyanobacterial cell and its complex light-guided regulation is in its infancy, and there is a persisting knowledge gap compared with established workhorses such as *E. coli* or *Saccharomyces*. Engineering cyanobacterial host strains is often tricky and time-consuming. Nevertheless, the field of cyanobacterial molecular biology, tool development, and successful engineering strategies is rapidly evolving, and it is only a question of time before this bottleneck is passed.

Technically, novel reactor systems are needed for genuinely scalable phototrophic bioprocesses. Reactors developed explicitly for phototrophic biofilms are essential to support cyanobacterial biofilm development, prevent clogging, and enable scale-up. Although several reactor types for cultivating cyanobacterial biofilms are available or being developed (Box 1), this area still needs a real breakthrough. Most systems have been demonstrated only on a small scale so far. Moreover, these reactors must control biofilm thickness to fulfill the 'zero-growth, maximal productivity' concept. Thin films are superior to thick biofilms with a lot of EPS as these will experience severe mass transfer issues and harbor a significant fraction of inactive cells in the middle or lower regions of the film.

Several outstanding questions remain for future research to pave the way for the broad application of cyanobacterial biofilms (see Outstanding questions).

(i) Light transfer into cyanobacterial cultures is challenging for cell suspension and biofilm cultures. This results in either low cell densities or flat, 2D systems with meager inner volume, necessitating a vast cultivation area. Innovative technologies are necessary to transfer light into

Outstanding questions

How can we solve the issue of light transfer into 3D systems?

Does the genetic stability of biofilm systems affect their overall performance in continuous systems?

How can we gain a better understanding of the internal mechanisms in a biofilm to make models of biofilm systems possible?

Which cyanobacterial strains are suited to be developed into biofilm workhorses?

3D structures or create self-illuminating materials with high surface area as attachment surfaces for the biofilm-forming cyanobacteria. This would allow reactor systems to have an improved area footprint.

(ii) The production strains must be genetically stable for long-term operation and production in a biofilm system. In the case of a mixed population, the consortium must be maintained in the desired proportions. It is still unknown how such stability issues may affect overall performance in a continuous system and how genetic drift will influence long-term productivity.

(iii) Modeling will be an indispensable tool for developing biofilm applications further and understanding already available systems. However, the internal mechanisms of cyanobacterial biofilms are poorly understood, and the system can be regarded as a black box. More knowledge on biofilm formation, development, and dynamics in reactor systems is needed to

Box 2. Methods for assessing cyanobacterial biofilm characteristics/performance

Biofilms are 3D complex structures with an intrinsic heterogeneity, which makes analysis challenging. Biofilm structure can be assessed via multiple imaging techniques, covering resolution and penetration depth from the nanometer to millimeter scale (Figure IA). While early studies of biofilms used simple light microscopy and various staining methods to visualize biofilm EPS (e.g., Alcian blue) [12], nowadays fluorescence microscopy and confocal laser scanning microscopy (CLSM) are commonly used as a noninvasive, high-resolution, live-cell imaging technique to unravel biofilm architectures, cell structures, and functions [39,90,91]. Targets such as nucleic acids or EPS compounds can be tagged specifically with fluorescence labels, or the autofluorescence of the photosynthesis pigments can be used without destroying the native biofilm structure during sample preparation. Optical coherence tomography (OCT) [92] and nuclear magnetic resonance (NMR) imaging [93] bridge the gap to the macro scale and provide excellent penetration depth. Other imaging techniques such as scanning electron microscopy (SEM) or transmission electron microscopy (TEM) [94], or helium-ion microscopy [90] provide a much higher resolution down to the nanometer scale but require special sample preparation, which also affects the native structure of the biofilms. Other technologies such as Raman microscopy [94], nanoscale secondary ion mass spectrometry (nanoSIMS) [95], and time-of-flight secondary ion mass spectrometry (TOF-SIMS) [96] provide information on the chemical composition of the biofilms, although especially the latter two require highly sophisticated and costly instrumentation.

On the cellular level, multiple omic strategies have been applied to characterize biofilm physiology on all intracellular organization levels (Figure IB–D) [85,86]. Because next-generation sequencing methods have become more available, obtaining transcriptome data of cyanobacterial biofilms is now feasible [97]. Capturing changes in the proteome [90] and the corresponding metabolome allows conclusions on the function of metabolic pathways and the fluxes within a cell.

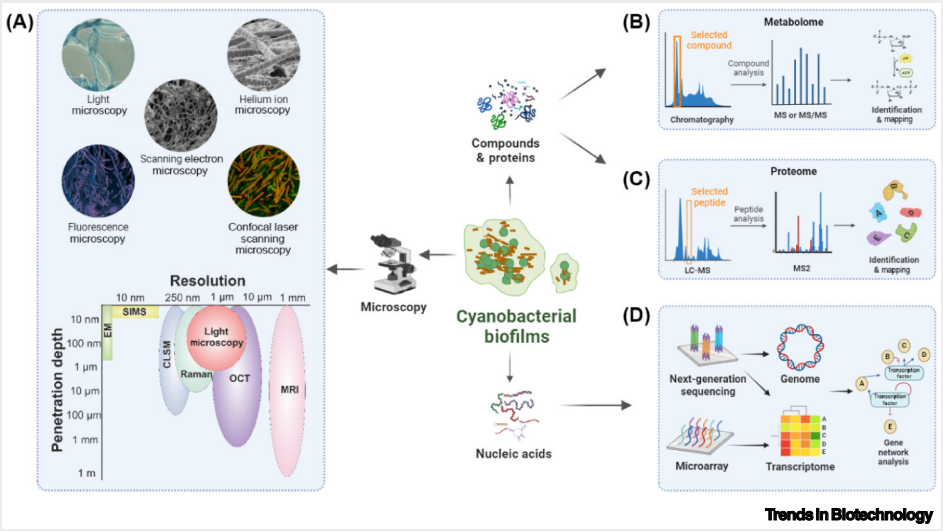

Figure I. Available biofilm analysis toolbox. Abbreviations: CLSM, confocal laser scanning microscopy; EM, electron microscopy; LC, liquid chromatography; OCT, optical coherence tomography; MRI, magnetic resonance imaging; MS, mass spectrometry; SIMS, secondary ion mass spectrometry.

construct models to support research and development of biofilm-specific applications. This includes knowledge of cell activity within the film, internal exchanges between cells, and exchange with the environment as functions of time and location within the film.

(iv) Different strains of cyanobacteria have very different lifestyles. Some unicellular model strains are more accessible to engineer as production hosts than others, which are more suitable for cultivation in biofilm reactors. Thus, not all cyanobacterial strains will be equally suited to function as biofilm production workhorses, and it may be necessary to engineer strains not only for producing target compounds but also to improve their biofilm attachment and stability properties. Further studies are required to determine which strains are the best candidates to develop for applications and how they can be engineered for this purpose.

This review highlights significant developments in cyanobacterial biofilms for application purposes. Pending challenges persist on both biological and technological levels. New light transfer technologies, learning more about the internal mechanisms of the biofilms in reactors, and understanding the selected host cells and how we can improve their properties for the scenario of zero growth and maximal productivity at a large scale are all major challenges that need to be solved to implement photobiofilms on a broad scale. We expect such development to unlock new potential for extended cultivation and enhanced space–time yields and to prove pivotal for developing efficient cyanobacterial production systems.

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Declaration of interests

The authors declare no competing interests.

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