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Review

Exopolysaccharides from Cyanobacteria: Strategies for Bioprocess Development

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Abstract: Cyanobacteria have the potential to become an industrially sustainable source of functional biopolymers. Their exopolysaccharides (EPS) harbor chemical complexity, which predicts bioactive potential. Although some are reported to excrete conspicuous amounts of polysaccharides, others are still to be discovered. The production of this strain-specific trait can promote carbon neutrality while its intrinsic location can potentially reduce downstream processing costs. To develop an EPS cyanobacterial bioprocess (Cyano-EPS) three steps were explored: the selection of the cyanobacterial host; optimization of production parameters; downstream processing. Studying the production parameters allow us to understand and optimize their response in terms of growth and EPS production though many times it was found divergent. Although the extraction of EPS can be achieved with a certain degree of simplicity, the purification and isolation steps demand experience. In this review, we gathered relevant research on EPS with a focus on bioprocess development. Challenges and strategies to overcome possible drawbacks are highlighted.

Keywords: cyanobacteria; exopolysaccharides; bioprocess

1. Introduction

Polysaccharides are complex polymeric carbohydrates that have a huge impact on the industry as hydrocolloids, bio-sourced materials, biological agents, among others [1]. These biopolymers are often used as raw materials in many industries and have a chemical diversity that can go from linear homopolymers to 3D structures of heteropolymers. The majority of commercialized polysaccharides are extracted from plants, seaweeds, and microorganisms [2]. Polysaccharides excreted from microbial sources hold a wide range of functionalities and are highlighted for easier downstream when compared to other natural sources [2,3]; however, the costs of production, mainly for carbon source, constant oxygenation, and intense energy demand, limit the generalized application of these technology [4]. Eco-friendlier cellular factories such as phototrophic organisms have been highlighted to raise the sustainability of the whole bioprocess [5]. Cyanobacteria can be light-powered, consume inorganic carbon to produce oxygen, and even fix nitrogen under diazotrophic conditions [6]. They are widely known for being rich in protein and a prolific source of secondary metabolites [7]. Nonetheless, their relative low carbohydrate content can be over-exceeded in the forms of sugars and polysaccharides when another relevant nutrient is missing for the production of proteins, nucleic acids, and other

biomolecules. This is commonly defined as photosynthesis "carbon overflow" and is similar to lipid accumulation in microalgae [8]. When produced in considerable amounts, the extracellular polysaccharides or exopolysaccharides (EPS) can be combined with proteins (proteoglycans) and lipids (glycolipids) [9]. There is no current consensus in regards to EPS classification in cyanobacteria [10]. Though many authors agree they should be classified into two main groups according to their location: (1) cell-bound polysaccharides (CPS) comprising slime, sheaths, and "capsular"; (2) released polysaccharides (RPS) [4]. A rule of thumb EPS from cyanobacteria are heteropolysaccharides of anionic nature due to uronic acids and are soaked in neutral sugars. In a nutshell, cyanobacterial EPS holds higher complexity among microbes [6]. Physico-chemical properties of cyanobacterial EPS have shown promising performances suitable for the industry as gums, bioflocculants, soil conditioners, biolubrificants, and biosorbents [11–13]. The presence of non-carbohydrate substituents is proven to act as biological weapons, showing anti-viral, anti-microbial, anti-tumoral activity [14–17]. Their potential has been already explored as drug delivery technology [18,19].

Cyanobacterial EPS, from the bioprocess point of view, is seen as a by-product of biomass and/or metabolite production such as proteins and to a lesser extent lipid [10]. The slimy texture of culture medium after EPS production hinders handling while structure elucidation and productivities gave a tough reputation to these classes of polysaccharides within the industrial sector; however, the uniqueness of the few resolved structures has opened markets to these polysaccharides.

The overlooked research of cyanobacterial EPS is dominated by their generic production and extraction which is then used to provide insights on EPS partial characterization, physico-chemical, and/or bioactive properties [10]. There is a lot of anticipation regarding their application as metal bioremediation [20] and soil restoration [21]; however, this review is focused on the bioprocess development, for wild type strains in particular. Within this scope, we approached the EPS cyanobacterial in terms of their chemical-diversity and what techniques were used for strain selection and EPS production. Main developments and strategies are outlined and proposed, respectively.

2. EPS from Cyanobacteria

2.1. Ecology

Cyanobacteria are a very diverse taxonomic group that can be found both in unicellular or multicellular forms of coccoid and/or filamentous [7]. These prokaryotic organisms have a bacterial like cellular envelope. They are structurally similar to Gram-negative bacteria, although some features and thickness of peptidoglycan resemble Gram-positive bacteria. In nature, cyanobacterial EPS can play a variety of functions such as adhesive, structural, protection against abiotic stress, bio weathering processes, gliding motility, and nutrient repositories in phototrophic biofilms or biological soil crusts [22–24]. The excretion of EPS, and in particular CPS, can occur via a junctional pore complex (JPC). The quantity and compactness of carbohydrates can help to sort the different types of CPS. Sheaths and capsules are more compact which aim to protect the cellular environment, from abiotic or biotic stresses, while the slime excretions are less compact and allow the cyanobacteria to have gliding motility. Some CPS may also contain aromatic pigments, like scytonemin or oligosaccharide mycosporine-like amino acids. These molecules which absorb UV light can be found in cyanobacteria located in highly exposed sunlight areas [25]. Interestingly, the RPS fraction is less noted in nature, whereas only under laboratory conditions can be easily detected and quantified [26].

2.2. Chemodiversity

The EPS in cyanobacteria is part of a complex network of extra polymeric substances, and it can also comprise proteins, nucleic acids, lipids, and secondary metabolites [10]. More than 13 different monosaccharides have been identified from C5 to C6 and underpinned on 40–50 glycosidic bonds tightly correlated to polysaccharide flexibility [9]. Glucose is the most common monosaccharide, however, some EPS strains were found to contain other dominant monosaccharides such as rhamnose,

xylose, arabinose, fucose, mannose, and uronic acids [27]. The latter have an exclusive presence in the cyanobacteria, being identified with a frequency of one or two units. Structurally monosaccharides can be grouped by their form: hexoses (glucose, galactose, mannose, and fructose); pentoses (ribose, xylose, and arabinose); deoxyhexoses (fucose, rhamnose, and methyl rhamnose); acidic hexoses (glucuronic and galacturonic acid). The EPS of *Arthrospira platensis* strain MMG-9 exhibited rich EPS diversity, the CPS fraction the most diverse (fucose, galactose, glucose, mannose, rhamnose, ribose, and xylose) [28]. Methyl, pyruvyl, and succinyl groups can be present as well as sulfate groups, which are only found in archaea and eukaryotes. Additional types of monosaccharides such as N-acetyl glucosamine, 2,3-O-methyl rhamnose, 3-O-methyl rhamnose, 4-O-methyl rhamnose, and 3-O-methyl glucose are also reported [29,30]. At the macromolecular scale, these polymers are characterized by high molecular weight, which can range from kDa to MDa, whereas more than 75% of those characterized are heteropolymers [6]. The physico-chemical role of these building blocks is extremely rich. Although some exhibit hydrophobic character, which promotes adhesion to solid surfaces, others exhibit hydrophilic character sticking to minerals, nutrients, and water molecules. Notwithstanding, the combination of both moieties can promote an amphiphilic character allowing cyanobacteria to react in different ways to the surrounding environment [30]. Cyanoflan, an RPS isolated from the marine *Cyanothece* sp. PCC 0010 has a high intrinsic viscosity and emulsifying activity in aqueous solutions [31]. Cyanobacteria constitute a prolific source of EPSs with physico-chemical properties. As a consequence of these natural complexities, a pitfall for structural elucidation burdens the number of available structures [9]. Non-exhaustive data are shown in Table 1.

Table 1. Isolated exopolysaccharides (EPS) from cyanobacteria.

Strain	EPS Fraction	Compositional Monosaccharides	Non-Carbohydrates Substituents	EPS Characteristic	Reference
Aphanothece sacrum	CPS	Glc, Gal, Man, Xyl, Rha, Fuc, GalA, GlcA	sulfate and carboxyl groups	anti-inflammatory; anti-allergic; adsorption of metal ions; liquid crystallization	[32]
Cyanothece PCC 0010	RPS	Man, Glc, uronic acids, Gal, Xyl, Rha, Fuc Ara	sulfate and acetate groups, peptides	thermostable, amorphous, pseudoplastic behavior	[31]
Cyanothece sp. 113	CPS	D-Glc		nd†	[33]
Cyanothece sp. ATCC 51142	RPS	2-C-Me-Glc, Ido-2-C-Carboxylic acid, 2-deoxy-Ido	calcium; sulfate groups	gel formation; adsorption of metal ions	[34]
Nostoc commune DRH-1	RPS	dXyl, dGlc, dGal, dRib, GlcA, Man		nd†	[35]
Nostoc flagelliforme	CPS	Glu, Gal, Xyl, Man, GlcA		anti-viral activity, negative antithrombin activity	[36]
Nostoc insulare	RPS	Ara, 3-O-Methyl-Ara, Glc, GlcA		Nd†	[37]
Nostoc sphaeroids kütz	CPS	Man, Glc, Xyl, Gal, GlcA		immunological activity	[38]
Spirulina platensis	CPS	Rha, Fru, Rib, Man, Gal, Xyl, Glc, GlcA, GalA	sulfate groups, calcium	anti-viral activity	[39]

nd†: non-defined.

3. Cyanobacterial Bioprocess (Cyano-EPS) Development

3.1. Strain Selection

The classical approach to bioprospecting an exemplar cyanobacterial EPS producer starts by looking at the growth rate and EPS content, to determine product titer and productivity. Cyanobacteria physiology is diverse and translates into a much wider strain-specific response to the stimulus applied during cultivation. Cultivation screenings can be a useful tool to compare the performance of different strains within a predefined environment. A compilation of cultivation screenings in cyanobacteria is presented in Table 2. Despite the diversity of habitats, we can observe that the same does not happen to the orders of strains studied. Most of the reports were done on Nostocales and Oscillatoriales orders, whereas the majority of tested organisms per study were from the *Nostoc* genus. In addition, the EPS fraction to be recovered varied among screenings to the methodologies applied. A study on forty *Nostoc* strains from Pasteur Culture Collection isolated from different habitats (soil, soil/water, water, and plant symbiosis), showing that the RPS production was not related to the habitat nor the morphology of the outermost investments. Still, PCC 7413, 8109, and 6720 were identified as the most productive strains 47, 30, and 28 mg/L/day respectively [40]. Moreover, a more comprehensive screening was recently performed in 166 marine algal strains, though only 16 were cyanobacteria. Despite the low number of cyanobacterial strains, the study comprised strains from five different orders some of them evaluated the EPS production potential for the first time. The authors opted to use the culture medium from the culture collection, which is often complex and used for maintenance; however, the screening conducted at 20 °C and 150 µE revealed *Synechococcus* sp. as a potential RPS producer (0.12 g/L) [41].

The cultivation screenings were characterized as being too long (30 days). This is explained by the cyanobacteria slow growth rate and also to EPS production associated with the stationary phase [10]. A dilution of N:P in BG11 to a factor of 7 was shown to anticipate the stationary phase in cultures of *Microcystis aeruginosa* and stationary phase characteristic EPS production was also observed under these conditions [42]. It is important to understand which is the cyanobacteria's physiological state throughout time and especially to define the period of highest EPS production. Although it is more relevant at the optimization stage, it can be used as a cut-off parameter. Applying a shorter production time (15 days) on four *Cyanothece* sp. strains from seawater in China's coast grown on modified f/2 with the addition of sea mud extract, aeration, and relatively strong inoculum for a small volume of cultivation was sufficient to highlight *Cyanothece 113* producing abundant quantities of a CPS (α-d-1,6-glucan).

The cyanobacteria inoculum quality (age and concentration) determines the period of acclimation, thus a lag phase in response to the transition of conditions was applied. The number of cells inoculated should be based on the steady-state of each strain, however, this methodology requires the previous knowledge of each strain's growth curve. The EPS screening studies have rarely provided information of inoculum, though some inoculated according to a determined concentration in terms of the number of cells, the mass of cells, chlorophyll *a* or even just a percentage. The culture medium provides the required nutrients for cyanobacterial growth, despite the existent formulations BG11 and $BG11_0$ (absence of nitrate source) were often applied. $BG11_0$ is used for nitrogen-fixing strains mostly from Nostocales order. Under diazotrophic conditions, growth is limited and the metabolism is re-directed towards nitrogen fixation with specialized cells and EPS production favored, namely CPS [40]. With respect to culture medium water sources, few studies have successfully applied seawater and other environmental resources to simulate habitats conditions and stimulate EPS production.

Light in the photoautotrophic metabolism is the energy provider for the cyanobacterial growth and it can be characterized in terms of light intensity (μE), light / dark cycles (L/Dh), and light quality. From Table 2, we can observe a heterogeneity of light conditions applied, while the majority opted for continuous illumination with low or high light intensity others opted for a balanced L/D cycle. No screening using other light sources such as natural light, different light qualities (monochrome) conditions were found. Temperature is a determinant factor for enzymatic activity and thus metabolism propeller. Cyanobacteria can be characterized by their adaptability to temperature as psychrophiles (below 15 °C), mesophiles, and thermophiles (above 40 °C). Very few studied strains belonging to the extremes of this classification, though they could have interesting industrial applications depending on the geographic location. Nonetheless the majority of studies applied constant temperature values within the mesophile range. In addition, no tests on temperature fluctuations were found. EPS production is known to affect the culture medium rheology; thus it is important to control the homogeneity of the cyanobacterial cultures in particular nutrients and pH. Culture mixing and/or aeration (air) were applied by the majority of the studies, though less often a CO_2–air mixture was intentionally applied to control pH. The volume of cultivation used varied along with studies (0.01–2 L) whereas it reduces the comparison between results; however, one study at the microplate level revealed a novel way to screen EPS production in 880 microalgal strains by correlating culture medium viscosity with sugar content. An overproducing bacterial strain was found in non-axenic *Mycrocistis aeruginosa f. flos-aquae* culture [43].

Table 2. Compilation of screening conditions applied to Cyano-EPS based on their origin and order.

Origin	Order (No. Strains)	Screening Conditions (Medium, Temperature, Light/Dark Cycle, Light Intensity, Air Supply/Mixing, Inoculum Conditions, Working Volume, Cultivation Days)	EPS Target	Reference
Soil, Soil/water, Water, Plant symbiosis	Nostocales (40)	$BG11_0$, 30 °C, L/D (24/0 h), 100 µE, 5% (*v*/*v*) CO_2-air, agitation, axenic, working volume: 0.4 L, 10–15 days	RPS	[40]
nd†	Nostocales, Chroococcales, Synechococcales (15)	$BG11_0$, 25 °C, L/D (16/10 h), 35 µE, L/D, axenic, working volume: 0.1 L, 44 days	RPS	[44]
Freshwater	Nostocales, Synechococcales, Oscillatoriales (25)	Z medium 2x concentrated, 20 °C, L/D (24/0 h) 15 W/m^2, 12-36 months	RPS	[45]
Marine	Oscillatoriales (4)	modified f/2 plus sea mud extract, 29 °C, L/D (24/0 h), 2700 Lux, aeration, inoculum 8×10^5 –9×10^5 cells/mL, working volume: 0.05 L, 15 days	CPS	[46]
Indo-Burma hotspot	Nostocales, Oscillatoriales (40)	$BG11_0$/ BG11#, 28 °C, L/D (14/10h), 54–67 µE, mixing 2x day, 50 mg of wet pellet, working volume: 0.1 L, 30 days	EPS	[47]
Freshwater (Indo-Burma hotspot)	Nostocales, Oscillatoriales (10)	$BG11_0$/ BG11#, 28 °C, L/D (14/10 h), 54–67 µE, mixing 2x day, 50 mg of wet pellet, working volume: 0.1 L, 30 days	CPS, RPS	[48]
Marine microbial mat French Polynesia	Oscillatoriales, Chroococcales, Synechococcales (6)	Conway (Fed-batch), 32 °C, L/D (12/12 h), 300 µE, 0.125 (*v*/*v*/min) pH 8.35 (CO_2 on demand), 250 rpm, 10% inoculum non-axenic, working volume: 2 L, 25–35 days	CPS, RPS	[49]
Soil contaminated, Gujarat, India nd†	Nostocales, Oscillatriales (4)	BG11 and ASN III, 27 °C, L/D (12/12 h), 3 kLux. Axenic inoculum (chlorophyll *a* concentration to ~2.0 mg/L), working volume 0.6 L, 30 days	RPS	[50]
Eroded soils; wastewater treatment plant; sediments; Cabras Lagoon	Nostocales, Oscillatoriales, Synechocchales, (7)	$BG11_0$/BG11#, 18 °C, L/D (14:10 h), 18 µE, working volume 0.3 L, 25–30 days (until stationary phase)	RPS	[51]
Freshwater lakes, Turkey	Synechocchales (3)	BG11, 25 °C, L/D (12:12 h), 1200 µE, 100rpm, working volume: 0.1 L, 20 days	CPS	[52]
Soil, garden	Nostocales (3)	$BG11_0$ and BG11, 30 °C, continuous illumination, 70–160 µE, aeration pH control (7–8.5) with CO_2-air, working volume: 0.25 L, inoculum: chlorophyll *a* concentration of 1.5 mg/mL, 8–15 days (until stationary phase)	RPS	[53]
Miscellaneous / Culture Collections; hard sands Pantelleria island, Italy; Antarctic lake, Antarctic	Nostocales, Oscillatoriales (16)	$BG11_0$/ BG11# or Allen and Arnon or alkaline medium, 25 °C or 11 °C (psychrophilic strains), 1.500 lux, aeration pH 7–8.5 (CO_2 on demand), inoculum chlorophyll concentration 1.5 or 3 mg/mL, working volume: 1.5 L, 30 days	CPS, RPS	[54]
Baltic Sea, Pacific Ocean, Atlantic Ocean, Mediterranean Sea, Red Sea	Synechocchales, Spirulinales, Pleurocapsales, Nostocales, Chroococcales (16)	PCR-11 medium, 20 °C, L/D (16:8 h), 150–300 µE, 120 rpm, working volume: 0.02 L, 30 days	RPS	[41]

Nd†—non-defined. $BG11_0$/BG11#—nitrate source was excluded ($BG11_0$) for all heterocystous strains or maintained (BG11) for non-heterocystous.

3.2. Production and Optimization

Even though strain selection can anticipate the success of the whole bioprocess development the performance will always need to be ameliorated. At this step, it will be determined how much product can the cell produce in a determined volume per period of time, i.e., productivity. Consequently, the moment that the cell produces the highest amount of EPS and/or how long it takes to achieve will determine the strategy applied [55]. With respect to stress conditions applied to cyanobacterial strains nitrogen, salinity, and light are reported as the most frequent inducers of EPS production. Single parameter optimization has been extensively applied to optimize EPS production, see Table 3. As observed in strain selection, Nostocales and Oscillatoriales are among the most studied orders.

3.2.1. Culture Medium

Culture medium optimization has a very important role in the economic feasibility of a bioprocess as well as to highlight and fine-tune the presence of certain nutrients. The combined presence of nitrogen in *Cyanothece* sp. CCY 0110 was able to improve RPS production (1.8 g/L) [56]. Although the highest levels of EPS on *Nostoc* sp. BTA97 and *Anabaena* sp. BTA990 were obtained in the absence of any combined nitrogen source [47]. The most common source of nitrogen used is $NaNO_3$ [57,58], though KNO_3 [59,60] has also shown a positive correlation with EPS production. Interestingly, among the four different sources of nitrogen, urea promoted the highest amount of RPS in *Nostoc flagelliforme* [61]. The absence of inorganic nitrogen, i.e., diazotrophic conditions, was reported to influence positively the production of EPS in certain heterocystous forming [62–64] and unicellular species [65].

The inorganic carbon source most commonly used is CO_2 (0.05–10% *v/v*) combined with air and used to control pH on demand. $NaHCO_3$ was found to positively influence EPS production in *Arthropira platensis* while at the same time it also promoted bioflocculation with almost 90% of harvest efficiency [66]. Some authors have evaluated the effect of using organic carbon sources. The use of glucose to induce mixotrophic growth on *Nostoc flagelliforme* was not found to increase EPS production [58,67]. Contrarily, a kinetics study on *Arthrospira platensis* tested in photoautotrophic, heterotrophic, and mixotrophic mode attained 290 mg/L of EPS in the presence of combined glucose and light [68].

The species *Cyanothece* sp. grown at very high salinities (70 g/L NaCl) was able to attain an EPS titer, namely CPS, of 22.34 g/L [33]. The increased EPS production, namely CPS fraction, on the freshwater *Synechococcus* under different salinities was shown to promote osmotic cellular balance. Cell-cycle phase duration was shown to be increased and thus a slowdown on growth rate was noted at higher salinities. Conversely, salinity stress marked an increase in EPS production [69]. Many authors reported the enhanced production of EPS through NaCl stress [46,52,60,62,67], though others reported no significant effect [54,56,59] or even its absence became beneficial [70].

Micronutrients such as $MgSO_4$ [46,62], K_2PHO_4 [54,57], and $CaCl_2$ [60] have been shown to produce significant effects on EPS, while no effect was found for $MgCl_2$ or NaH_2PO_4 [56,65]. A less common stress applied in *Cyanothece epiphytica* was the exposure to ozone for 50 s at 0.06 mg/L resulting in a relatively high EPS yield. The EPS produced showed biolubrificant potential, while the effect of ozone did not provoke any damage to cell structure [62].

3.2.2. Process Conditions

Process conditions and respective control are important to assure the reproducibility and feasibility of the bioprocess. Nevertheless, these settings can possibly influence the desired outcome of the process.

Cyanobacteria can grow in pH ranging from 6–12, while the initial pH was shown to affect the number of EPS produced [47,48]. CO_2 is thought to increase EPS production in planktonic cyanobacteria [71]. Setting a pH range to control the cultivation period either through injection of CO_2 or the use of acid/basic solutions avoids the limitation of growth before light, which is the ultimate limiting factor for photoautotrophic cultures like cyanobacteria [49,53,54,70,72].

The growth rate of cyanobacteria has been observed to increase with shear stress, probably by promoting orientation and increasing the contact area of the microorganisms and nutrients [73,74]. For the Cyano-EPS a certain degree of shear stress will be necessary once the rheology of the medium is altered and the mass and gas transfers become less efficient throughout the time. The evolution of culture medium rheology combined with shear stress highly affects the bacterial EPS production at the industrial scale [55]; however, reports have shown a variety of shear stress applied most of the times by default, single aeration [33,58] or agitation [47,59,60,69], aeration with CO_2 supplementation [53,54,64,70,75,76], simultaneous aeration, and agitation [49,56,57]. Optimization studies demonstrated a low number of reports studying the effect of shear stress on EPS production [54,56,65,70], whereas the majority of them found it highly relevant.

Temperature is also reported to have a positive effect on growth and EPS production [56,57,65,70], though others have reported a dissimilitude [75,76]. When combining the effect of temperature and light intensity the settings for EPS enhancement are antagonists [75]. A petri dish work using crossed gradients of temperature and light intensity have found the optimal range for growth and EPS for *Scytonema tolypothrichoides* and *Tolypothrix bouteillei.* These two strains from the same microhabitat had different light and temperature optima, supporting, even more, the strain specificity on Cyano-EPS control. Moreover, the temperature was shown to be the most significant parameter [77].

Light/Dark (L/D) cycles highly affected the production of insoluble carbohydrates per cell [78]. The optimization studies found positive correlation by applying L/D cycles [54,56,60,64], while continuous illumination was found favorable for EPS production in *Anabaena* genus [54].

Much work has been done on *Nostoc flagelliforme* [11,61,63,79–84]. This strain is an edible terrestrial cyanobacterium that is used as food in China for more than 2000 years. The EPS from *N. flagelliforme* are reported to be bioactive and possess interesting physico-chemical properties. Light quality experiments have shown that *N. flagelliforme* has a higher growth rate and EPS production under monochromatic light, namely red and blue light when compared to white light [79,80]. However, no relationship between monosaccharidic composition and the quality of light applied was found [80]. The same was found for another *Nostoc* sp. [85]. Culture medium optimization was found to enhance the antioxidant activity of CPS fraction [67]. Different light intensities of red light induced photoinhibition which possibly stimulated the protection of the cell by the production of EPS, namely CPS [80]. Optimization of carbon, nitrogen source, and light quality reinforced the fact that growth and EPS have their own requirements. In addition, these culture conditions also affected the EPS-associated enzymes, showing a correlation with the number of EPS produced [63]. More recently, a transcriptomic analysis revealed that light quality regulated EPS biosynthesis via the intracellular reactive oxygen species (ROS) level directly other than oxidative stress of *N. flagelliforme* [83].

Meta-bibliographic research was conducted to infer which were the cultivation factors that most influence the EPS and glycogen production in *Arthropira platensis*. The results showed a considerable heterogeneity of authors' results explained by different operating conditions and extraction/purification methodologies applied. The authors found light intensity as the most preponderant factor in their desk-research and experimented with different light intensities (100, 400, 800, and 1200 μE). The ratio EPS/glycogen was found higher with lower light intensities. In addition, EPS monosaccharides composition was significantly different among different light intensities applied [86].

For those cases, where there was no correlation between growth and RPS production, a two-stage cultivation might be the most appropriate way to attain high biomass and then obtain a higher amount of the RPS at a second stage. This was already suggested in [51]. Playing with intensities of multiple wavelengths (red, blue, and green) and nitrogen source a strategy was defined and an increase in 66% and 217.3% in *N. flagelliforme* growth an RPS production was attained, respectively. The growth phase was set with white light for nine days while the EPS phase was followed by nine days of mixed wavelength (red/blue/green = 12:5:5) using urea as a nitrogen source [61]. The use of light as a way to control the production of cyanobacterial products has already been reported [87,88]. Moreover, lower light intensity was also utilized as a strategy to increase EPS production [61,89]. *Arthrospira*

sp. was grown in two-stage cultivation wherein biomass production (Zarrouk medium, 30 °C, 80 µE) was favored at first. Secondly, the combined effect of light intensity and salinity (NaCl) was found antagonistic on EPS enhancement. Optimal conditions were found (10 µE and 39 g/L of NaCl) allowing EPS production to have a 1.67-fold increase when compared to optima growth conditions [89].

Applying outdoor cultivation systems can potentially reduce production costs by reducing light and temperature control [62,90–92]. Relevance was found in an outdoor pilot scale cultivation of *Spirulina* sp. LEB-18 on 250 L of open raceways in the summer of Salvador, Bahia, Brazil. The crude RPS demonstrated pseudoplastic behavior and high thermal stability. At day 30, the biomass reached a titer of 1.01 g/L while the crude EPS production was shown to attain 9.5 g/L, wherein the highest productivity happened on day 10 (0.6 g/L/day); however, it is known that outdoor conditions can affect productivities due to variable climate conditions and geographic location [93].

3.2.3. Modes of Cultivation and PBR Design

Cultivation modes are used as a strategy to increase biomass and EPS production [94]. The majority of Cyano-EPS are reported in batch mode followed by almost unexplored fed-batch [51,72] and continuous mode [70]. *Anabaena* sp. ATCC 33047 grown under different dilution rates was found optimal EPS/biomass ratio of higher than 1.5 and EPS productivity attaining 1.1 g/L/day at 0.03 $hour^{-1}$. In addition, EPS and biomass production phases were shown to be disassociated [70]. Other authors have claimed the same disassociation of the kinetics [76,77,79].

Photobioreactor (PBR) design technology has been highly evolving due to extensive research applied to microalgal biofuels; however, their application to EPS production does not seem adequate [95]. Immobilizing cyanobacteria in cotton toweling showed the basic limitations of biomass production due to high lateral pressure [96]. A flow-lane incubator in fed-batch mode was used to grow biofilm-forming cyanobacteria. The results showed that the planktonic growth was far more productive. The same was observed for EPS production in a 25 L open pound system with *N. flagelliforme* under batch and fed-bath cultivation mode. The comparison demonstrated higher biomass and EPS production under fed-batch mode [72]. *Arthrospira platensis* was batch-grown in 30 m^2 PBR located at a coal power plant in Taiwan for flue gas consumption. This novel PBR consisted of multiple 1 L stacked vessels individually controlled. The outdoor conditions were supplied with sunlight and an extra 10 h of LED illumination during the night. Temperature levels ranged from 30–34 °C during 15 days of experiment. The system was able to produce 2.36 g L^{-1} and produce a CPS with immunogenic activity [92].

3.2.4. Design of Experiments

Optimization refers to the discovery of the conditions of a system, a process, or a product to obtain the maximum benefit from it [97]. The classical methods involve the optimization of just one condition at a time, a methodology highly applied in Cyano-EPS (see Table 3). The optimization method must take into account all the interactions and the optimum conditions obtained are validated. The design of experiments is highly used in bioprocess optimization, though the examples in Cyano-EPS are less frequent. A central composite design (CCD) was applied to *Cyanothece epiphytia* to evaluate the combined effect of micronutrients magnesium and iron [62]. A full factorial design was applied *Arthrospira* sp. for the combination of NaCl and light intensity factors [89]. The same experimental design was applied to *A. platensis* strain "Compére 1968/3786" under photoautotrophic [98] and mixotrophic modes [68]. Temperature highly affected growth and EPS production when compared to light intensity; however, the combination of temperature and light were also found to be significant, while growth and EPS production had different optima settings [98]. Light intensity and glucose concentration were investigated under mixotrophic conditions. In this case, EPS titer and yield were mostly affected by glucose concentration with divergent optimal set points [68].

Table 3. Cyanobacterial EPS optimization studies on culture media and process parameters organized by order. Bold effects correspond to positive/or significant effect on EPS production.

Strains	Optimization Factor		EPS Titer/Productivity /Yield	Literature
	Culture Media	Process Parameters		
Nostocales				
Anabaena augstmalis VRUC163	nd†	Fed-batch; Film forming PBR	14.73 mg/g (CPS)	[51]
Anabaena cylindrica 10 C	N source (**$NaNO_3$**) Mixotrophy	nd†	2.36 mg/L (RPS)	[58]
Anabaena WSAF	N source (absence; **$NaNO_3$**); P source (**K_2PHO_4**);	L/D cycle (**continuous**); Shear stress (**aeration**);	1.86 mg/L/day (EPS)	[54]
Anabaena sp. *ATCC 33047*	N source (**N_2**, KNO_3; NH_4Cl); Salinity (NaCl/**absence**)	Temperature; Light intensity (**medium**); Shear stress (**high aeration**); **Dilution rate** (0.03 h^{-1})	1100 mg/L/day (RPS + CPS)	[70]
Anabaena sp. BTA997	nd†	Initial pH (**8.5**)	1.7 g/L (RPS)	[48]
Anabaena turolosa	N source (absence; **$NaNO_3$**); P source (**K_2PHO_4**);	L/D cycle (**continuous**); Shear stress (**aeration**);	0.73 mg/L/day	[54]
Cyanospira capsulata	C flux metabolism (**glyoxylate**; nitrogen inhibitor)	nd†	7.5 mg/L/day	[99]
Nostoc flagelliforme	N source (**$NaNO_3$**); P source (**K_2PHO_4**);	Temperature (low)**; Light intensity (high); Initial pH (alkaline)**	14.29 mg/L/day (RPS)	[57]
Nostoc flagelliforme	nd†	Cultivation mode (**Fed-batch**); pH (8–9)	8.86 mg/L/day (CPS)	[72]
Nostoc flagelliforme	nd†	light quality (monochromatic **red**, yellow, green, **blue**, purple)	47.39 mg/g (RPS)	[79]
Nostoc flagelliforme	nd†	light quality (white fluorescent and monochromatic **red**, yellow, green, blue, purple); Red light intensity (**medium**)	275 mg/g (CPS)	[80]
Nostoc flagelliforme	C source (absence; **$NaHCO_3$**); N source (**absence**; $NaNO_3$)	light quality (monochromatic red, **blue**)	nd†	[63]
Nostoc flagelliforme	N source (**Urea**, $NaNO_3$, NH_4Cl; Arginine)	Light intensity (**low**); Light quality (mixed wavelengths, **red**, **blue**, green); **Wavelength shift**	5.42 mg/L/day (RPS)	[61]
Nostoc flagelliforme	C source (glucose); Salinity (**NaCl**)	nd†	234.82 mg/g (CPS)	[84]
Nostoc sp.	nd†	Light intensity **(high)**	134.26 mg/g DW (RPS)	[85]
Nostoc sp. BTA97	N source ($NaNO_3$, **absence**);	Initial pH (**alkaline**)	53.3 mg/L/day (RPS + CPS)	[47]
Nostoc sp. PCC 7413	N source ($NaNO_3$; **presence**/absence)	Light intensity (low, **high**)	150 mg L/day (RPS)	[53]
Scytonema tolypothrichoides	nd†	**Temperature** and light intensity crossed gradients	310–360 mg/L (CPS)	[77]
Tolypothrix bouteillei	nd†	**Temperature** and light intensity crossed gradients	186–216 mg/L (CPS)	[77]
Oscillatoriles				
Arthrospira platensis PCC 8005	nd†	**Light intensity (low)**	nd†	[86]
Arthrospira platensis	C source (**$NaHCO_3$**)	nd†	nd†	[66]
Arthrospira platensis "Compére 1968/3786"	nd†	**Temperature; Light intensity**	11.76 mg/L/day (RPS)	[98]
Arthrospira platensis "Compére 1968/3786"	Photoautotrophic (light), Mixotrophic **(light, glucose)**, Heterotrophic (glucose)	nd†	26.4 mg/L/day (RPS)	[68]

Table 3. *Cont.*

Strains	Optimization Factor		EPS Titer/Productivity /Yield	Literature
	Culture Media	Process Parameters		
Cyanothece epiphytica AUS-JR/DB/NT-021	N source (**absence**; $NaNO_3$); Salinity (**NaCl**); Micronutrients (**$MgSO_4$**); **Ozone**	nd†	9.66 mg/L/day (RPS + CPS)	[62]
Cyanothece sp. 113	N source (**absence**; $NaNO_3$); Salinity (**NaCl**); Micronutrients (**$MgSO_4$;** NaH_2PO_4)	**Aeration; Temperature; Light intensity; Time course**	1300 g/L/day (CPS)	[46,65]
Cyanothece sp. CCY 0110	C source (glycerol); N source (absence/**combined**); Salinity (NaCl); Micronutrients ($MgCl_2$)	Temperature; **Light intensity**; L/D cycle; shear stress (aeration)	42.86 mg/L/day (RPS)	[56]
Microcoleus vaginatus	nd†	**Light intensity**;	139 mg/g (RPS)	[100]
Synechococcales				
Limnothrix redekei PUPCCC 116	N source (**KNO_3**); Salinity (NaCl)	nd†	14.48 mg/L/day	[59]
Oscillatoria formosa	N source (**KNO_3**); Salinity (**NaCl**); Micronutrients (**$CaCl_2$**)	Temperature **(high)**; L/D cycles **(14/10)**	9.88 mg/L/day (RPS)	[60]
Synechococcus sp.	N source (N_2, nitrate, combined/**absence**)	Light intensity; L/D cycle	330 mg/L/day	[64]
Synechocystis sp. *BASO*	Salinity (**NaCl**)	nd†	500 mg/L (CPS)	[52]
Chroococcales				
Cyanobacterium aponinum	C source (5% CO_2; $NaHCO_3$)	Temperature (**high**); Light intensity (**high**)	20 mg/L/day (RPS)	[76]
Spirulinales				
Spirulina sp.	N source (**$NaNO_3$**); P source (**K_2PHO_4**); Salinity (NaCl)	Temperature	1.83 mg/L/day (EPS)	[54]

nd†—non-defined. Levels of process parameters are in agreement with the associated literature in question. In some cases, Nitrogen did not produce significant effects which can be justified by the fact the control had a defined nitrogen concentration, for instance in situations where BG11 was used as control.

3.3. Downstream Processes and Cyano-EPS Global Market

Interest for cyanobacteria EPS has increased significantly during the last two decades as they offer some structural originalities compared to those extracted from marine microalgae and to hydrocolloids from macroalgae and plants [10,50]. They have wide putative applications such as: food additives, soil (water holding capacity), wastewater treatments (removal of heavy metals), bioactive agents for nutraceutic, pharmaceutical, and cosmetic fields [101]. The development of strategies for their extraction and purification is strongly correlated with their cellular location. EPS should be employed only to reference polysaccharides excreted by microorganisms in their extracellular environment and not having covalent links with their cellular envelope. Considering the abundant literature focusing on polysaccharides from cyanobacteria they can be divided into three groups: biopolymers poorly associated with the cell surface and usually encompassed under the term of slime or released polysaccharides (RPS); polysaccharide structured as a sheath, which is a thin layer next to the outer cell membrane and containing fibers; capsular polysaccharides (CPS) intimately associated with the envelope cells [102]. The extraction and purification of these polysaccharides from cyanobacterial culture media is a major concern before the investigation of their structure and/or to find commercial applications. The main question before developing a strategy for their purification is what kind of EPS is the objective of the extraction: slime, sheath, or capsular polysaccharides? Several articles or reviews detail the processes available for the extraction of these three kinds of biopolymers, often calling them with different appellations, increasing the confusion between these different classes [10,35,40,102–105]. All the protocols are summarized in Figure 1. Firstly, the extracellular medium of cyanobacteria

cultures is collected generally by centrifugation, but tangential microfiltration may be an interesting alternative as described by [106] for red microalgae or by [10]. Note that [105] suggested suspending the pellet in Milli-Q water and to incubate them at 4 °C during 12 h before to centrifuge them again to collect, by depletion, the maximum of slime. The cyanobacteria free supernatants or permeates are then treated by polar alcohols (methanol, ethanol, or isopropanol) or acetone to precipitate the EPS. The volume ratios are different depending on studies but two or three volumes of cold ethanol (or isopropanol) is probably a good compromise. Sometimes the supernatant is concentrated under vacuum at low temperatures (50–60 °C) to limit the volume of used alcohol or acetone [10,50]. This sole EPS precipitation is generally not enough to remove salts and/or low molecular weight metabolites from extracted polysaccharides as they co-precipitated during alcohol/acetone treatment. So, two protocols are generally used by authors to increase the purity level of polysaccharides, measured by Dubois assay [107]. The first one is a solubilization of dried polysaccharides in Milli-Q water before their precipitation using the same alcohol or acetone volume ratio. The second one is to dialyze them against water. Finally, the EPS are freeze-dried or dried under vacuum. The second extraction strategy focuses on polysaccharides designed as a sheath (or CPS) and concerns the main extraction protocols described in the literature. Numerous cyanobacteria such as *Nostoc* genus form macroscopic colonies embedded in extracellular polysaccharides protecting them from environment [108].

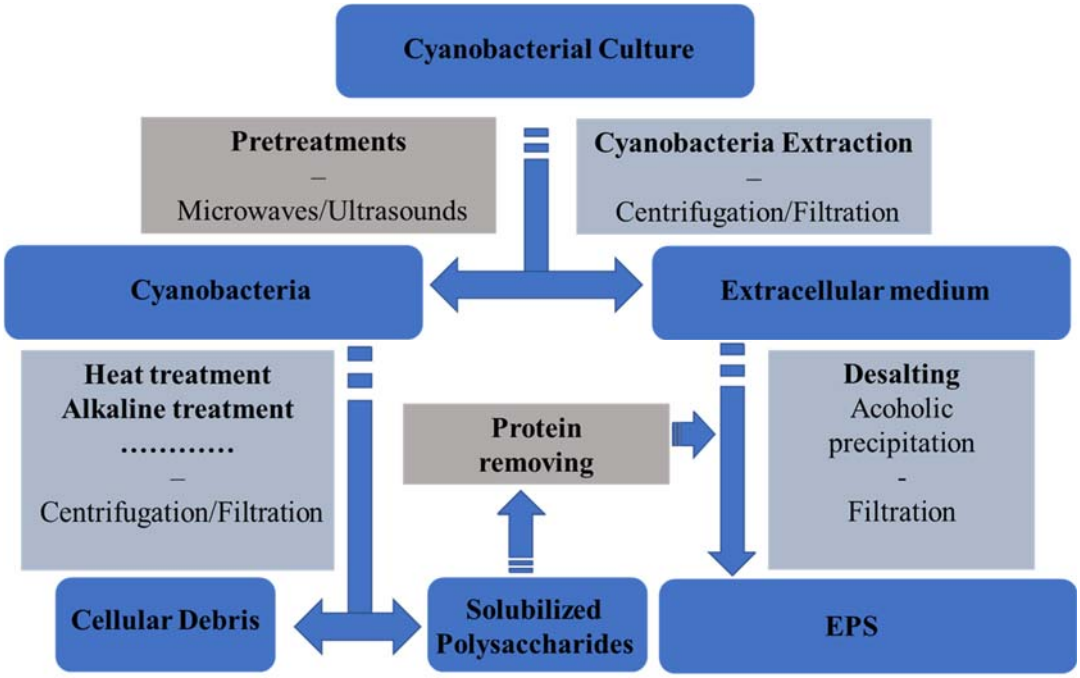

Figure 1. Process flow chart for the extraction of EPS from cyanobacteria.

Even if these polysaccharides do not have covalent linkages with the cyanobacteria cell wall, they interact strongly with it, which requires specific processes to dissociate them. Note that in numerous publications, authors include sheath and capsular polysaccharides as a unique fraction. These processes present some differences but can be resumed by collecting biomass using centrifugation and the extraction of EPS using hot distilled water (50–121 °C) in varying times (0.5–6 h). In other protocols, the extraction is achieved by differential sucrose gradient centrifugation of cyanobacterial biomass or after re-suspending it in a low ionic strength buffer at 100 °C [22,105,108]. After the extraction, the suspension is filtered through a filter paper, of 0.2 µm membrane or centrifuged to collect the permeate or the supernatant. The retentate or the pellets are extracted again several times using the same process to deplete the biomass (up to 5–6 times). Note that with this extraction numerous non-polysaccharidic compounds and notably proteins are co-extracted needing a purification supplementary step. Another way to start is by a 50 °C concentration under vacuum (not always

necessary or done) followed by an optional protein extraction using the Sevag method or another. Then, an alcoholic (isopropanol or ethanol (2–3 *v/v*)) precipitation of polysaccharides is applied. Finally polysaccharides are freeze- or oven-dried [10,14,90,105]. Sometimes, the lipids and pigments of the biomass can be previously extracted using an organic solvent such as hexane, acetone, ethanol, or other [14,92]. This kind of process may be improved using microwave or ultrasound treatments as applied to *Arthrospira* biomass [109,110]. Finally, the last class of extracellular polysaccharides is that of capsular polysaccharide needing stronger conditions for their extraction. Often considered as EPS in literature, their extraction implies the use of alkaline treatments of biomass such as a reflux in NaOH 0.1 M for 5 h at 90 °C [111]. The polysaccharides are precipitated using two volumes of isopropanol and dissolved in Milli-Q water. A second precipitation with alcohol is performed to increase their purity level.

In their excellent review entitled « Exocellular polysaccharides from cyanobacteria and their possible applications » published in FEMS Microbiology Letters in 1998, R. De Philippis and M., Vincenzini [108] were very careful considering the industrial exploitation of EPS from cyanobacteria despite their potential. Cyanobacterial EPS have to compete with cheaper hydrocolloids from seaweeds, terrestrial plants, and non-photosynthetic bacteria, wherein some have authorizations for their use in food. What is the situation 21 years later? The number of publications focusing on the rheological and biological potential of these biopolymers increases dramatically [10,22,101,105,112] but is not correlated with the arrival on the market of EPS from cyanobacteria. Currently and to our knowledge, only four EPS from Cyanobacteria are commercially available and exploited in niche markets mainly in the cosmetic and nutraceutical area. They are Spirulan, Immulan, Nostoflan, and Emulcyan extracted from respectively *Arthrospira platensis*, *Aphanotece halophytica*, *Nostoc flagelliforme*, and *Phormidium* [22,113–115].

4. Considerations for Cyano-EPS Development

In light of bioprocess development, Cyano-EPS has still a long way to become a trustable supplier of polysaccharides for the hydrocolloids industry. For that, three key steps must interplay: strain selection, production process, and downstream processing.

Increased cell robustness is a concept elaborated in [116], wherein the selection of the organism takes into account the relevant process parameters as well all the whole bioprocess stages until the final product performance. According to [116] the stability of a bioprocess depends on the following factors: shear stress resistance; tolerance against temperature and/pH; low sensitivity to infections; robustness against contaminations; no sticking or biofouling. To date no comprehensive screening taking into account the sustainability of bioprocess was published for cyanobacterial EPS. Interesting examples are being applied to the microalgae field by: the selection of desert-adapted strains for commercial application and CO_2 sequestration [117]; cold-adapted microalgae strains for the production of fatty acids and proteins [118]. Application of such strategies in Cyano-EPS might bring a new possibility, whereas it will be determinant the polymer functionality. Other possibilities include the use of genetic engineering technologies for the development of cyanobacterial tailor-made polymers [119].

For the production process, much can be done around optimization, especially by applying statistically-based experimental design methods. Culture medium components, pH, mass, and gas transference rate, light, and inoculum conditions are some of the factors to consider in Cyano-EPS optimization. For this, it is important to understand the kinetics of biomass and EPS production for the cultivation conditions applied and opt for the most convenient strategy. The use of omics as a support tool can along with cultivation unroll unknown factors [120]. One stage cultivation seems practical for cases in which growth and EPS are associated, whereas two-stage cultivation will fit for the decoupled cases and biomass can be produced followed by the EPS production phase. PBR design has still considerable space for improvement with special needs for the mass and gas transfer within the culture vessel due to culture medium viscosity increase.

The location of the EPS should be taken into account since it will strongly affect the downstream processing. RPS can be easily recovered from the culture medium allowing the possibility to valorize the biomass produced. Conversely, CPS attached to the cell surface involve more destructive methodologies that might restrict the valorization of the produced biomass. However, an alternative to this was found through the combination of methodologies for the extraction of CPS and pigments. This methodology was confirmed in four cyanobacterial strains and proposes the CPS extraction followed by freeze-drying of the biomass, extraction of phycobiliproteins, and extraction of chlorophyll *a* and carotenoids [121].

Water and nutrients sources must be highly considered since the beginning of the bioprospection process not only for their sustainability but also for their future impacts on the EPS purification.

5. Conclusions

Cyano-EPS can offer unique structures, and thus, new functionalities; however, bioprospection needs more diversity of strains and methodologies to discover reliable EPS producers. Integrative methodologies of production and extraction can make this bioprocess more attractive to the industry through the increase of productivities and efficiency, respectively. Cyano-EPS have the potential to be a natural polysaccharide supplier to niche markets.

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