EPS Cyanobacterial Bioprocess Development

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Cyanobacteria have the potential to become an industrially sustainable source of functional biopolymers. Their exopolysaccharides (EPS) harbor chemical complexity, which predicts bioactive potential. 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. 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. To develop an EPS cyanobacterial bioprocess (Cyano-EPS) three steps are highlighted: the selection of the cyanobacterial host; optimization of production parameters; downstream processing.

Keywords: Exopolysaccharides ; Cyanobacteria ; Bioprocess ; Cyano-EPS

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 1. 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.

Table 1. 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	[1]
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	[2]
Freshwater	Nostocales, Synechococcales, Oscillatoriales (25)	Z medium 2x concentrated, 20 °C, L/D (24/0 h) 15 W/m^2, 12-36 months	RPS	[3]
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	[4]
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	[5]
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	[6]

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
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	[7]
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	[8]
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	[9]
Freshwater lakes, Turkey	Synechocchales (3)	BG11, 25 °C, L/D (12:12 h), 1200 µE, 100rpm, working volume: 0.1 L, 20 days	CPS	[10]
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	[11]
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	[12]
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	[13]

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

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 [14]. 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 [15]. 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 [1]. 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 1, 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 [16].

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 [17]. 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 2. As observed in strain selection, Nostocales and Oscillatoriales are among the most studied orders.

Table 2. 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)**	[9]
***Anabaena cylindrica* 10 C**	**N source ($NaNO_3$) Mixotrophy**	**nd†**	**2.36 mg/L (RPS)**	[18]
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)**	[12]
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)**	[19]
***Anabaena* sp. BTA997**	**nd†**	**Initial pH (8.5)**	**1.7 g/L (RPS)**	[6]
Anabaena turolosa	**N source (absence; $NaNO_3$); P source (K_2PHO_4);**	**L/D cycle (continuous); Shear stress (aeration);**	**0.73 mg/L/day**	[12]
Cyanospira capsulata	**C flux metabolism (glyoxylate; nitrogen inhibitor)**	**nd†**	**7.5 mg/L/day**	[20]
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)**	[21]
Nostoc flagelliforme	**nd†**	**Cultivation mode (Fed-batch); pH (8–9)**	**8.86 mg/L/day (CPS)**	[22]

Strains	Optimization Factor		EPS Titer/Productivity /Yield	Literature
	Culture Media	Process Parameters		
Nostoc flagelliforme	nd†	light quality (monochromatic red, yellow, green, blue, purple)	47.39 mg/g (RPS)	[23]
Nostoc flagelliforme	nd†	light quality (white fluorescent and monochromatic red, yellow, green, blue, purple); Red light intensity (medium)	275 mg/g (CPS)	[24]
Nostoc flagelliforme	C source (absence; $NaHCO_3$); N source (absence; $NaNO_3$)	light quality (monochromatic red, blue)	nd†	[25]
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)	[26]
Nostoc flagelliforme	C source (glucose); Salinity (NaCl)	nd†	234.82 mg/g (CPS)	[27]
Nostoc sp.	nd†	Light intensity (high)	134.26 mg/g DW (RPS)	[28]
Nostoc sp. BTA97	N source ($NaNO_3$, absence);	Initial pH (alkaline)	53.3 mg/L/day (RPS + CPS)	[5]
Nostoc sp. PCC 7413	N source ($NaNO_3$; presence/absence)	Light intensity (low, high)	150 mg L/day (RPS)	[11]
Scytonema tolypothrichoides	nd†	Temperature and light intensity crossed gradients	310–360 mg/L (CPS)	[29]
Tolypothrix bouteillei	nd†	Temperature and light intensity crossed gradients	186–216 mg/L (CPS)	[29]
Oscillatoriles				
Arthrospira platensis PCC 8005	nd†	Light intensity (low)	nd†	[30]
Arthrospira platensis	C source ($NaHCO_3$)	nd†	nd†	[31]
Arthrospira platensis “Compére 1968/3786”	nd†	Temperature; Light intensity	11.76 mg/L/day (RPS)	[32]
Arthrospira platensis “Compére 1968/3786”	Photoautotrophic (light), Mixotrophic (light, glucose), Heterotrophic (glucose)	nd†	26.4 mg/L/day (RPS)	[33]
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)	[34]
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)	[4][35]
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)	[36]
Microcoleus vaginatus	nd†	Light intensity;	139 mg/g (RPS)	[37]
Synechococcales				
Limnothrix redekei PUPCCC 116	N source (KNO_3); Salinity (NaCl)	nd†	14.48 mg/L/day	[38]
Oscillatoria formosa	N source (KNO_3); Salinity (NaCl); Micronutrients ($CaCl_2$)	Temperature (high); L/D cycles (14/10)	9.88 mg/L/day (RPS)	[39]
Synechococcus sp.	N source (N_2, nitrate, combined/absence)	Light intensity; L/D cycle	330 mg/L/day	[40]

Strains	Optimization Factor		EPS Titer/Productivity /Yield	Literature
	Culture Media	Process Parameters		
Synechocystis* sp. *BASO	**Salinity (NaCl)**	**nd†**	**500 mg/L (CPS)**	[10]
Chroococcales				
Cyanobacterium aponinum	**C source (5% CO_2; $NaHCO_3$)**	**Temperature (high); Light intensity (high)**	**20 mg/L/day (RPS)**	[41]
Spirulinales				
***Spirulina* sp.**	**N source ($NaNO_3$); P source (K_2PHO_4); Salinity (NaCl)**	**Temperature**	**1.83 mg/L/day (EPS)**	[12]

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.

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, namely nitrogen [5][36][21][18][38][39][26][34][25][40][35], carbon [18][31][42][33], salinity [43][4][10][12][38][39][34][42][44][19] and micronutrients [4][12][36][21][39][34][35].

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.

Among them, pH [7][11][12][19][22], shear stress [43][5][7][12][36][21][38][39][40][19][45][41], temperature [36][21][35][19][41][29], Light/Dark (L/D) cycles [12][36][39][40][46] have been highly addressed.

Much work has been done on *Nostoc flagelliforme* [47][26][25][23][24][48][49][50][27]. 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 [23][24]. However, no relationship between monosaccharidic composition and the quality of light applied was found [24]. The same was found for another *Nostoc* sp. [28]. Culture medium optimization was found to enhance the antioxidant activity of CPS fraction [42]. Different light intensities of red light induced photoinhibition which possibly stimulated the protection of the cell by the production of EPS, namely CPS [24]. 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 [25]. 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* [50].

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 [30].

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 [9]. 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 [26]. The use of light as a way to control the production of cyanobacterial products has already been reported [51][52]. Moreover, lower light intensity was also utilized as a strategy to increase EPS production [26][53]. *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 [53].

Applying outdoor cultivation systems can potentially reduce production costs by reducing light and temperature control [25][54][55][56]. 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 [57].

2.1. Modes of Cultivation and PBR Design

Cultivation modes are used as a strategy to increase biomass and EPS production [58]. The majority of Cyano-EPS are reported in batch mode followed by almost unexplored fed-batch [9][22] and continuous mode [19]. *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 [19]. Other authors have claimed the same disassociation of the kinetics [41][29][23].

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 [22][56][59][60].

2.2. 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 [61]. The classical methods involve the optimization of just one condition at a time, a methodology highly applied in Cyano-EPS (see Table 2). 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 [34][33][53][32].

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 [14][8]. 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 [62]. 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 [63]. 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 [14][64][1][63][65][66][67]. 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 [68] for red microalgae or by [14]. Note that [67]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 [14][8]. 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 [69]. 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 [70].

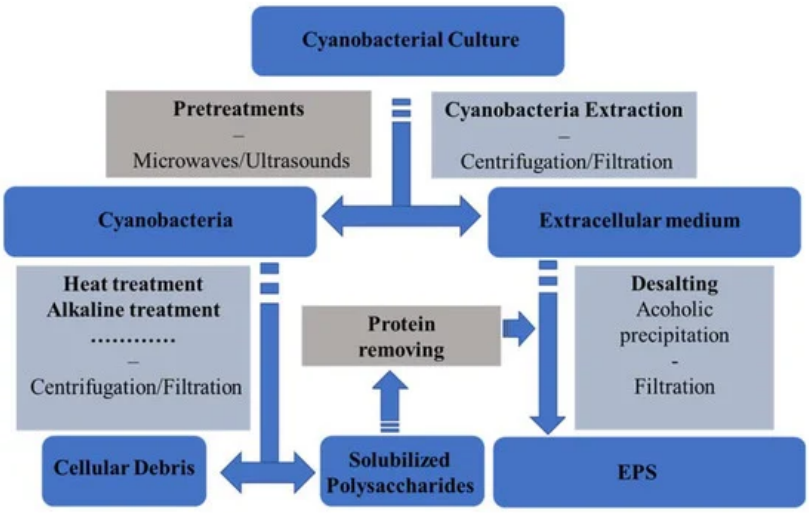

Figure 1. Process flow chart for the extraction of EPS from cyanobacteria.

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 [71][67][70]. 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 [14][72][54][67]. Sometimes, the lipids and pigments of the biomass can be previously extracted using an organic solvent such as hexane, acetone, ethanol, or other [72][56]. This kind of process may be improved using microwave or ultrasound treatments as applied to *Arthrospira* biomass [73][74]. 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 [75]. 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.

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 [14][71][62][67][76] 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* [71][77][78][79].

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 [80], wherein the selection of the organism takes into account the relevant process parameters as well all the whole bioprocess stages until the final productperformance. According to [80] 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 CO2 sequestration [81]; cold-adapted microalgae strains for the production of fatty acids and proteins [82]. 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 [83][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 [84]. 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 [85]. 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.

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