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Review

Extracellular Polymeric Substances (EPS) as Microalgal Bioproducts: A Review of Factors Affecting EPS Synthesis and Application in Flocculation Processes

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Abstract: Microalgae are natural resources of intracellular compounds with a wide spectrum of applications in, e.g., the food industry, pharmacy, and biofuel production. The extracellular polymeric substances (EPS) released by microalgal cells are a valuable bioproduct. Polysaccharides, protein, lipids, and DNA are the main constituents of EPS. This review presents the recent advances in the field of the determinants of the synthesis of extracellular polymeric substances by microalgal cells and the EPS structure. Physical and chemical culture conditions have been analyzed to achieve useful insights into the development of a strategy optimizing EPS production by microalgal cells. The application of microalgal EPS for flocculation and mechanisms involved in this process are also discussed in terms of biomass harvesting. Additionally, the ability of EPS to remove toxic heavy metals has been analyzed. With their flocculation and sorption properties, microalgal EPS are a promising bioproduct that can potentially be used in harvesting algal biomass and wastewater management.

Keywords: extracellular polymeric substances; microalgae cultivation; flocculation; harvesting; biomass

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1. Introduction

Microalgae are a widespread and diverse group of photosynthetic microorganisms. Since microalgal biomass is a rich source of many compounds, e.g., carotenoids, polysaccharides, proteins, fatty acids, lipids, and vitamins, these organisms have a wide range of applications in the food and feed, pharmacy, and cosmetics industries as well as biofuel production [1,2]. Besides valuable intercellular components, microalgae also secrete high-molecular polymers outside the cell referred to as extracellular polymeric substances (EPS). The mechanism of microalgal adaptation to certain stress factors, e.g., light intensity and continuity, temperature, pH, nutrient deficiency, and toxic substances, involves secretion of extracellular polymeric substances [3].

Extracellular polymeric substances are macromolecules with a highly diverse chemical composition and structure related to species and growth conditions. Polysaccharides and proteins are the main components. Additionally, fatty acids, nucleic acids, humic acids, amino acids, and other molecules are present in smaller amounts [4].

Due to their various physiochemical properties, EPS are applied in many branches of industry, e.g., food, paper, textile, biotechnological, pharmaceutical, and cosmetics industries and for water retention [5,6]. Furthermore, EPS exhibit many biomedical properties: antimicrobial, anti-tumor, anti-inflammation, or antioxidative activity; hence, they can be applied in medicine [2,6]. Microalgal EPS have different properties determined by the strain, growth medium composition, environmental stress, and culture age [2,6].

This review describes the latest findings about the determinants of the synthesis of microalgal bioproducts: extracellular polymeric substances and their structure. This

manuscript presents a comprehensive review comparing studies on the individual and mutual effects of culture conditions, cultivation systems, and nutrients on the EPS synthesis with reference to optimization of EPS bioproduction. The use of microalgal extracellular polymeric substances synthesized by microalgae in flocculation and the mechanisms involved in this process in terms of biomass harvesting has been discussed sporadically; therefore, this manuscript provides an overview of relevant reference data published so far.

2. Characteristics of Extracellular Polymeric Substances

Extracellular polymeric substances (EPS) are a group of macromolecular compounds secreted outside the cell. These molecules create a slimy coating around cells, which acts as a protective barrier against some environmental stresses. EPS synthesis in microalgae takes place in the Golgi apparatus [2].

Extracellular polymeric substances are characterized by a variety of chemical structures and a homopolymeric or heteropolymeric structure [7]. Microalgal EPS have a complex structure, which has evolved in response to diverse biotic and abiotic environmental conditions [8]. Depending on their properties and ability to bind to cells, several types of EPS have been reported in the literature. EPS that are weakly bound with cells and are secreted into the medium are referred to as soluble EPS (SL-EPS). In turn, bound EPS represent a type that is bound with cells [3]. This fraction is divided into loosely bound EPS (LB-EPS) and tightly bound EPS (TB-EPS). LB-EPS and TB-EPS show a different composition and functionalities. As reported by Li et al. (2020), LB-EPS show the highest Pb(II) adsorption abilities in comparison to TB-EPS and SB-EPS (soluble EPS) [9]. The role of LB-EPS seems to be more important in response to adverse environmental changes than the role of other EPS fractions; hence, they serve a protection function in algal metabolism [10]. Both EPS fractions exert a different effect on the flocculating ability, as described in Section 5.1. EPS may be involved in nutrient distribution, as the extracellular matrix contains some metabolites released by algal cells, e.g., nucleic acids, enzymes, lipopolysaccharides, and phospholipids. EPS components (proteins and humic substances) may be involved in redox processes as well. Wang et al. (2020) suggest that proteins may be transferred from the inner layer (TB-EPS) to the outside of the cell (LB-EPS and SB-EPS). They also observed a decrease in protein content in TB-EPS in the death phase, whereas the protein content was comparable during the logarithmic growth phase in all EPS fractions [11].

However, the terminology of EPS is inconsistent; therefore, other terms for total EPS can be found in the literature, for example, exopolysaccharides, extracellular polysaccharides, extracellular proteoglycan (EPG), exopolymeric substances, or extracellular polymers. Both fractions exhibit different properties and composition [4,12]. However, Rossi et al. (2016) claim that there are no significant differences. This review focuses on a released fraction referred to as EPS [13].

EPS are composed primarily of polysaccharides, proteins, lipids, and nucleic acids [4]. Extracellular polymeric substances may occur as polysaccharides (with the predominance of sugar components), proteoglycans (containing proteins in sugar chains), or glycoproteins (with proteins as the major elements of the polymer) or they exhibit a different polymer type [14]. EPS have high molecular weight, but there is also a fraction of low molecular weight polymeric substances [15]. The weight of a polymer extracted from *Chlorella vulgaris* JSC-7 was 9.86×10^3 g mol^{-1} [16]. Whereas EPS from *Dictyosphaerium* strains contained a high molecular weight fraction (which was from 1×10^6 g mol^{-1} to, more than 2×10^6 g mol^{-1}), as well as low molecular weight compounds in the range of 9000–56,000 g mol^{-1} [14]. They are mostly negatively charged (hydrophobic) due to the presence of hydrolyzed functional groups. FTIR spectra show the presence of carboxyl, hydroxyl, and amino groups [14,16,17].

Polysaccharides are one of the main components of EPS, accounting for 45–95% of EPS [3]. The sugar units observed in microalgal EPS include glucose, galactose, mannose, arabinose, rhamnose, and fucose (Table 1), with glucose and galactose as the most frequent

sugars. An exception are the EPS derived from *Dunaliella salina*, which are composed mainly of fructose [18].

Table 1. Monosaccharide composition (% *w*/*w*) of extracellular polymeric substances (EPS) produced by Chlorophyta.

Species	fuc	gal	ara	glc	man	xyl	rib	rha	fru	UA	glcA	galA	glcN	Other	Source
Botryococcus brauni [c] CCALA 778	13	26	2	42	15	2		1			<1	2	1		[19]
Botryococcus brauni CCALA 778	31	51		4							3	9		OMe	[20]
Chlamydomonas mexicana	23.7	18.4	14.8	27.8	4.5	5	2.3	3.3		12.8					[21]
Chlamydomonas sajao		77.6	13.5	3.8	1.	7.5		2.3		12.9					[21]
Desmococcus olivaceus		28.8	13.1	27.6	5.9	12.4		7			trace	trace			[22]
Dictiosphaerium chlorelloides	trace	42 [b]	2.4	8 [b]	13.7	4 [b]		18.5						UH 12	[23]
Dictiosphaerium tetrachlorum Ruzicka	11.6	46.6	8	1	7.8	2.9		12				4.8 [a]	1.6 [a]	OMe	[14]
Dictiosphaerium tetrachlorum Fott	15.7	32.8 [b]	1.3	8.9	2.2	18.7		2.5			12.4 [a]		0.8 [a]	OMe	[14]
Dictiosphaerium pulchellum	6.9	35 [b]	2.2	6.4	2.9	9.8		16.6			5.2 [a]		1.4 [a]	OMe	[14]
Dunaliella tertiolecta UTEX LB 999				89											[24]
Graeciella sp.	32	16.3	12.5	12.1	11.5	10.3	2.7	2.3		23					[25]
Neochloris oleoabundans		18.9	4.6	40.7	19	8.7	6.9	1.2							[26]

ara—arabinose, fru—fructose, fuc—fucose, gal—galactose, glc—glucose, man—mannose, rha—rhamnose, rib—ribose, xyl—xylose, UA—uronic acids, glc-A—glucuronic acid, gal-A—galacturonic acid, glcN—glucosamine. [a] Measured by HPAE chromatography; [b] with methyl derivatives; [c] approximate values (read from graph); UH—unidentified methyl hexoses; OMe—methylated sugars.

Monosaccharides can be modified by non-sugar residues such as pyruvate, sulfates, acyl groups, phosphates, O-acetyl-, and O-methyl groups [2,14,16,27,28]. EPS from *Porphyridium* sp. are mainly composed of xylose, glucose, and galactose. Protein accounts for 5.5% of the polymer. The negative charge of EPS is associated with the presence of negatively charged sulfate groups and glucuronic acids [29]. EPS from Chlorophyta is characterized by high contents of galactose [8].

Polysaccharides may form linear or branching structures with regular or irregular morphology related to the presence of various glycosidic bonds [30]. As reported by Delattre et al. (2016), α-(1,3), β-(1,3), β-(1,4), and (2,1)-β are linkages observed in EPS. Most EPS are heteropolymers [2]. However, homopolysaccharides are observed in some cases, for example, the EPS synthesized by *Dunaliella tertiocelata* are (1→ 4)-α-D-glucans [24].

Proteins are one of the main components of microalgal EPS. In terms of their functions, there are non-enzymatic structural proteins, which stabilize the EPS matrix [6], and enzymatic proteins degrading exopolysaccharides, which can be a source of carbon and energy for cells [31]. As reported by Xiao and Zheng (2016), the content of extracellular total protein per mg L^{-1} of supernatant in different microalgae (green microalgae, diatoms, and red algae) is in the range from 0.5 mg L^{-1} to 11 mg L^{-1}; when expressed as weight % of total EPS, it ranges from 0.5% to 16.9% [6]. The analysis of experimental results of the protein content shown in literature reports reveals a large variation among Chlorophyta, which suggests that the protein content in EPS is not a species-specific trait. Analyses of

the composition of *Chlorella vulgaris* EPS showed protein and carbohydrate contents of 25.6 mg L^{-1} and 31.1.mg L^{-1}, respectively [32]. In turn, EPS synthesized by *Chlamydomonas reinhardtii*, i.e., another representative of Chlamydomonadales, contained 42.1% of proteins [33]. Investigations conducted by Liu and Miao (2017) on the effect of $MgSO_4$ on EPS production by *Heynigia riparia* (Chlorophyta) showed a protein level in EPS in the range from 15.27% to 24.19% [34]. The EPS of Chlorophyta typically contains glycoproteins [35].

3. Effect of Culture Conditions on the EPS Synthesis Process

The process of EPS synthesis by microalgal cells is influenced by many factors: culture conditions, such as light, temperature, culture aeration, age of culture, as well as the availability of nutrients, including nitrogen and carbon sources, and their concentration, type of nutrition, and salinity. Optimal conditions for EPS production are different from the optimal conditions for the growth of microalgal cells. Maximum EPS production is accompanied by a decline in the specific growth rate [36]. Therefore, appropriate adjustment of culture conditions may promote EPS synthesis.

3.1. Effect of Light

Light and the light regime are two crucial factors determining microalgal growth and metabolic pathways in autotrophic conditions [37]. The production of EPS by microalgal cells is a light-dependent process. The synthesis of extracellular polysaccharides is a function of the photosynthetic activity and reproduction of autotrophic algal cells; therefore, factors that exert an impact on the photosynthesis process also influence the synthesis of EPS [38].

It has been shown that increasing light intensity can enhance EPS production. As demonstrated by Liqin et al. (2008), EPS production by *Porphyridium cruentum* was enhanced with an increase in light intensity up to the light saturation point, above which the production declined (Table 2) [39]. The highest EPS production level was achieved at the light intensity of 80 μ $m^{-2}s^{-1}$ [39]. These results are in agreement with those reported by You and Barnett (2004), who observed an increase in extracellular polysaccharide production by *Porphyridium cruentum* in the light intensity range from 39 to 70 μEm^{-2} s^{-1} [40]. Above this level, i.e., at the light saturation point, the polysaccharide production was found to decline. However, in a study conducted by Clément-Larosière (2014), specific EPS production of *C. vulgaris* (excreted per cell) decreased with increasing light intensity (50, 120, 180 μmol m^{-2} s^{-1}). The highest specific EPS production was recorded at the lowest light intensity of 50 μmol m^{-2} s^{-1} [41].

Research on the influence of light intensity on EPS production was also carried out on cyanobacteria (Table 2). Continuous light and high light intensity were found to favor EPS production by Cyanobacteria [42]. *Cyanobacterium aponinum* is able to synthesize ESP at light intensity of 500 μmol photons m^{-2} s^{-1} [43], likewise *Anabena* spp. with maximum EPS production were detected at 460 μmol photons m^{-2} s^{-1} [44]. Chentir et al. (2017) observed an increase in EPS production by Cyanobacteria with increasing light intensity [45]. However, after salt stress application, there was no such a relationship or the amount of EPS was decreasing [45]. Similarly, other studies confirm the increase in the EPS yield accompanying enhanced light intensity. In investigations of *Nostoc* sp. cyanobacteria, an increase in light intensity from 40 μE m^{-2} s^{-1} to 80 μE m^{-2} s^{-1} contributed to intensified EPS production and higher protein content in EPS [46].

Table 2. Effect of light intensity on EPS production.

Species	Light Intensity	EPS Production	References
Porphyridium curentum	30 μ Em^{-2} s^{-1}	0.56 ± 0.06 (g L^{-1})	[39]
	40 μ Em^{-2} s^{-1}	0.66 ± 0.08 (g L^{-1})	
	50 μ Em^{-2} s^{-1}	0.79 ± 0.06 (g L^{-1})	
	60 μ Em^{-2} s^{-1}	0.82 ± 0.10 (g L^{-1})	
	80 μ Em^{-2} s^{-1}	0.95 ± 0.09 (g L^{-1})	
	100 μ Em^{-2} s^{-1}	0.77 ± 0.07 (g L^{-1})	
	150 μ Em^{-2} s^{-1}	0.31 ± 0.06 (g L^{-1})	
Porphyridium cruentum	39 μ Em^{-2} s^{-1}	0.18 (g L^{-1})	[40]
	48 μ Em^{-2} s^{-1}	0.30 (g L^{-1})	
	60 μ Em^{-2} s^{-1}	0.55 (g L^{-1})	
	70 μ Em^{-2} s^{-1}	0.95 (g L^{-1})	
	90 μ Em^{-2} s^{-1}	0.45 (g L^{-1})	
Chlorella vulgaris	50 μmol m^{-2} s^{-1}	6.79 ± 0.3 (pg CO_2 $cell^{-1}$)	[41]
	120 μmol m^{-2} s^{-1}	5.58 ± 0.27 (pg CO_2 $cell^{-1}$)	
	180 μmol m^{-2} s^{-1}	4.58 ± 0.32 (pg CO_2 $cell^{-1}$)	
Cyanobacterium aponinum	15 μmol m^{-2} s^{-1}	~84 (mg g_{DW}^{-1})	[43]
	40 μmol m^{-2} s^{-1}	~83 (mg g_{DW}^{-1})	
	70 μmol m^{-2} s^{-1}	~80 (mg g_{DW}^{-1})	
	100 μmol m^{-2} s^{-1}	~70 (mg g_{DW}^{-1})	
	150 μmol m^{-2} s^{-1}	~60 (mg g_{DW}^{-1})	
	300 μmol m^{-2} s^{-1}	~60 (mg g_{DW}^{-1})	
	500 μmol m^{-2} s^{-1}	~70 (mg g_{DW}^{-1})	
	650 μmol m^{-2} s^{-1}	~63 (mg g_{DW}^{-1})	
Anabaena sp. ATCC 33047	115 μ Em^{-2} s^{-1}	~2 (g L^{-1})	[44]
	185 μ Em^{-2} s^{-1}	~3 (g L^{-1})	
	345 μ Em^{-2} s^{-1}	~2.5 (g L^{-1})	
	460 μ Em^{-2} s^{-1}	~12.5 (g L^{-1})	
	920 μ Em^{-2} s^{-1}	~12 (g L^{-1})	
	1840 μ Em^{-2} s^{-1}	~11 (g L^{-1})	
Spirulina sp.	10 μmol m^{-2} s^{-1}	0.152 (g g^{-1})	[45]
	65 μmol m^{-2} s^{-1}	0.192 (g g^{-1})	
	120 μmol m^{-2} s^{-1}	0.454 (g g^{-1})	

The irradiation time is another factor influencing EPS production by *P. cruentum* [39]. The maximum polysaccharide production in the analyzed range of light/dark cycle periods (24:0 h, 16:8 h, 18:6 h, 12:12 h, 10:14 h) was observed under the 18:6 h light/dark cycle. The beneficial impact of light: dark cycles on EPS productivity in comparison with the use of continuous illumination was reported in cultivation of *Botryococcus braunii* under high light intensity (2000 μmol m^{-2} s^{-1}) [20]. In turn, a study conducted by Lupi et al. (1994) indicated that the concentration of exopolysaccharide in *Botryococcus braunii* UC 58 decreased after introduction of a photoperiod (14 h light/10 h dark) in comparison with the use of continuous illumination [47]. In turn, a similar level of specific production of exopolysaccharide was found when the same phase of growth was considered. The light intensity was lower than that used by García-Cubero et al. (2018) [20], i.e., it was 250 μmol m^{-2} s^{-1} [47].

Another factor that has an impact on the EPS yield is the light wavelength. Results showed that blue light (400–500 nm) and red light (600–700 nm) increase extracellular polysaccharide production [40]. In a study on *Porphyridium purpureum*, the application of multi-chromatic LED wavelengths (red, green, and blue: 620–625, 520–525, and 465–468 nm, respectively) in the culture induced an increase in the EPS yield [40]. In turn, the use of a specific narrow light-emitting diode: red (620–625 nm), green (520–525 nm), and blue (465–468 nm) wavelengths did not cause differences in the EPS yield [48]. The results of a

study conducted by Medina-Cabrera et al. (2020) indicated white light as the most suitable type for EPS production in *Porphyridum* strains [49].

Studies on the effect of light intensity on EPS production demonstrate that this factor is associated with other environmental variables. Dayananda et al. (2007) tested the impact of continuous light and photoperiod (16 h light: 8 h dark) on EPS synthesis in two *B. braunii* strains, SAG 30.81 and LB 572, cultivated in shaken and non-shaken cultures on different growth media [50]. Cultures growing in the continuous light in the shaking variant were characterized by higher production of exopolysaccharide than cultures conducted with the photoperiod and continuous light without shaking [50]. These investigation results are in agreement with the findings reported by Cordoba-Castro et al. (2012), who showed an effect of light intensity, agitation, and carbon source availability on EPS production by *Scenedesmus obliquus* [51]. The highest concentration of EPS was observed at the highest of the three light intensity values applied in the study (80, 130, 180 μE m^{-2} s^{-1}). The increase in the light intensity from 80 to 180 contributed to an increase in the EPS concentration from 16.17 mg L^{-1} to 24 mg L^{-1} in the variant based on the use of the maximum values of the three variables tested [51]. The effect of the mixing time, light, and glycine on the production of EPS by *Chlorella vulgaris* was confirmed by Shen et al. (2015) [52]. The maximum levels of bound EPS production were 47.3 mg g^{-1} at 250 μmol m^{-2} s^{-1} light intensity (mixing time 3 days, glycine concentration of 0.1 g L^{-1}) and 44.2 mg g^{-1} at 125 μmol m^{-2} s^{-1} light intensity (mixing time 3 days, glycine concentration of 1 g L^{-1}) [52]. Light intensity is an important determinant of EPS synthesis by photoautotrophic microalgal cells. Most studies have shown enhanced EPS production by higher light intensity up to the light saturation point. The enhancement of EPS production in the conditions of increasing light intensity may be related to the protection of the cell against the harmful effects of the external factors. Results of investigations of the effect of light intensity also indicate that the EPS production cell response at high light intensity is species-specific. An accurate comparison is difficult, as different values are defined as high light intensity in different studies; additionally, investigations of EPS production often do not take into account the cellular biomass productivity.

3.2. Effect of Temperature

Temperature is one of the key parameters influencing metabolic processes in microalgal cells. Temperature can also have an impact on EPS production, and its optimal value for EPS synthesis is species-specific. In investigations of the effect of light intensity and temperature on EPS production by a thermophilic *Graesiella* strain, the highest EPS yield was obtained at the highest temperature tested, i.e., 40 °C, and at the lowest light intensity of 20 μmol m^{-2} s^{-1} (Table 3) [53]. As indicated by the authors, the increase in temperature was the main determinant of the higher EPS production, whereas no correlation was found between the amount of EPS and light intensity. The increase in the EPS synthesis by the thermophilic strain induced by the temperature rise from 20 to 40 °C is indicated as a process of protection of cellular metabolic pathways [53]. The effects of irradiance and temperature on EPS production were also shown by Kumar et al. 2017 in their study of *Dictyosphaerium chlorelloides* [36]. The maximum production of EPS was observed at a moderate temperature (25.7 °C) and light intensity of 50.3 μmol m^{-2} s^{-1}. At the optimal temperature ensuring maximum EPS production, a decline in the specific growth rate of *D. chlorelloides* was observed [36]. Investigations of the production of soluble EPS by *B. braunii* showed the highest EPS productivity in the temperature range of 25–30 °C, which was consistent with the optimum range for algal growth [54].

Temperature seems to be an important factor in EPS production, and its optimum value for EPS synthesis is species-specific. To optimize EPS production, it is important to select an appropriate strain with a temperature range that is optimal for both algal growth and EPS production.

Table 3. Effect of temperature on EPS production.

Species	Temperature (°C)	EPS Production	References
Graesiella sp.	40	11.7 mg L^{-1} day^{-1}	[53]
Dictyosphaerium chlorelloides	25.7	1075 mg L^{-1}	[36]
Botryococcus braunii UC 58	25–30	4500–5500 mg L^{-1}	[54]

3.3. Effect of Growth Phase

One of the determinants of the amount of EPS synthesized by algal cells is the growth phase. The largest amount of synthesized EPS was detected during the stationary phase or the death phase [28,55]. The impact of the growth phase (logarithmic and stationary phases) on the rate of polysaccharide release was also shown in studies of marine diatoms [56]. The end of the growth phase is often correlated with nitrate starvation conditions, which induce changes in the C/N ratio, and the beginning of extracellular polysaccharide production [29,57]. Li et al. (2020) reported a gradual increase in the EPS content during the logarithmic growth phase in *Porphyridium purpureum* [58].

3.4. Cultivation Systems

One of the strategies to optimize the production of exopolysaccharide is the two-stage cultivation system. In the first stage, algal cells are cultivated in optimum conditions to allow an increase in the biomass concentration. The second stage consists in application of a stress factor or introduction of changes in culture conditions to enhance the production of the target metabolite. Medina-Cabrera et al. (2020) showed that the two-stage cultivation system strategy enhanced the production of EPS in *Porphyridium* strains *P. sordidum* and *P. purpureum* [49]. The use of semi-continuous culture was found to lead to an increase in EPS productivity in comparison with batch cultures of *Porphyridium marinum* [59].

Another solution to increase the production of EPS may be provided by the co-culture system. In the study conducted by Angelis et al. (2012), microalgae (*Chlorella* sp.) and cyanobacteria (*Spirulina* sp.) were cultivated with *Basidiomycetes* [60]. Algal and fungal substrate mixed at a 1:1 ratio was the growth medium in this method. The results showed that the co-culture increased and accelerated EPS synthesis, in contrast to monocultures. Each of the strains used in those experiments was able to produce EPS, but the polymer obtained from the co-culture was a mix of both EPS synthesized by the microalgae and fungi separately, with predominance of fungal EPS. The presence of fungal strains is a stress factor for algae [60]. As underlined by the authors, the increased EPS production in the co-culture conditions may be associated with nutrient competition, response to toxins, growth factors released by other microorganisms, or increased amounts of carbon dioxide and vitamins [13,61,62]. Co-cultures of microalgae and other microorganisms could be a promising and useful biotechnological tool to enhance production of numerous valuable products.

4. Optimization of the Growth Medium for Production of Extracellular Polymeric Substances

4.1. Nitrogen

Investigations on the optimization of the medium to increase EPS production highlight the role of sodium nitrate. As shown by Bafana (2013), $NaNO_3$ and $CaCl_2$ significantly increased EPS production by *Chlamydomonas reinhardtii* [63]. A pH value of 7 was the optimum value for EPS production. The addition of EDTA exerted a negative effect on this process [18]. Similarly, other studies conducted on the red microalga *Porphyridium* sp. with the use of potassium nitrate, ammonium hydrocarbonate, and ammonium nitrate resulted in the highest productivity of EPS in the presence of sodium nitrate [64]. Among the different sources of nitrogen (potassium nitrate, urea, and ammonium carbonate), the highest production of EPS was detected in the presence of nitrate and the lowest yield was obtained upon the application of urea, where the pH value was approximately 5 in

the presence of urea and ammonium carbonate and 7 for potassium nitrate [47]. This may suggest that the pH value of the growth medium determined by the nitrogen source has some influence on the EPS synthesis.

One of the factors contributing to an increase in EPS synthesis by microalgal cells is the nitrogen nutrient limitation. Overproduction of EPS in nitrogen-deficient conditions (BG11 without $NaNO_3$; 2%CO_2) was confirmed by Sasaki et al. (2020) in investigations of microalgae *Parachlorella* sp. BX1.5 [65]. A large amount of acidic EPS was produced in such conditions. The effect of the reduced nitrogen content in the medium on higher EPS production by *B. braunii* was confirmed by Bayona and Garcés (2014) [66]. Lupi et al. (1994) compared the impact of three concentrations of potassium nitrate (0.5, 2, and 8 mM) on EPS synthesis in *B. braunii* [47]. The highest EPS-specific productivity (g g^{-1} dry biomass) was obtained in the presence of 2 mM KNO_3. Cultures with 0.5 mM addition contained a similar amount of EPS, but the production ceased after 20 days due to nitrogen depletion (while both other cultures were cultivated up to day 36). The lowest specific production of EPS was detected in a culture supplied with 8 mM KNO_3. However, the highest EPS yield (g L^{-1}) at the end of the culture time was noted in the presence of 8 mM KNO_3 [47]. The nitrogen (nitrate) limitation increases specific EPS production. However, it also induces a strong decrease in the biomass concentration; thus, the yield of EPS may be much lower. In turn, it has been shown that high N concentrations in wastewater result in high protein content in EPS [67].

In addition to N availability, the carbon-to-nitrogen ratio was found to improve EPS production. An increased C/N ratio (in nitrogen limitation conditions) in the medium was reported to promote EPS release by *Porphyridium purpureum* [58]. From the tested 0.96 (low), 3.84 (medium), and 12.82 (high) ratios, the most effective productivity at the end of culture time (day 20) was estimated at 3.84 [58]. However, the high C/N ratio accelerated the EPS synthesis, and the amount of secreted polysaccharides on day 12 was approximately 40% higher than at the medium ratio. In turn, the EPS productivity at the low C/N ratio was significantly lower than in the other variants, but strongly increased in the last days of cultivation and finally reached 1.25 g L^{-1}. As observed by the authors, a high C/N ratio may affect protein degradation. The resulting new nitrogen source can be used for EPS and lipid synthesis [58]. Supplementation with glycerol to increase the C/N ratio under nitrogen limitation additionally increased the EPS yield [60]. In turn, a decline in the C/N ratio led to changes in the EPS composition. In investigations of mixed microalgae (with predominance of *Scenedesmus* sp.), a fivefold increase in the NO_3 concentration in the municipal effluent resulted in reduction of C/N, from 41.4 to 2.9, which contributed to an increase in the protein content in bound EPS [64]. Data shown by Razaghi et al. (2014) indicate that the optimal N/P ratio for carbohydrate synthesis is less than 4.9:1, whereas biomass production requires a 35:1–50:1 ratio [68]. The results are similar to those obtained by Soanen et al. (2016), who achieved optimal EPS production at an N/P ratio of 3.97 [59]. In turn, Nur et al. (2019) showed that, in the case of *Phaeodactylum tricornutum*, the N/P molar ratio had no significant influence on EPS production in contrast to the nitrogen source and temperature [69].

4.2. Phosphorus

The influence of phosphate limitation on the growth and EPS synthesis by the diatom *Cylindrotheca fusiformis* was studied by Magaletti et al. (2004) [70]. The results indicate that a low phosphate concentration has an impact not only on the increase in total EPS synthesis but also on the composition of the dissolved carbohydrates [70]. A comparison of the influence of one-stage and two-stage phosphorus depletion on EPS synthesis by cyanobacteria *Anabaena variabilis* and *Microcystis aeruginosa* demonstrated that the two-stage phosphorus depletion resulted in an approximately threefold higher EPS yield in comparison to the one-stage depletion in the case of both species [71]. Similar to nitrogen depletion, phosphorus starvation causes inhibition of culture growth; hence, two-stage cultivation contributes to higher EPS yields [71]. As shown in a study of marine

diatoms, under nutrient limitation (variations in the nitrogen/phosphorous/silicon ratio), the process of extracellular polysaccharide excretion is species-specific and none of the limiting factors influences EPS release in the same way [56].

4.3. Carbon

An important determinant of EPS release by cells is the carbon metabolic mode [13]. In mixotrophic and heterotrophic growth conditions, carbon contained in the medium contributes to increased accumulation of intracellular polysaccharides and soluble extracellular polysaccharides [29]. This was confirmed by investigations conducted by Zhang et al. (2019) demonstrating higher EPS production by *Chlorella vulgaris* in mixotrophic conditions, i.e., 364.3 mg L^{-1} (BG11 with glucose 5 g L^{-1}), in comparison with EPS amounts produced in autotrophic conditions: 235 mg L^{-1} [72]. Cheirslip et al. (2016) reported that *Chlorella* sp. showed higher EPS production upon an increase in the glucose concentration in the growth medium from 0.2% to 1% (w/v) [73]. In the semi-continuous cultivation system, the amount of EPS in each cycle ranged from approximately 1.25 g L^{-1} in the first three cycles to 1.45 g L^{-1} in the fourth and fifth cycles (on days 5 and 6, respectively). Higher EPS production in heterotrophic (0.5 g L^{-1} EPS) vs. autotrophic conditions (0.39 g L^{-1} EPS) has been reported [73]. Investigations of the impact of the photoautotrophic, mixotrophic, and heterotrophic cultivation modes on EPS production by *Neochloris oleabundans* demonstrated that only mixotrophic cultivation (with 20% lactose and CO_2 from air as a carbon source) ensured production of large quantities of EPS: 5 g L^{-1} [74]. No EPS were detected in the same mixotrophic conditions with glucose as a carbon source; likewise in the heterotrophic and photoautotrophic conditions (5% CO_2). The authors highlight the role of lactose as a precursor for the biosynthesis of EPS [74]. In turn, in the case of *Nostoc flagelliforme* cultured in heterotrophic conditions with glucose, sucrose, and fructose as a carbon source, the highest levels of EPS production were found in the glucose-supplemented variant [75].

In turn, in the study conducted by Přibyl and Cepák (2019), EPS production by *Dictyosphaerium chlorelloides* did not differ between the mixotrophic and photoautotrophic modes [76]. The highest level of algal organic matter (which is closely related to EPS) produced by *Scenedesmus obliquus* in photoautotrophic conditions was reported by Choi et al. (2019) [77].

Carbon sources were found to exert an effect on EPS production by *Chlorella* sp. [78]. Under 0.1% salinity, *Chlorella* sp. was found to produce the largest amounts of EPS in cultures with ethanol and glycine as a carbon source. In turn, at a higher salinity level, i.e., 3.5%, the concentration of EPS increased, and the highest EPS concentration (31 mg/L) was achieved in glycine-supplemented cultures [78]. Fabregas et al. (1999) cultivated red microalga *P. cruteum* using 15% of potato extract as an organic carbon source [79]. The mixotrophic growth yielded 0.33 g L^{-1} EPS, whereas the EPS level in autotrophic conditions reached 0.13 g L^{-1} [79]. Literature provides few reports on mixotrophy and heterotrophy in microalgal cultivation. Nevertheless, the data cited above show that supplementation with an organic carbon source may be a promising factor increasing EPS productivity.

In the photoautotrophic mode, microalgal cells utilize carbon dioxide as a carbon source. At low carbon dioxide concentrations, the carbon-concentrating mechanism leads to concentration of inorganic carbon from the environment to optimize carbon acquisition [13]. A study conducted by Clement-Larosiere et al. (2014) demonstrated that an increase in the CO_2 concentration from 2% to 13% was accompanied by a 1.4-fold increase in the total exopolymer excretion by *Chlorella vulgaris*, but the EPS concentration per cell was not significantly altered [41]. This may have been related to the limited availability of light in the culture with higher carbon availability (13%), which led to limitation of further carbon fixation and EPS secretion [41]. In their study, Li et al. (2016) highlighted the synergistic effect of CO_2 and temperature on EPS productivity in the green alga *Scenedesmus acuminatus*, the diatom *Cyclotella meneghiniana*, and the cyanobacterium *Microcystis aeruginosa* [38]. The amount of extracellular carbohydrates in the *S. acuminatus* culture increased with the rising temperature and CO_2. In the case of *C. meneghiniana* and *M. aeruginosa*, the level of

extracellular carbohydrates increased at the higher temperature values [38]. The secretion of exopolysaccharides by microalgal cells in excess carbon dioxide conditions may be a result of overflow metabolism, which is defined as the excess binding of carbon dioxide in relation to growth requirements [80].

4.4. Wastewater

Investigations of microalgal EPS of *Chlorella* sp. in wastewater cultivation conditions demonstrate the effect of higher initial concentrations of nutrients and/or organic substrate on higher production of soluble EPS [81]. The increase in their concentration was accompanied by a decline in the concentration of carbohydrates and an increase in the protein concentration in bound EPS [81]. Wang and Park (2015) cultured *Micractinium* sp. and *Chlorella* sp. on secondary effluent mixed with primary effluent [82]. The use of wastewater for the algal culture resulted in relatively higher protein content in EPS [82].

4.5. Metals

As reported in literature, environmental stresses lead to increased production of extracellular polysaccharides, while there are few reports on the effect of metals on EPS production [83]. Chen et al. (2015) studied the influence of cadmium stress on the growth rate and EPS production in *C. vulgaris* [84]. In the culture supplied with 1 mg L^{-1} Cd^{2+}, the amount of soluble and bounded EPS was higher in comparison to the control [84]. Mona and Kaushik (2015) examined the effect of chromium and cobalt on EPS production by *Nostoc linckia* [85]. The results showed that the presence of the tested metals significantly increased the synthesis of EPS, i.e., about 5 times by cobalt and 4 times by chromium. The highest EPS concentration was achieved at 40 mg L^{-1} of Co^{2+} and Cr^{6+} [85]. It has been found that a cyanobacterial *Synechocystis* sp. strain produces increased amounts of EPS when exposed to chromium, cadmium, and combination of the metals (chromium + cadmium) applied at 35 ppm [83].

4.6. Effect of pH

EPS synthesis in unicellular algae begins in the stationary phase when pH of the culture medium rises to 8–9. This is a natural change resulting from culture aging. De Philippis et al. (1991) did not observe an effect of more alkaline pH compared with the physiological value [86]. However, in investigations of the effect of different nitrogen sources on EPS production, the pH value of a growth medium with urea and ammonium carbonate addition was about 5, whereas pH 7 was determined in the presence of potassium nitrate. The results showed that EPS synthesis was more effective in a culture with KNO_3 addition. Differences in the EPS yield may be connected with pH of the growth medium rather than the nitrogen source [54].

5. Flocculation

Due to the large amount of acidic functional groups and long-chain molecules, EPS can be used as natural flocculants [6]. Nowadays, microalgae are mostly cultured for biomass production. As reported by Aljuboori et al. (2016), the addition of $ZnCl_2$ to EPS-producing *Scenedesmus quadricauda* suspension is sufficient to induce flocculation and thus biomass harvesting [87]. Nowadays, the use of microalgal EPS is limited to high-value products, e.g., pharmaceuticals and cosmetics. This is caused by the high cost of algal biomass harvesting and the low concentration of EPS in the growth medium [8]. The development of an effective and low-cost method for EPS-dependent flocculation may potentially reduce these costs and increase the use of microalgal EPS in industry. Flocculation is one of the methods of separation of the solid fraction from liquid suspensions. The process consists in association of small particles in the colloid solution to form larger aggregates with high molecular weight, allowing the particles to settle [88]. The flocculation process is applied in algal harvesting [89], wastewater treatment [84], paper manufacture, clarification of

sugarcane juice in the sugar industry [90,91], metal binding [92], and removal of color from textile wastewaters [93].

Substances used as flocculants must have specific properties, e.g., molecular weight, type, and ionic strength. Large amounts and small sizes of colloidal particles as well as their low distribution increase flocculation efficiency [91]. Synthetic polymers (polyacrylamide) cause contamination of harvested biomass with metals, metal hydroxides, and non-biodegradable polymers. Additionally, polyacrylamide-based flocculants may be contaminated by acrylamide monomers, which exhibit neurotoxicity and carcinogenicity [94]. This excludes further processing in food, feed, or other applications requiring contamination-free biomass [95,96].

In turn, since they are non-toxic, biodegradable, and inexpensive, natural polymers are the most environmentally friendly group of this type of compound [91]. Natural polymers used in the flocculation process comprise EPS, chitosan, cellulose, starch, and natural gums with their derivatives. They are characterized by high molecular weight, fixed molecular constitution, and mostly long chains. One of the prerequisites for flocculation of microalgal biomass to occur is the positive charge of the polymer (since the surface charge of algal cells is negative); however, it is not common in the environment [91,95]. Inorganic salts (ferric chloride, aluminium sulphate) are coagulants and may induce polymer flocculation [95].

5.1. Extracellular Polymeric Substances in Flocculation

Extracellular polymeric substances have high molecular weight, long chains, and numerous functional groups that are crucial for effective flocculating activity [6]. EPS are mainly negatively charged due to the dominance of carboxyl and hydroxyl residues, mostly from uronic acids and proteins [97]. Badireddy el al. (2010) showed the role of O-acetylated and acidic carbohydrates and the secondary structure of proteins, which are changing during the exponential and stationary phases, in bioflocculation of activated sludge microorganisms [28]. An essential issue is the use of an appropriate biopolymer concentration to achieve flocculation. As suggested by Liu et al. (2015), an insufficient concentration of the bioflocculant is ineffective in the flocculation process, as the polymer chains cannot bind to all cells [98]. In turn, an excessive dose of the negatively charged biopolymer increases repulsion between particles and results in greater stability of the suspension [98].

The flocculating properties are associated with the type of EPS. It has been reported that TB-EPS have much higher flocculating abilities than LB-EPS [17]. A relatively low amount of LB-EPS should be provided to trigger the flocculation process; otherwise, the free polymer may impede cell aggregation. Another explanation is based on the DLVO theory. According to this theory, TB-EPS have no interaction energy and the LB-EPS fraction is the determinant of the flocculating abilities [99].

The process of flocculation with EPS depends on the presence of metal ions, which partly neutralize the negative charge of the cell surface and facilitate polymer adsorption. Divalent cations may also cross-link polymers attached to different cells [100]. The most common and effective are Ca^{2+} [101], Mg^{2+} [98], and Zn^{2+} [87,102]. The addition of $ZnCl_2$ was reported to increase the flocculating efficiency of EPS derived from *Scenedemus quadricauda* from 26.5% to 82.7% [87]. In contrast, Liu et al. (2014) observed no influence of Mg^{2+} and Ca^{2+} ions on flocculation of *Chlorella zofingiensis* and *C. vulgaris* [103]. Therefore, the main mechanism involved in EPS bioflocculation is cation bridging [99]. The pH value is another factor influencing the flocculating activity. Biopolymers differ in the dissociation of functional groups at different pH values. This determines the number and type of dissociated groups and the effectiveness of polymer adsorption [104]. The thermal properties of the biopolymer require adjustment of temperature. It has been found that EPS mostly consisting of sugars have high thermal stability; however, when the content of proteins increases, their stability decreases [100]. For example, Surendhiran and Vijay (2013) observed the highest bioflocculation efficiency of *Chlorella salina* at 30.63 °C (tested

temperature range: 20–40 °C) with a microbial flocculant [105]. There are few reports on self-flocculating microalgae synthesizing EPS with flocculating abilities [16,17,87,106].

5.2. Mechanism of EPS-Dependent Flocculation

Aggregate formation in microalgal culture is difficult due to the small size and low density of cells [107]. Additionally, most functional groups on the cell surface are anionic (carboxyl, hydroxyl, and phosphate); hence, even if the cells are relatively close to each other, the electrostatic repulsive forces keep them dispersed [108].

The mechanism of flocculation depends on different factors, including the molecular weight, concentration, and type of the flocculant, pH, ionic strength, and the size of particles in the solution [89,91]. To initiate this process in algal culture, it is necessary to neutralize the negatively charged cell surface [109]. There are several ways of formation of flocculants: double layer compression, charge neutralization (patching and sweeping), and bridging [88].

5.2.1. Double Layer Compression (DLVO Theory)

Particles in colloid suspensions are coated with an electrical double layer. This structure consists of two layers. The first (stern) layer surrounding a negatively charged cell is created by cations, which adhere to the particle strongly. In the second layer (diffuse), the concentration of ions decreases as the distance from the molecule increases (Figure 1). Hence, the force of the interaction with the opposite ion decreases and it can diffuse to the suspension. Finally, the charge equalizes with the environment. The potential on the outer side of the diffusion layer is called zeta potential (ζ). It determines repulsion between particles and sustains the dispersion of the suspension [110]. According to the DLVO theory, which explains the double layer compression, the total energy is the sum of the electrical double layer (repulsion) and Lifshitz-van der Waals energy (attraction). Attractive forces depend on the ionic strength of the suspension. When the suspension has high ionic strength, the thickness of the double layer is reduced. For aggregation to occur, the force of attraction must be higher than repulsion. This can be achieved by addition of Na^+, K^+, Ca^{2+}, or Fe^{2+} cations to the suspension with low ionic strength [99,110]. This type of flocculation can occur, for example, as a consequence of mixing seawater with freshwater, which have high and low ionic strength, respectively [88]. In algal cells there are LB-EPS and TB-EPS, which may be considered as stern and diffuse layers [95].

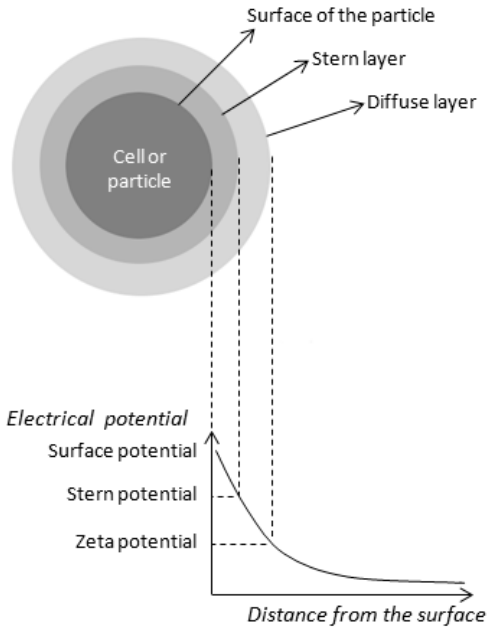

Figure 1. Scheme of double layer compression (DLVO theory).

5.2.2. Charge Neutralization

Negatively charged cells are surrounded by a double layer, which keeps them dispersed. In the presence of positively charged polymers or hydrolyzing metal salts (coagulants), the zeta potential changes to values close to zero. This reduces repulsion forces and facilitates interactions between cells. The effectiveness of this type of flocculation depends on the charge density of the polymer. The amount of the flocculant used should be quite precisely suited to the biomass concentration and charge [111]. One of the types of flocculation involving charge neutralization is the electrostatic patch model (Figure 2).

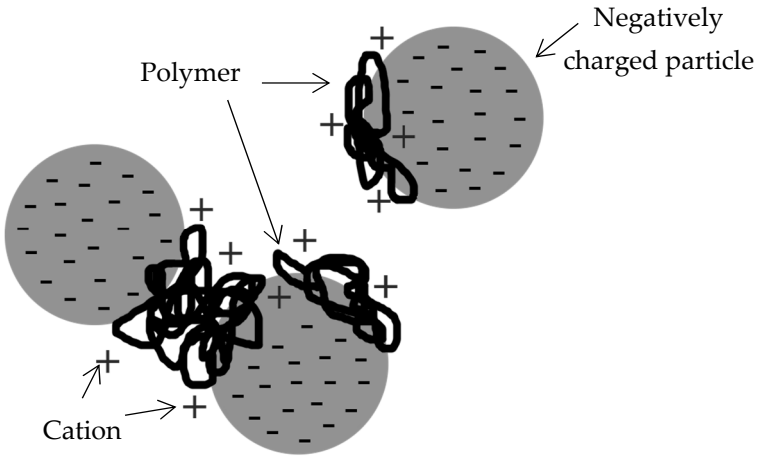

Figure 2. Mechanism of patching flocculation.

Polymers with high charge density attach strongly and locally to the cell surface, thereby forming positively charged patches, which are tightly fitted to the surface and can match other cells. In this way, aggregates are gradually becoming larger and heavier and finally sediment. Short-chain polymers are often engaged in this mechanism [89].

5.2.3. Bridging

The mechanism of linking particles by the adsorption polymer to the cell surface involves long-chain EPS. The functional groups of the polymer attach to the surface locally in several areas to form specific structures: loops, trains, and free tails (Figure 3) [88,112].

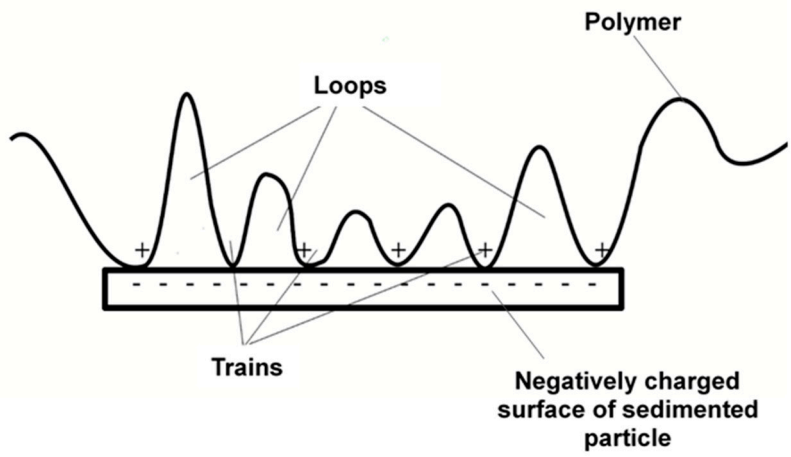

Figure 3. Polymer adsorption on surface of solid particle.

The affinity of a single train may not be very strong, as there are more sites of interactions. Long-chain non-branched polymers are preferred for effective bridging, as this increases the possibility of interactions with more than one particle in a dispersed suspension (Figure 4) [88].

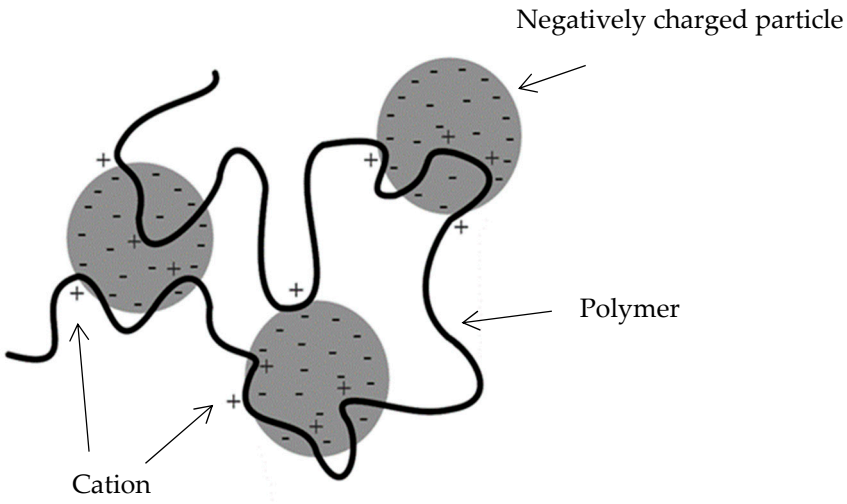

Figure 4. Mechanism of bridging flocculation.

With time, the distance between cells decreases and the same polymer can aggregate more particles using the free part of the chains or loops, resulting in an increase in the flocculant size and weight. In addition, aggregates formed in this way are more stable than others. The bridging mechanism occurs in the presence of non-ionic polymers with high molecular weight and low charge density polyelectrolytes with high molecular mass [88,99,112].

Bridging can be also an effect of the cation bridging mechanism. Divalent cations bind to the anionic groups of the biopolymer surrounding microorganisms and cross-link the matrix. This kind of flocculation depends on the ratio of monovalent to divalent cations, which should be equal to or less than 2 [110,113]. Bridging with divalent cations is non-specific, whereas these cations bind to the polymer in a specific way and may form a regular structure, according to the alginate theory.

5.3. Bioflocculation

Bioflocculation is a method in which other microorganisms (microalgae, bacteria, fungi) or their products (extracellular polymeric substances) are used to induce particle aggregation [114]. In contrast to biopolymers, which are costly, bioflocculation can be used for large-scale applications [115]. The first report on microalgal bioflocculation was presented by Schuessler [116]. The investigations demonstrated potential flocculating properties of EPS [116]. *Chlorophyta* microalgae and their flocculation efficiency are presented in Table 4. Most of them are effective in the first 30 min. Flocculation efficiency (FE) [%] is the amount of settled algal biomass as a result of autoflocculation [16,27,106,116–118], EPS addition [17,119], the use of autoflocculating algae to harvest non-flocculating species [120], or the presence of $ZnCl_2$ [87]. Aljuboori et al. (2016) investigated the role of EPS surrounding *Scenedesmus quadricauda* cells in flocculation [87]. It was shown that EPS-free cells were unable to flocculate in the presence of a coagulant ($ZnCl_2$) [87]. In turn, the use of untreated cells resulted in flocculation of 80–85% of non-flocculating *C. vulgaris* CNW-11 and *S. obliquus* FSP-3 biomass [17]. Similar results were observed for self-flocculating *Desmodesmus* sp. PW1. In this case, the addition of *Desmodesmus* sp. PW1 to non-flocculating microalgae *C. vulgaris*, *S.obliquus*, and *N. oceanica* resulted in an increase in flocculation efficiency from 10.5%, 12.3%, and 9.1% to 80.5%, 89.4%, and 54.7%, respectively [118].

Table 4. Culture conditions and flocculation efficiency (FE) of Chlorophyta.

Species	Culture Conditions	FE [%] *	Settling Time [min]	Source
Chlorella vulgaris JSC-7	BBM medium, 28 °C, 11/13 h light/dark cycle, light intensity 25 µmol $m^{-2}s^{-1}$	76.3	30	[16]
Chlorococcum sp. GD	Simulated secondary effluent, 25 °C, 14/10 h light/dark cycle, light intensity 3000 lux	84.4 47.7	180 30	[106]
Scenedesmus obliquus AS-6-1	DM medium, 28 °C, 14/10 h light/dark cycle, light intensity 60 µmol $m^{-2}s^{-1}$	80–85	30	[17]
Scenedesmus quadricauda	BG-11 medium, 26 °C, continuous illumination, light intensity 10,000 lux, aeration min^{-1}, initial pH 7.1, $ZnCl_2$, photobioreactor	86.7	30	[87]
Neochloris texensis	Freshwater medium, 25 °C, aeration 3 L min^{-1} with 2% CO_2, light intensity 50 µmol m^{-2} s^{-1},	55	20	[120]
Tetraselmis suecica	Marine medium containing NaCl, 25 °C, aeration 3 L min^{-1} with 2% CO_2, light intensity 50 µmol m^{-2} s^{-1}, 100 rpm	72	20	[120]
Ankistrodesmus falcatus	Freshwater, 25 °C, aeration 3 L min^{-1} with 2% CO_2, 16/8 h light/dark, light intensity 50 µmol m^{-2} s^{-1}, 100 rpm	50	20	[120]
Chlorococcum sp.	MA medium, pH 6.8, 30 °C, 14/10 h light/dark cycle, 3 weeks	75	10	[119]
Nannochloropsis oculata	F medium, 20 °C, 12/12 h light/dark cycle, light intensity 500 µmol m^{-2} s^{-1}, 8 days, aeration with 400 ppm CO_2, 110 rpm (flocculating pH 10.4 at the end)	90	10	[117]
Scenedesmus rubescens SX	Synthetic wastewater, pH 7.8, 25 °C, 14/10 h light/dark cycle, light intensity 55.5 µmol m^{-2} s^{-1}, 160 rpm, 8 days	81–90.9	180	[27]
Desmodesmus sp. PW1	Piggery wastewater, 25 °C, 150 rpm, continuous illumination	~90	150	[118]

* FE was calculated with the following formula FE [%] = (A − B)/A * 100%, where A is initial suspension density and B is suspension density after flocculation.

Bioflocculation can be carried out by other microorganisms, e.g., fungi [115], bacteria [121], and autoflocculating microalgae [89]. Flocculating microorganisms can be cultured together with the main species (as co-culture) or separately and can be added just before harvesting [95].

Fungal hyphae are positively charged and may act as a cationic flocculant entrapping microalgal cells. Additionally, the mycelium has a self-pelletization capability enhancing microalgal flocculation [115]. Addition of *Aspergillus niger* [122] and *Aspergillus oryzae* [123] to *C. vulgaris* resulted in more than 90% and 93% efficiency of flocculation, respectively. Bacteria are known for production of large quantities of EPS in a short time, which is desirable for large-scale applications. Bacterial bioflocculation has been well examined and shown to be very effective [100,124]. Ndikubwimana et al. (2014) reported that a flocculant from *Bacillus licheniformis* harvested 99% of *Desmodesmus* sp. biomass [125]. The polymer consisted of D- and L- glutamic acid (poly-gamma-glutamic acid). The optimal flocculation efficiency was achieved at pH 2–3.3 after 4 h. Moreover, the bioflocculant was more effective at a higher biomass density (0.5–1.5 g L^{-1}). The most effective flocculant concentration was 2.5 mL L^{-1}, as it harvested 99.5% of *Desmodemsus* sp. biomass at a

concentration of 0.5 g L^{-1} [125]. Similar results were obtained after addition of poly-gamma-glutamic acid derived from *Bacillus subtilis* to *Chlorella salina* culture [125]. In turn, the effectiveness of *Shinella albus* EPS in *C. vulgaris* biomass harvesting was estimated at 85.6% [121]. The bioflocculant from *S. albus* was the most effective (>80%) in *C. vulgaris* biomass harvesting at a temperature of 60–80 °C. The activity decreased only at 121 °C to values < 70%. Flocculating agents contain some proteins, but due to their high thermal stability point, they are not engaged in flocculation. Moreover, neutral and alkaline pH values are more favorable for this process. However, the flocculation efficiency sharply decreased after dialysis from almost 80% in the control to 4.9%. This indicates that *S. albus* produce low molecular weight agents with high flocculation potential [121]. A limitation of the application of bacterial bioflocculation for algal biomass harvesting is the necessity of supplying organic carbon for bacterial growth, which increases costs.

One of the most promising methods for bioflocculation is the use of autoflocculating microalgae to induce flocculation of non-flocculating species. Salim et al. (2011) used autoflocculating microalgae *Ankistrodesmus falcatus*, *Scenededesmus obliquus*, and *Tetraselmis suecica* to achieve flocculation of non-flocculating *Chlorella vulgaris* and *Neochloris oleoabundans* [89].

5.4. Autoflocculation

The process of autoflocculation is a result of interactions between cells and depends on such cell characteristics as the cell size (large cells settle more easily), morphology, cell wall composition, culture aging, cell density, and changes in the pH value during culture growth [107,126]. The autoflocculation process is influenced by the substrate pH value. The ratio between CO_2 and metal ions keeps pH stable. Growing predominance of calcium and magnesium ions in culture broth decreases repulsion between cells, and cations start binding to negatively charged functional groups. Alkaline pH promotes generation of magnesium and calcium hydroxide and calcium phosphate precipitates, which contributes to formation of larger aggregates [108].

Microalgae producing flocculating EPS may spontaneously form aggregates using these biopolymers. In this case, the flocculation efficiency and the time of harvesting may be induced by changing culture conditions, including irradiance and temperature [126]. To date, several species of green microalgae have been reported as bioflocculant producers. These include *Chlorococcum* sp., *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Scenedesmus quadricauda* [16,17,87,106].

Both pH-induced and EPS-assisted autoflocculation occur at the end of the exponential phase or in the stationary phase when the flocculating factors have been accumulated [126]. The process of autoflocculation has not been elucidated to date, and it is necessary to know the mechanism so that the process can run in a controlled manner [108,109].

6. Applications of Extracellular Polymeric Substances in Biomass Harvesting and Heavy Metal Removal

One of the main applications of flocculating EPS is algal biomass harvesting. This process carried out with conventional techniques is extremely costly. It can constitute even 30% of the total biomass production costs. Centrifugation is the most effective method for harvesting within a short time, but it is the most expensive technique [127]. The sedimentation process is a more cost-efficient method; however, it is characterized by low efficiency. Biomass can also be harvested in the processes of filtration and flocculation. Filtration is not an appropriate solution when the biomass should not be contaminated with particles from the filter (cellulose or diatomaceus earth) [127]. The most economically satisfying solutions are autoflocculation or bioflocculation through co-culture with spontaneously flocculating algal species or the use of EPS. However, co-culture cannot be employed when pure culture has to be obtained. The most promising method for algal biomass separation that causes no additional contamination is the use of EPS or other biopolymeric substances. The level of self-flocculation of *C. vulgaris* JSC-7 was 76.3% or 85% when it was added to non-flocculating algae [16]. *S. obliquus* AS-6-1 EPS was reported to harvest 88% of non-

flocculating *S. obliquus* FSP-3 [17]. EPS play an important role in sludge dewatering [128], flocculant stability and removal of heavy metals [92,129].

The ability of EPS to bind metals can be used for removal of heavy metals from wastewater and soil. The capability of metal binding by exopolysaccharides is related to the content of acidic groups (-COOH) derived from galacturonic and glucuronic acid as well as proteins. Other functional groups engaged in metal binding are -NH, -OH, -CO-, phosphate, pyruvate, succinyl [130], and sulfate [131]. It is also suggested that mannose and rhamnose play an important role in metal complexing [6,130]. The interactions between EPS and metal ions include electrostatic attraction or covalent bonding [130]. The capability of copper binding by *Scenedesmus acuminatus* was examined by Lombardi et al. (2005) [130]. This microalga produces EPS that are a strong copper-complexing ligand. The authors have found that the results are comparable with those obtained for Cyanobacteria, which are known to have better metal complexing abilities than Chlorophyta. The polymer contains 76% of mannose and 12% of uronic acids, which are crucial for the ability to bind metals by EPS [130]. In earlier studies, Lombardi and Vieira (1999) showed the copper and lead complexing ability by EPS produced by *Kirchneriella aperta*, which contained 19% of acidic sugars [132]. Kaplan et al. (1987) observed the metal binding capacity of *Chlorella stigmatophora* EPS at the level of 0.215, 0.310, 0.03, and 1 mg g^{-1} in the case of Zn^{2+}, Cd^{2+}, Pb^{2+}, and Cu^{2+}, respectively [131]. EPS extracted from *C. vulgaris* accumulated 0.13 µg mg^{-1} of silver ions, which was 69% of the total metal concentration in the tested system [133]. EPS-coated *Chlorella pyrenoidosa* cells adsorbed 36.7% and 22.8% higher amounts of As(III) and As(V), respectively, than cells without EPS [134]. Similar results were obtained by Xie et al. (2020) for *C. vulgaris*. EPS-coated cells adsorbed about twofold higher amounts of cadmium ions than EPS-free cells [135]. The authors also investigated the role of proteins in the process of cadmium adsorption. The results showed that deproteinized EPS reduced adsorption capacity by 70% [135]. In other studies, Freire-Nordi et al. (2005) found the highest manganese sorption (8.52 mg g^{-1}) by EPS in cyanobacteria *Anabaena spiroides*. This polymer has a high content of mannose (which is one of the three main sugars) and 8% of uronic acid [129].

7. Conclusions

Currently, the interest in algal biomass is mainly focused on its commercial use, whereas algal post-culture media, which are a source of extracellular polymeric substances released by algal cells, are unused. The analysis of the studies presented in this review shows that the physical and chemical culture conditions are largely involved in increasing productivity and in the properties of microalgal extracellular polymeric substances. Additionally, many studies have reported that EPS production is influenced simultaneously by several factors and the cell response is also species-specific. Given the induction of EPS by the presence of metals and their sorption properties, EPS can be applied in wastewater management. Bioflocculants, such as microalgal extracellular polymeric substances, significantly increase the safety of harvested biomass, as they are biodegradable but not toxic. Moreover, they enrich biomass or can be extracted in further processing and used in accordance with their properties. Research on extracellular polymeric substances is important for application thereof in the processes of wastewater bio-management and microalgal harvesting.

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