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Investigation and Optimization of Exopolysaccharide (EPS) Production by Microalgae

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Summary

Exopolysaccharides (EPSs), are carbohydrate polymers excreted by microbes to their surroundings. Their importance has increased rampantly; due to many applications in various fields. Furthermore, EPS' biogenic nature and properties makes them a renewable alternative to replace petroleum based polymers. Currently, bacteria are the main producers of EPS, followed by fungi. In the quest of novel and distinctive EPSs, microalgae have been identified as a promising source.

Porphyridium sordidum and *Porphyridium purpureum* were selected and characterized in this investigation. The characterization was used to determine the effect of the initial cultivation conditions on both strains. While the initial conditions favor *P. purpureum* growth and EPS production, it was determined that cultivation medium and parameters should be modified to better fit *P. sordidum* needs. EPSs were also characterized, identifying the same carbohydrate monomers: xylose, galactose, glucose and glucuronic in both. Differences were also detected: a 3-methyl xylose, 4-methyl galactose and a higher sulfate concentration (179 ± 38 mg L^{-1}) in *P. sordidum* EPS and a methyl hexose uronic acid dimer and lower sulfate content in *P. purpureum* EPS (122 ± 0.3 mg L^{-1}).

White light was identified as the most suitable parameter for growth and EPS production in both strains. Plackett-Burman and Taguchi design were used to optimize the cultivation medium and parameters. The optimal conditions were used in a two-stage cultivation system, first to enhance growth and then to induce EPS production. *P. sordidum* growth and EPS production was increased (3.4 times) by using an improved medium, 20 °C and 24 hours of light exposure in the EPS production-phase. Differently, *P. purpureum* EPS production was improved by using two different media: one for the cultivation-stage and a customized medium fixed at pH 6 and maintaining 12 h of light exposure in the EPS induction-phase.

Optimal cultivation conditions and a 4.5 L cultivation system were used to produce *Porphyridium* EPSs for a rheological evaluation. Both *Porphyridium* EPSs showed a strong rheological stability in 85 mM NaCl and $CaCl_2$ solutions and high salinity medium, elevated temperature (75 °C) and wide range of pH values.

Phycobiliproteins were extracted from the biomass left from EPS production step; this by changing the NaCl concentration of the medium to disrupt both *Porphyridium* strains cells in a single extraction step.

Future investigation should focus on four aspects. Identification of novel microalgae EPS producers, further increase EPS yields, elucidation of EPS chemical and rheological characteristics and development of a suitable biorefinery to produce other biogenic compounds in addition to EPS from a single cultivation run. The combination of those research themes would allow to develop a feasible microalgae EPS production bioprocess.

List of publications

Medina-Cabrera E. V., Rühmann B., Schmid J. & Sieber V. (2020): Characterization and comparison of *Porphyridium sordidum* and *Porphyridium purpureum* concerning growth characteristics and polysaccharide production. *Algal Research*. Volume 49. Pages 101931-101940. DOI: 10.1016/j.algal.2020.101931.

Medina-Cabrera E. V., Rühmann B., Schmid J. & Sieber V. (2020): Optimization of growth and EPS production from two *Porphyridium* strains. *Bioresource Technology Reports.* Volume 11. Pages 100486-100495. DOI: 10.1016/j.biteb.2020.100486.

Medina-Cabrera E. V., Gansbiller M., Rühmann B., Schmid J. & Sieber V. (2021): Rheological characterization of *Porphyridium sordidum* and *Porphyridium purpureum* exopolysaccharides. *Carbohydrate Polymers*. Volume 253. Pages 117237-117246. DOI: 10.1016/j.carbpol.2020.117237.

1. Introduction

Several Industries utilize petroleum as substrate to produce different products, generate energy and power motorized vehicles. Those activities exert an enormous impact on the environment; as large amounts of carbon dioxide (CO_2) are emitted polluting the air and considerable quantities of residues are generated contaminating water, air and soil [1]. Thus, several approaches are devoted to decrease petroleum dependence by replacing their derivatives with less harmful compounds for the environment [1].

Biotechnology, which is the use of living organism or their derivatives as biocatalyst to generate renewables compounds, is one of the most promising approaches that are currently investigated to replace petroleum derived products [2]. To reach this goal it is important to choose and characterize the most adequate organism to produce a target compound. Furthermore, an inexpensive substrate needs to be used for the cultivation process. If it is possible the feedstock should be a byproduct of the petroleum industry like CO_2 to decrease its concentration from the environment [3]. For all those reasons, photosynthetic microorganisms like microalgae could be a viable host to produce highly valuable biogenic compounds using CO_2 as substrate, fulfilling the requirements of a truly green bioprocess [3, 4].

1.1. Microalgae

Microalgae are a group of microorganisms with photosynthetic capability that produce similar storage compounds and defense mechanism to plants [5]. Because, of their wide morphological and genetical diversity and the inability of some microalgae strains to perform photosynthesis; it is difficult to frame them in a single definition [5, 6].

Similarly, its definition; their classification is not straightforward. Generally microalgae are divided in prokaryotic and eukaryotic phyla [7]. Building from that division the most spread classification is the following: There are two prokaryotic phyla: cyanophyta and prochlorophyta and eleven eukaryotic phyla: charophyta, chlorarachniophyta, chlorophyta, cryptophyta, dinophyta, euglenophyta, glaucophyta, haptophyta, heterokontophyte, ochrophyta and rhodophyta [5, 7-11]. At this point it is important to discriminate microalgae from macroalgae and filamentous algae which are multicellular beings with similar characteristics to the ones described for microalgae (photosynthetic capability, storage compound, defense mechanism and classification) [5, 7].

Microalgae diversity allows them to colonize many types of environments. The most common are the aqueous ones like estuaries, rivers, lagoons, lakes and oceans. In addition, microalgae live in unlikely environments such as soil and even ice [12, 13].

The many microalgae strains provide a fruitful source for numerous applications. This is evidenced by the increasing trend of microalgae related scientific publication, reaching 2,700 research articles published in 2017 [14]. This rapid increase is based in exploiting microalgae's special characteristics for different applications and the generation of products. The most important characteristics will be mentioned in the following lines.

1.1.1. Special characteristics of microalgae

Microalgae are microbes that display characteristic from plants and other microorganism. In this section, those characteristics are classified in three groups: the ones displayed by plants and microalgae; the ones observed only in microorganism and microalgae and common characteristics between microalgae, plants and microorganisms.

The majority of the before mentioned similitudes are shared between plants and microalgae which is described in this section:

Photosynthetic capability. It is the most well-known similitude between plants and microalgae. Almost all microalgae and plants perform photosynthesis to fix CO_2 and harvested sunlight energy; contrary a small group of bacteria are also capable of performing photosynthesis [15]. Furthermore, it is reported that bacterial photosynthesis (mechanism and pigments) is slightly different to the ones used by microalgae and plants [15-17]. A brief description of the photosynthetic mechanism is the following. Light is harvested by chlorophylls (A and B); and in certain microalgae this is complemented by phycobiliproteins (rhodophyta and cyanobacteria) [18]. Then, light is uptake by photosystems II (PSII), to hydrolyze water producing O_2 and triggering an electron flux. The electrons travel through a cascade of proteins generating adenosine triphosphate (ATP) as energy storage until reaching the photosystems I (PSI) that also absorbs light and reduces nicotinamide adenine dinucleotide phosphate (NADPH), which is an enzymatic cofactor. NADPH and ATP are directed to the Calvin Benson cycle, where atmospheric CO_2 is captured to power microalgae metabolism and to generate biomolecules such as lipids, protein, nucleic acids, carotenoids and carbohydrates [19-21]. Microalgae metabolism oxidases NADPH generating nicotinamide adenine dinucleotide phosphate ($NADP^+$) and dephosphorylated ATP forming adenosine diphosphate (ADP). ADP and $NADP^+$ are redirected to the before mentioned electron cascade and PSI to regenerate ATP and NADPH [12, 22, 23]. A graphical representation of microalgae photosynthesis is presented in Figure 1.1.

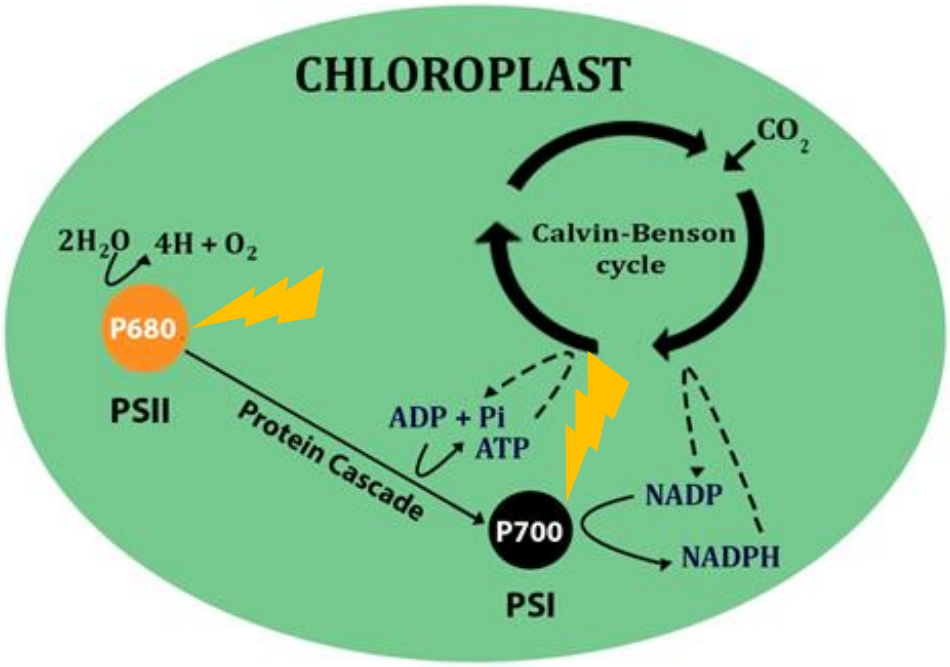

Figure 1.1. Photosynthetic mechanism in microalgae chloroplast. After light is captured by PSII (photosystems II) also known as P680, electrons are transported via a cascade of proteins until reaching PSI (photosystems I), producing ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate). The Calvin-Benson cycle consume ATP and NADPH generating ADP (adenosine diphosphate) and $NADP^+$ (nicotinamide adenine dinucleotide phosphate), which are regenerated in ATP and NADPH by the electron cascade and PSI.

Currently, strong efforts are done to sequestrate CO_2 produced from fossil fuels [12, 23, 24]. In this sense, microalgae are important microorganism as they can capture atmospheric CO_2 for their growth and valuable biogenic compounds generation.

Cultivation in non-arable land. Microalgae and plants are agricultural crops because similar techniques and nutrients are utilized for their cultivation. Yearly, more arable land is needed to produce more food for a fast growing population [12]. Contrary, reactors can be built on non-arable land for intense microalgae cultivation [25, 26]. This feature is highly beneficial as it secures agriculture soil for producing food while microalgae would be cultivated in nonagricultural fields [27, 28].

Valuable Industrial biogenic compounds. Similarities between plants and microalgae also converge in the compounds that they produce. In this sense, highly valuable industrial biogenic compounds such as carotenoids (β carotene and xanthophylls), carbohydrates (starch), lipids (polyunsaturated fatty acids, PUFAs) and proteins, are harvested from microalgae and plants sources [5, 19]. In addition, microalgae

produce some biogenic compounds that are not commonly produced by plants like: phycobiliproteins, high protein biomass, astaxanthin, methyl and sulfate carbohydrates [29-34].

Microalgae share similar characteristics exclusively with microbes; the following are most important ones:

Similar growth behavior to unicellular microbes: Microorganism have been thoroughly investigated, allowing to potentiate their growth and biogenic compounds production [35, 36]. Similar approaches are been used on microalgae to potentiate their growth and valuable compound production [37-39]. The most well know examples are the generation of long chain fatty acids and astaxanthin [34, 40]. In this regard, microbial growth behavior can be improved to then produce valuable biogenic compounds. However, microalgae photosynthetic nature (light irradiance and photoperiod duration) must be taken into account to set cultivation parameters as they are not commonly investigated in other microbes [38, 41].

Cultivation in controlled environments. Microbes have the potential to be cultivated in controlled environments (bioreactors). Those system enable a higher, reproducible and stable generation of biomass and biogenic compounds [42]. Also, control environments allow to utilize different cultivation modes (batch, fed-batch, and continuous) further enhancing microalgae growth and secondary metabolite production [39, 43-46].

Also, microalgae display common characteristics with plants and microbes at the same time:

Microalgae capacity to uptake CO_2 and organic carbon: Because microalgae are at the verge of plants and microbes, they can uptake both CO_2 and organic carbon. In this sense, microalgae's photosynthetic capability allows them to uptake atmospheric CO_2 like plants (autotrophic cultivation) [47-50]. As well, some microalgae strains have the potential to exclusively uptake organic compounds like other microbes (heterotrophic cultivation) [51-55]. Some organic carbon that can be uptake by microalgae are glycerol, glucose and diverse organic compounds in wastewater to name a few examples [56-59]. Furthermore, microalgae strains can uptake CO_2 and organic carbon at the same time (mixotrophic cultivation), potentiating their growth rate and biogenic compound production in comparisons to autotrophic and heterotrophic cultivation settings [60-63].

1.1.2. Microalgae cultivation system

Open system. These types of systems are made of open vessels. Because of their simple geometry and low construction cost, they are the most commonly used system for microalgae cultivation [64, 65]. Open systems can be classified in three main types.

There are various systems used to cultivate microalgae, which are classified in two main groups, open and closed systems [66, 67]. A comparison of the advantages and disadvantages of open and closed cultivation system, of are presented in Table 1.1.

Table 1.1.
Comparison of open and closed microalgae cultivation systems. Common features share by open and closed system are listed in this table.

	Systems			
	Open		Closed	
	Aspect	References	Aspect	References
Advantages	Their construction is inexpensive (materials and instruments).	[64, 68]	External contamination is unlikely as the cultivation broth is separated by the vessel from the environment.	[65]
	Temperature is regulated by cheap procedures.	[65, 69, 70]	Evaporation almost null in closed systems.	[65, 71]
	Easy to scale up due to simple geometry of the vessel.	[65]	Better control of the cultivation parameters.	[72]
	Open system operations and are inexpensive.	[68, 73]	Dense cultivation broth and larger secondary metabolite yields can be generated.	[69]
Disadvantages	Evaporation alters the volume and nutrient concentration, affecting microalgae growth.	[65, 70]	Expensive materials and instruments are needed for their construction.	[69]
	Cultivation broth can be easily contaminated from external sources.	[70, 74]	Complex cooling systems are needed to control temperature in closed systems.	[65]
	Limited production of biomass and secondary metabolite.	[65]	Artificial light could be need to enhance microalgae growth.	[69]
	Vessel depth limits microalgae growth due to photoinhibition.	[43, 75]	Difficult to scale up due to their geometry.	[65]
	Industrial production required large construction areas.	[70, 76]	Expensive to operate and maintain them.	[65, 69]

Unstirred pond (Figure 1.2.A): It is the simplest microalgae cultivation system. Although, these systems are easy to operate, the slow growth rate, low biomass generation and a poor secondary metabolite production makes them unsuitable for large scale production [64, 75].

Circular pond (Figure 1.2.B). An axial rotating arm is used to stir the cultivation broth in a circular pond. Although, higher growth rate and biomass are generated in circular ponds if they are compared to unstirred systems; but still low productivities are obtained [64, 75].

Raceway ponds (Figure 1.2.C). It is an artificial shallow system in which the cultivation broth is mixed with a paddle mechanism. The raceway shape and paddles enhance growth rate, biomass generation and

biogenic compounds production in comparison to the before described open system [43, 68, 74, 77]. For that reason, this is the most widespread system used for microalgae cultivation [43, 68, 74, 77].

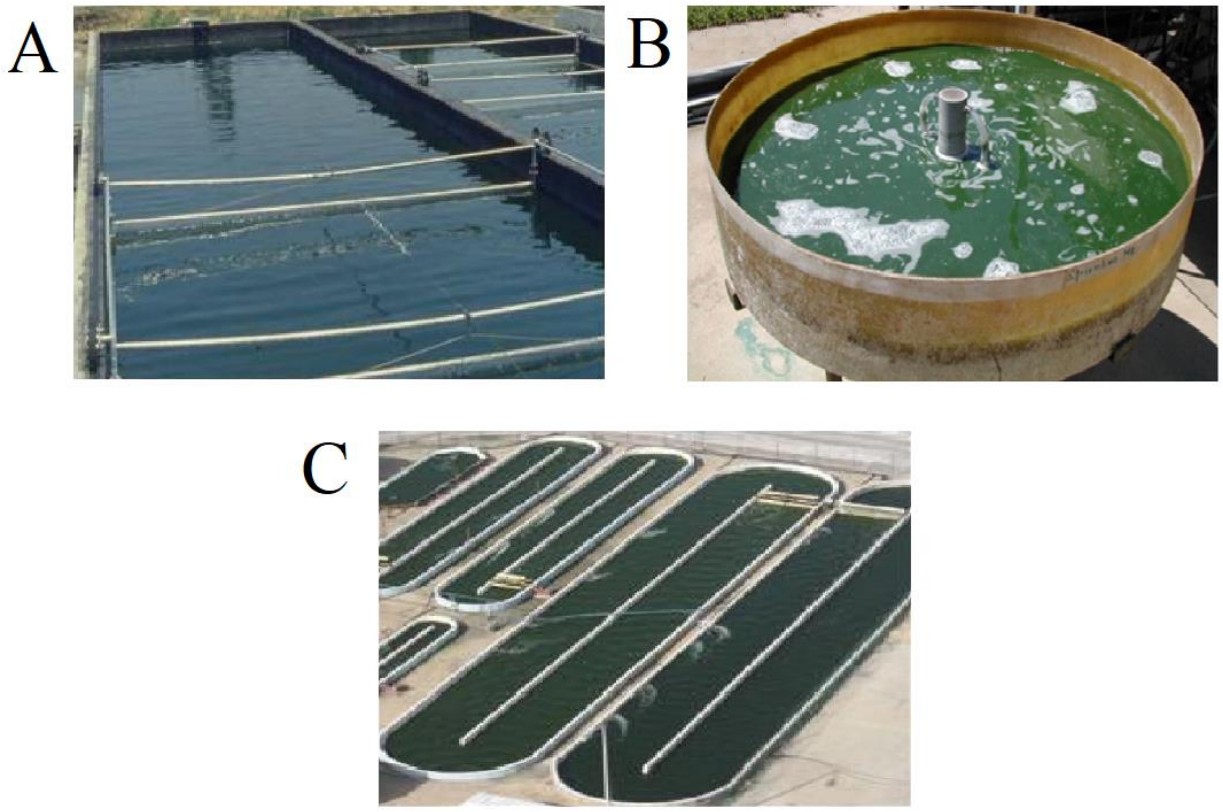

Figure 1.2. Pictures of open systems used for microalgae cultivation. Pictures were adapted for reprint with permission of the authors. A. Unstirred pond, [75] B. Circular pond [76] and C. Raceway pond [75].

Closed system. There are several types of closed systems used to cultivate microalgae; the most important are following.

Tubular photobioreactors (Figure 1.3.A). These reactors are made of transparent tubular vessels, which can be placed in different configurations (horizontal, vertical, incline, fence-like and helical); to better harvest sunlight [75, 78]. The vessel geometry and bubbling system generates an efficient aeration, mixing, heat and mass transfer, which enhances microalgae growth [64, 77, 79, 80]. They are the most common closed system used for microalgae cultivation as they are simple to scale up (adding parallel tubes to the system) [77, 78, 81].

Flat panel photobioreactor (Figure 1.3.B). The reactor vessel in this system is rectangular and can be placed in vertical, angle or horizontal positions [77, 81]. Because the vessel length and wide are greater

than its depth, this system are also known as thin-layer reactors [65]. These systems are extensively used because of the large surface area of the reactor vessel; which allows microalgae to receive high illumination rates, enhancing their growth [75, 77, 79]. Furthermore, their scale up process is easily achieved; only by installing reactor panels one next to another [80]. On the other hand, these systems have an important drawback; their rectangular shape prevents an optimal mass transfer generating temperature gradients, which can negatively affect microalgae growth. To eliminate the temperature gradient, external heat exchanger or sprinkler can be installed outside the reactor vessel [64].

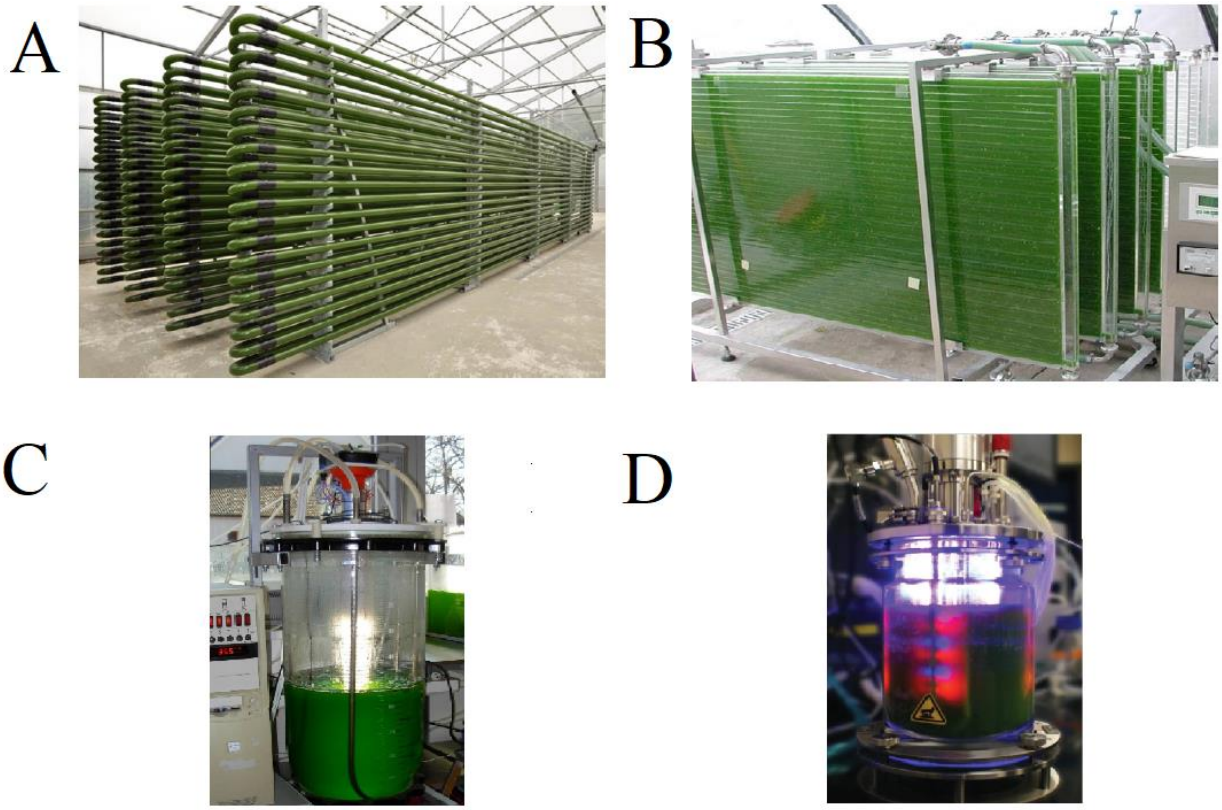

Figure 1.3. Pictured of closed cultivation systems used for microalgae cultivation. Pictures were adapted for reprint with permission of the authors. A. Tubular photobioreactor (horizontal display) [65], B. Flat Panel photoreactor (vertical configuration) [65], C. Airlift photobioreactor [76] and D. Stirred tank photobioreactor [72].

Airlift photobioreactor (Figure 1.3.C). Also known as bubble-columns [77]. These systems are made by two vertical concentric tubes; one is called “riser” as the cultivation broth moves upwards pushed the air inject into the system and a second called “downcomer”, in which the cultivation broth comes done to initial

position [77, 80, 82, 83]. Airlift systems have several features that enhances microalgae growth rates. Firstly, the circular mixing pattern, which is generated when the cultivation medium passes through the concentric tubes [80]. Also, when an opaque inner tube is used a flashing light effect (fast day/night cycle) is triggered, enhancing microalgae growth [84]. On the other hand, this cultivation system has two important disadvantages. Their construction is challenging, as complex agitation systems are needed to move the cultivation broth upwards [84]. And, because the column height and diameter are modified in the scaling up process, the cultivation parameters changes altering microalgae growth [77].

Stirred tank photobioreactor (Figure 1.3.D). These reactors are similar to fermenters used for several microbial strains. Stirred tank efficient agitation, mass transfer and aeration, enable a robust microalgae growth [64, 79]. On the other hand, this system had an important disadvantage: Large amounts of energy are need to power the agitation mechanism, and many need artificial light [64, 77].

Other closed photobioreactors. There are other closed reactors, which are not so extensively used for microalgae cultivation [78, 84, 85]. For example, bag reactors, which are plastic units placed next to a light source [69, 86, 87]. Helical reactor: transparent tube coiled next to an artificial light [80]. And hybrid reactors, which are a combination of two or more of the before described cultivation system [80, 86].

1.1.3. Microalgae harvesting procedure

The final cost of a target biogenic compound can be significantly altered by the harvesting procedure used to obtain it. So, developing an efficient procedure can minimize production cost [65, 88]. Because the majority of biogenic compounds are produced inside the microalgae cell, several harvesting procedures focus in separating the biomass from the cultivation medium [88, 89].

Microalgae biomass harvesting techniques can be classified in five groups: biological, chemical, electrical, magnetical and physical [65, 90-92]. The most common operations are the physical ones, followed by the biological, chemical, and electrical. Most recently magnetical procedures were used to harvest microalgae. The advantages and challenges of the most prominent unit operations used to harvest microalgae biomass are summarized in Table 1.2.

After harvesting microalgae biomass, different biogenic compounds can be extracted, separated, concentrated and purified. The harvesting strategy used to obtain a specific biogenic compound must be developed for each one of them; as their chemical nature, location inside the biomass and final product purity must be taken into account to develop an adequate harvesting strategy [93-98]. Specific harvesting strategies for a target molecule are so vast that should be described in specialize investigation for that particular compound.

Table 1.2.
Unit operation utilized to harvest microalgae biomass. A definition, advantages and disadvantages of each unit operation are also presented in this table. Adapted from reference [65].

Unit operation	Advantages	Disadvantages	References
	Biological		
Auto-flocculation: Sunlight, photosynthesis and increase in pH trigger microalgae interactions forming flocs.	Flocculants are not required for this procedure. Inexpensive technique.	It is only suitable for some microalgae strains. Very slow process.	[99-101]
Bioflocculation: Microorganisms or biological compounds are added to the cultivation broth to interact with microalgae forming flocs.	Bioflocculants (Fungi, plant based and animal based) are not harmful for the environment.	Difficult to control the flocculation process. High amounts of carbon-based flocculants are needed. Bioflocculants could be as feedstock for by other microorganisms triggering contamination.	[78, 90, 91, 99, 102]
	Chemical		
Flocculation/Coagulation: Flocs are formed by the addition of salt or polymers, which interact with microalgae biomass.	Fast method, low cost, inorganic and organic compound are used. (If biopolymeric compound such as EPS it could be considered as biological method).	Chemical flocculants could be toxic for microalgae. Supernatant is contaminated by flocculants.	[89, 101]
	Electrical		
Electro-flocculation/coagulation: Flocs are formed using electricity. Sacrificial electrodes (electro-coagulation) and no sacrificial electrodes (electro-flocculation).	Flocculants are not required to perform this operation. Electricity has the potential to be used in all microalgae strains. Electricity is eco-friendly.	High energy is required. Expensive to operate. Metallic ion (electrode) contaminates the supernatant. Electrodes need to be periodically changed. It is not currently applied in large scale.	[79, 90, 92, 103]
	Magnetical		
Magnetic separation: Microalgae biomass is separated using magnetic fields,	Very fast unit operation. High biomass recovery.	Biomass need modifications in order to use magnetism to separate them from the media.	[79, 92, 104]
	Physical		
Filtration: Biomass is retained in a semi-permeable membrane from a liquid phase. Pumps are required to assist this operation.	High microalgae biomass recovery. Microalgae are unlikely to be destroyed. Several types of membrane and pore sizes are available.	It is an expensive operation. Small microalgae strains are not separate by this operation. Membrane clogging is highly possible.	[88, 104, 105]
Flotation: Small bubbles interacted with microalgae biomass carrying them to the surface.	Low-cost operation. Short time are need. Small operation areas are needed. Microalgae are unlikely to be damaged by this operation.	Chemicals are often required to assist this operation. Medium is difficult to be re-used due to flocculants contamination.	[78, 79, 104]
Sedimentation: Microalgae biomass steeling in an aqueous solution mediated by gravity.	Low energy consumption is required.	It is a very slow process. It is specific for certain species. Large operation areas are needed.	[102, 105, 106]

1.2. Valuable biogenic compounds obtained from microalgae

Microalgae have been used as a food source and to treat diseases for several millennia [107-109]. However, industrial biomass production has not been reported until 1950 [21]. From that point biomass productivity has increased several folds in order to produce a broad range of valuable products [107]. The most prominent products obtain from microalgae biomass are presented in Table 1.3.

1.2.1. Commercial products obtained from microalgae

From the various microalgae strains, four genera are most used: *Arthrospira spp., Chlorella spp., Dunaliella spp*. and *Haematococcus pluvialis* [110]. The most important commercial products derived from microalgae are summarized in Table 1.3.

Biomass base products. Dry microalgae biomasses are used to supplement animal and human nutrition (nutraceutical). This is because animal and human require lipids, proteins, carbohydrates, carotenoids, vitamins and minerals found in microalgal biomass for their growth and to power their metabolism [111-114]. Also, microalgae biomass could be used as biofertilizer for different crops [115-118].

Lipid base products. Biodiesel is the most important lipidic product generated and commercialized from microalgae biomass [27, 119]. However, high cost and low energy efficiency of biodiesel compared to petroleum makes its industrial production unfeasible [112, 120]. Other lipidic products obtained from microalgae biomass are polyunsaturated fatty acids (PUFAs) [121]. Docosahexaenoic acid (DHA) is the most commonly produced PUFA from microalgae biomass (Table 1.3). As well, microalgae produced other PUFAs that are not so extensively commercialized, like: eicosapentaenoic acid (EPA), γ-linolenic acid (GLA) and arachidonic acid (AA) [21, 40, 107]. Microalgae oil PUFAs can be used in physiological and structural functions in animal and human cells [121]. Furthermore, microalgae oils are superior than their plant counterparts; as they have a higher PUFAs concentration. And are superior than fish oil as they do not have heavy metals contamination [107, 110].

Carotenoids base products. In addition to β-carotene and astaxanthin (Table 1.3) other carotenoids are produced in microalgae biomass such as: lutein, zeaxanthin, fucoxanthin, violaxanthin, antheraxanthin and neoxanthin [109, 122]. Carotenoids are currently used as pigments and antioxidants, for cosmetics, pharmaceutical and nutraceuticals application, rendering an important market for products derived from microalgae [19, 122-124].

Table 1.3.
Commercial products obtained from microalgae. The most prominent products by microalgae, the strains from the products, the application and the approximate price are presented. This table is adapted from the original focusing in products derived from microalgae [111].

Strain	Product	Annual production*	Application	Approximate price (US$) kg^{-1}	References
Arthrospira spp.	Biomass	3,000	Nutraceutical	10 – 20 (bulk powder), 35 (powder) 68 – 112 (tablets)	[111, 125, 126]
Arthrospira platensis	Phycobiliproteins		Food colorant, Nutraceutical	370 (food grade), 5,000 (reagent grade) **	[19, 125, 127]
Chorella spp.	Biomass	2,000	Nutraceutical	100 – 120 (tablets)	[111]
Dunaliella salina	β-carotene	1,200	Pigment, Food additive, Health supplement	300 – 3,000	[107, 125, 127]
Haematococcus pluvialis	Astaxanthin	300	Pigment, Food additive, Pharmaceuticals, Health supplement	2,000 – 2,500 (food), >100,000 (nutraceutical)	[107, 125, 128]
Crypthecodinium cohnii,	Docosahexaenoic acid (DHA)	240***	DHA oil	80 – 160	[107, 129]
Amphora sp., Chaetoceros sp., Cyclotella sp., Isochrysis sp., Nannochloropsis sp., Navicula sp., Nitzschia sp., Pavlova sp., Phaeodactylum sp., Skeletonema sp., Tetraselmi sp.	Biomass	-	Used as food in aquaculture	50 – 300	[111, 130]
Several strains	Isotopes	-	Biotechnology	260 – 5900**	[19, 107, 127, 131]

* Tons of dry weight | ** Price in US$/g | *** Tons of oil

Protein based products. Phycobiliproteins are water soluble colored proteins produced by cyanobacteria, and rhodophyta strains. Allophycocyanin, phycocyanin and phycoerythrin are the most common phycobiliproteins produced by microalgae [111, 132-135]. Phycobiliproteins' applications exploit their antioxidant, anti-inflammatory, neuroprotective and hepatoprotective properties in the food and pharmaceutical industries [111, 136].

Other products derived from microalgae. There are several products produced by microalgae, which are not classified in the before mentioned groups. Some examples are: biohydrogen, bioethanol, toxins and isotopes [19, 21, 107, 109, 111].

1.2.2. Carbohydrates from microalgae

Although microalgae generate several types of biogenic compounds (Table 1.3), carbohydrate polymers are not been reported to be industrially produced. There are several carbohydrate polymers produced from microalgae the most prominent ones are the following:

Starch and cellulose. Both biopolymers are made by glucose monomers; starch is used as storage molecule and cellulose as part of their cell walls. Those polymers are used as substrate for bioethanol production. Hence, microalgae strains with a high concentration of starch were screened and selected (50 % of starch in their biomass) [137]. Then the biomass of selected strains (*Chlorococcum littorale, Chlamydomonas sp., Scenedesmus obliquus, Chlorococcum humicola* and *Arthrospira platensis*) were hydrolyzed and fermented reaching 50 % of carbohydrate conversion into bioethanol [138-141]. Unfortunately, bioethanol produced from microalgal biomass cannot compete with the yields obtained from plant lignocellulosic residues, making its industrial production unachievable [137, 142].

Sulfated carbohydrates polymers. Currently, these are industrially produced from macroalgae biomass and animal tissue [143-148]. Another source for sulfated carbohydrate polymers is microalgae but the production yields are far from industrial scale production [8, 149]. The importance of sulfate carbohydrate polymers is rooted in the many properties described for this molecule [29, 30, 150]. The most important ones are the following:

Pharmaceutical and medical properties. Sulfated carbohydrate polymers show several properties such as: anticoagulant, anti-parasitic, antioxidant, and antibacterial behavior [29, 30, 151]. The most important sulfate carbohydrate polymers are carrageenan derivatives, glycosaminoglycan and chondroitin, the first one produced from macroalgae biomass and the other two are extracted from animal tissue samples. The production of the before mentioned polymers have important challenges to overcome. The chemical composition of each batch is slightly different due to uncontrollable changes in the cultivation parameter

of macroalgae and the different tissues used for their extraction; which can alter sulfated carbohydrate composition [148, 152, 153]. This could be solved by culturing microalgae in controlled environments to produce similar sulfated carbohydrate polymers batches [153].

Antiviral activity. Currently, modified carrageenan molecules and carbohydrate polymers obtained from microalgae have been tested as antiviral agents against various viruses like: herpes simplex (HSV-1 and HSV-2), human immunodeficiency viruses (HIV), influenza A virus (IFV-A), showing promising results [29, 153-159].

Methylated carbohydrates. In addition to sulfate groups, microalgae carbohydrates polymers have methyl groups in their structure [8, 31, 160, 161]. Although no biological activities have been reported for methylated carbohydrates, those residues have the potential to increase the hydrophobicity of carbohydrate polymers.

Classification of microalgae carbohydrates polymers. Several carbohydrate polymers are produced by microalgae; their classification depends on the location where they are found:

Intracellular polysaccharides. Polysaccharides generate inside microalgae cytoplasm such as: starch and different types of sulfated polymers [29, 154, 162-164].

Structural polysaccharides. Polysaccharides that constitute microalgae cell wall. Cellulose is a common structural polysaccharide, between other carbohydrate polymers that are intertwined in the cell wall structure [163, 165-168],

Capsular polysaccharides (CPS). Carbohydrate polymers exported to the cell membrane outer layer where they are attached without been released into the cultivation medium [169, 170].

Exopolysaccharides (EPS). Carbohydrate polymers that are excreted outside the microalgae cell wall into their surroundings [8, 29, 171].

1.3. Exopolysaccharides (EPS)

Intracellular, structural and capsular polysaccharides have been studied as part of the biomass. Contrary EPS have been less studied as they are exported outside microalgae cell. EPS importance lies in the various applications in which they are currently utilized (Table 1.4). Furthermore, they are a viable ecofriendly alternative to synthetic polymers [172].

1.3.1. EPS physicochemical properties

In addition, to being excreted outside the cell, EPS have chemical, physical and biological properties [173].

Chemical composition. Generally, EPS contains carbohydrate monomers and non-carbohydrate decorations in their molecules.

Carbohydrate monomers. They constitute the biggest fraction of the EPS molecules. The most common monomers in EPSs are neutral carbohydrates such as: glucose, galactose, mannose, fucose, rhamnose, xylose and fructose. Also, special carbohydrates are sometimes embedded in EPS structure been the most recurrent carbohydrate acids monomers like: glucuronic acid, mannuronic acid, guluronic acid, and galacturonic acid [174-176]. EPS homopolymers are made of one type of carbohydrate monomer; while heteropolymers contain at least two different carbohydrate monomers in their structure [176, 177]. Noticing that heteropolymers can group two, seven and even up to fourteen monomers in each repeating unit [175, 177-179].

Non-carbohydrate decorations. Several EPS contain a non-carbohydrate fraction in their structure; some of the most common substituents are acetate, pyruvate, succinate, methyl and sulfate groups [8, 176, 180]. The non-carbohydrate fraction can alter EPS properties, so their evaluation is very important during the characterization [181].

Physical characteristics. Molecular weight and rheological properties are two most important physical characteristics that are investigated in EPS molecules.

Molecular weight. EPS are macromolecules with a wide range of molecular weight, ranging from 100 to 1000 kDa [177].

Rheological properties. EPS contain a solid and liquid fraction (viscoelastic behavior), which are studied using rheology. The flow curve identifies a shear thinning or pseudoplastic behavior in carbohydrate polymers [182]. In plain terms a shear thinning behavior is the decrease in viscosity (η) in inversive proportion to shear rate ($\dot{\gamma}$) [29, 183-185]. Then, the solid/elastic behavior or storage modulus (G') and liquid/viscous behavior or loss modulus (G") are measured by the amplitude sweep analysis [182]. By doing that a constant shear stress (viscoelastic region or LVE) is determined; to be used in subsequent evaluations [181]. The frequency sweep detected, which fraction (solid or liquid) dominates the rheological measurements [181, 184-186]. Predominantly, microbial carbohydrate polymers including EPS show a gel behavior (G'>G") [181, 184-186]. Temperature sweep is performed by measuring G' and G" while temperature is first increased and then decreased. Generally, EPS shows a decrease in G' and G" while temperature increases and restauration of the initial values when the temperature decreases. Lastly, a thixotropy evaluation is performed; identifying a structural regeneration of carbohydrate polymer after a strong shear stress [181]. Remarking that carbohydrate polymers rheological behavior are stabilized through the addition of mono- and divalent salts [181, 184].

Biological functions. EPS natural properties are not completely elucidated but they have been assigned some general putative functions.

Carbon and energy reserve. Microbes excrete carbohydrate polymers as a reserve molecule in normal and stress conditions to later use them when nutrients are depleted from the cultivation medium [8, 187].

Biofilm formations. Microorganism produce a biofilm for several reasons. Assisting microbial adherence to surfaces in order to colonize different ecological niches [188-191]. Also, biofilm facilitates communication between microorganism in the biofilm [192]. Furthermore, microorganism produce biofilm as protection against viruses, enzyme, toxic metallic ions, detergents and antibacterial compounds [8, 193-197]. In addition, biofilms shield microorganisms against environmental changes like water evaporation and changes in salt content [197-201].

The characteristics described above allow EPS to use in several application in various industries (Table 1.4).

1.3.2. Microorganism described as EPS producer

A summary of most important microbial EPSs, their monomeric units and the applications in which they are currently used are presented in Table 1.4.

EPSs are used in multiple applications exploiting their chemical, physical and biological characteristics before described. The production yield and market price of the most prominent EPS are the following. 165,000 metric tons (mt) of xanthan are produced with a market price between 3.5 - 10 US$ kg^{-1}; gellan production reach 4,000 mt with a price between 25 - 50 US$ kg^{-1}; alginate annual production is reported to be 30,000 tons with a price of 5 - 20 US$ kg^{-1}; 300 mt of welan is produced with a price of 12 - 25 US$ kg^{-1}; 200 mt of diutan are produced with a market price between 12 - 25 US$ kg^{-1}; pullulan production is reported to be 300 mt with a market price of 20 - 25 US$ kg^{-1}; scleroglucan has a market price of 12 - 20 US$ kg^{-1} and dextran annual production reaches a production of 2,000 mt [176, 202-206]. This list is complete with curdlan, which is priced in 55 US$ kg^{-1} and cellulose price that ranges between 5.8 - 12 US$ kg^{-1} [176]. The most expensive EPS is hyaluronic with a price market price of 1 billion US$ [206].

There are other microbial strains described as EPS producer but there are in their nascent stage, requiring more evaluations to reach the information summarized in Table 1.4 [207-212]. Furthermore, strong efforts are currently made to identify new EPS producer using novel methodologies and platforms [213].

Table 1.4.
Exopolysaccharides produced by different microorganism (bacteria and fungi). A summary of the EPS components, the applications in which they are currently used are presented in this table. The information in this table compiled the information of different studies [175-177, 180, 214-216].

EPS	Strains	Components	Application	References
Alginate	*Azotobacter spp., Pseudomonas spp.*	Glucuronic acid, mannuronic acid, acetate	Food hydrocolloid, medical products, water treatment	[217-219]
Cellulose	*Agrobacterium tumefaciens, Gluconacetobacter spp., Pseudomonas spp., Rhizobium spp., Sarcina ventriculi*	Glucose	Emulsifier, foods additive, food packaging	[220-222]
Colanic acid	*Citrobacter sp., Enterobacter spp., Escherichia coli, Salmonella spp., Shigella spp.,*	Fucose, glucose, glucuronic acid, galactose, acetate, pyruvate	-	[223-225]
Curdlan	*Alcaligenes faecalis, Agrobacterium spp., Cellulomonas spp., Rhizobium spp.,*	Glucose	Concrete additive, food additive, heavy metal removal, pharmaceutical industry	[226-228]
Dextran	*Leuconostoc spp.*	Glucose	Chromatographic medium, confectionery, cosmetic, food additive, pharmaceutical field	[229-231]
Diutan	*Sphingomonas sp.*	Glucose, rhamnose, glucuronic acid, acetate	Construction chemistry	[232-234]
Emulsan	*Acinetobacter spp.*	Glucose	Oil recovery	[235, 236]
FucoPol	*Enterobacter sp.*	Fucose, galactose, glucose, acetate, pyruvate, succinate	Cosmetic products, fucose and fuco-oligosaccharides, food additive, pharmaceutical products, medical products	[172, 237]
Galactan	*Sporobolomyce sp.**	Galactose, galactose 6-phosphate	-	[238, 239]
GalactoPol	*Pseudomonas oleovorans*	Galactose, mannose, glucose, rhamnose, acetate, succinate, pyruvate	Coatings, cosmetics products, cultivation medium, food additive, medical products, oil recovery, packaging, pharmaceuticals products	[240-242]
Gellan	*Sphingomonas spp.*	Glucose, rhamnose, glucuronic acid, acetate, glycerate	Food additive	[243-245]
Glucuronan	*Gluconacetobacter hansenii, Sinorhizobium meliloti*	Glucuronic acid	Cosmetics products, food additive	[246-248]
Hyaluronic acid/ Hyaluronan	*Streptococcus pyogenes*	Glucuronic acid, N-acetyl-glucosamine	Cultivation medium additive, medical products	[249-251]

Table 1.4. continuation

EPS	Strains	Components	Application	References
Levan	*Arthrobacter ureafaciens, Bacillus spp., Erwinia herbicola, Geobacillus stearothermophilus, Halomonas spp., Lactobacillus reuteri, Microbacterium laevaniformans, Pseudomonas fluorescens, Saccharomyces cerevisiae*, Serratia levanicum, Xanthophyllomyces dendrorhous, Zymomonas mobilis*	Fructose	Cryo-protector agent, cosmetics products, encapsulating agent, emulsifier agent, food (prebiotic), glue, osmoregulatory, medical products, stabilizer agent, thickener agent	[252-254]
Mutan	*Streptococcus mutans, Streptococcus sobrinus*	Glucose	Dental products, surgical procedure	[255, 256]
Pullulan	*Aureobasidium pullulans**	Glucose	Antitumor agent, drug delivery carrier, gelling agents, stabilizing agent, texturizing agent, thickening agent	[205, 257, 258]
Schizophyllan	*Schizophyllum commune*	Glucose	Immunomodulating agent	[259-261]
Scleroglucan	*Slerotium spp.*, Schizophyllum commune*, Botrytis cinereal*, Epicoccum nigrum**	Glucose	Agriculture stabilizer, cosmetic products, pharmaceutical formulation, food additive	[262-264]
Succinoglycan	*Agrobacterium tumefaciens, Alcaligenes faecalis, Rhizobium mellioti*	Glucose, galactose, acetate, pyruvate, succinate	Food additive, oil recovery	[265-267]
Welan	*Alcaligenes spp*.	Glucose, rhamnose, mannose, glucuronic acid, acetate	Construction chemistry, oil operation	[268-270]
Xanthan	*Xanthomonas spp.*	Glucose, mannose, glucuronic acid, acetate, pyruvate	Food additive, petroleum industry, pharmaceuticals products, cosmetics products, personal care products	[271-273]

* Fungal EPS producers

1.4. Microalgae as EPS producer

In addition to bacteria and fungi, some photosynthetic microorganisms are reported to produce EPS [274-276]. Although, EPS producing microalgae strains have not been studied as deeply as other microbial counterparts; some important features have been already described.

1.4.1. Advantages and challenges of microalgae as EPS producer

Microalgae as source of new EPS, have several advantages; the most important are the following.

Utilization of supernatant for EPS production. As it was described in Introduction 1.2, microalgae biogenic compounds are mainly obtained from their biomass, leaving the cultivation medium unprocessed. Furthermore, harvesting EPS, which are excreted into the cultivation medium do not interfere with strategies used to obtain other biogenic compounds from microalgae biomass. This feature makes it possible to obtain from a single bioprocess, EPS and other valuable compounds from microalgae biomass.

CO_2 as a backbone for EPS synthesis. In autotrophic conditions microalgae primordial carbon source is CO_2, which is used for biomass generation and growth [12, 23]. As well, CO_2 is used as a building block for the catabolism of organic compounds such as: lipids, protein, nucleic acids, carotenoids and carbohydrate including EPS [33, 277-279]. So, EPS generation in microalgae can be considered a truly green process because CO_2 as the main carbon source for their production. This feature put microalgae in the spotlight as nowadays there are strong efforts devoted to decrease CO_2 emissions from the atmosphere [12].

Utilization of low-cost medium for EPS production. CO_2 is used as carbon source in autotrophic cultivation; the rest of nutrients are inorganic salts provide by the cultivation medium [5]. In this sense, unexpensive plant fertilizers (inorganic salts) can be used to prepare low cost cultivation medium for EPS production [280-282]. As well, low cost medium could utilize wastewater or subproducts from other bioprocess in a heterotrophic or mixotrophic cultivation mode [58, 85, 283-285].

Uniformity of EPS batches produced by microalgae. Sulfated carbohydrate polymers are industrially produced from macroalgae and animal tissues samples. However, slight changes in the chemical composition of each sulfated carbohydrate polymer batch could be triggered, by uncontrollable changes in the environment where seaweed growth or biological contaminates found in animal tissue samples [145, 286]. In this regard, microalgae could be cultivated in a control environment producing similar EPS (sulfated carbohydrate) batches as it is currently done in bacteria and fungi [29, 207, 262].

Although EPS production in microalgae has several advantages, there are important challenges that need to be addressed.

Low EPS yield production. Nowadays, EPS with high yield like scleroglucan (30 g L^{-1}) and xanthan (50 g L^{-1}) are commercially produced [180, 263]. Unfortunately, microalgae EPS production is reported to be in the mg L^{-1} range. In this regard, 4 g L^{-1} is the highest EPS yield produced by a microalgae (*Porphyridium marinum*) [287]. Those yields are far away from the ones obtained from fungal and bacterial strains, making microalgae EPS production currently unviable [288-290].

Long cultivation time. Bacteria and fungi EPS generation requires hours or maximum days, contrary EPS production in microalgae needs days and even weeks to reach the production stage [263, 287-289, 291-294]. The combination of low EPS yields and long cultivation times puts microalgae EPS far away from yields required to reach industrial production.

Harvesting technology. As it as described in Table 1.1 the majority of harvesting technologies focus on microalgae biomass. Conversely, because EPS are excreted into the cultivation medium, other harvesting strategies must be implemented to separate, concentrate and purify them.

EPS contamination. It is common that microalgae samples contain bacteria and/or fungi because of the symbiotic interactions between those microbes [295-298]. In this sense, microalgae low growth rate can be surpassed by bacterial and fungal strains when an organic carbon source is placed into the cultivation medium to enhance growth and EPS production [33, 61, 299-302]. This makes, microbial infection a serious problem as it has the potential to reduce microalgae and even completely stop the growth and EPS production. And even if EPS is produced despite of microbial contamination, the number of unit operations used to purify it could drastically increase.

1.4.2. Microalgae described as EPS producers

It is important to remark that only few microalgae strains compared to fungi and bacteria are reported as EPS producers. Furthermore, EPS investigation in microalgae is behind bacterial and fungal counterpart. Carbohydrate monomer composition and sulfate content are known for some microalgae EPS. However, their structure, molecular weight and rheological properties have been barely study [8, 10, 303-307]. For that reason, deeper investigation is required to fully understand the composition and properties of microalgae EPS. Remarking that some microalgae strains exudate lipids and proteins in addition to carbohydrates (exopolymeric substances) [201, 308]. It is not clear if exopolymeric substances are analogous to EPS. This can be determined by elucidating exopolymeric substances chemical composition and rheological properties [181, 184]. In Table 1.5 a list of the microalgae reported as EPS producers is presented.

Table 1.5.

Microalgae strains described as EPS producer. The information was gathered from several investigations [8, 10, 303-307].

Phylum	Strains	References	Phylum	Strains	References
Chlorophyta (green algae)	*Botryococcus spp.,*	[306, 309]	Charophyta	*Cosmarium sp.*,*	[307]
	Chlamydomonas spp.,	[310, 311]		*Netrium spp.,*	[307, 312]
	Chlorella spp.,*	[313, 314]		*Penium spp.,*	[307, 315]
	Chlorococcum oleofaciens,	[306]		*Pleurotaenium trabecula*,*	[307]
	Chlorosarcinopsis sp.,*	[10]		*Tetmemorus brebissonii**	[307]
	Desmococcus olivaceus,	[160]	Cryptophyta	*Cryptomonas spp.,*	[316, 317]
	Dictyosphaerium spp.,*	[10]		*Chroomonas sp.*	[11]
	Dunaliella spp.,*	[318, 319]	Dinophyta (Miozoa)	*Amphidinium carterae,*	[320]
	Dysmorphococcus globosus,	[306]		*Cochlodinium polykrikoides,*	[321]
	Gloeocystis vesiculosa,*	[10]		*Crypthecodinium cohnii,*	[322]
	Graesiella spp.,	[323, 324]		*Gyrodinium impudicum*	[325]
	Hormotilopsis gelatinosa,	[306]	Haptophyta	*Isochrysis spp.*	[305, 306]
	Neochloris oleoabundans,*	[326]	Ochrophyta	*Nannochloropsis oculate,*	[306]
	Neocystis mucosa,	[327]		*Trachydiscus minutus**	[10]
	Pseudo-coccomyxa ellipsoidea,	[328]	Rhodophyta (red algae)	*Flintiella sanguinaria,*	[288]
	Scenedesmus spp.,	[329, 330]		*Porphyridium spp.,*	[151, 287, 331-333]
	Tetraselmis spp.	[305, 306]		*Rhodella spp.,*	[151, 290, 334, 335]
Cyanophyta (Prokaryotic algae)	*Anabaena spp.,*	[306, 309]		*Rhodosorus marinus*	[336]
	Aphanocapsa halophytia,	[310, 311]	Heterokontophyta (Diatoms)	*Achnanthes longipes,*	[337]
	Aphanothece spp.,	[313, 314]		*Amphipleura rutilans,*	[303]
	Arthrospira spp.,	[306]		*Amphora spp.,*	[304, 338]
	Cyanobacterium aponinum,	[339-342]		*Chaetoceros affinis,*	[343]
	Cyanospira capsulate,	[344]		*Coscinodiscus nobilis,*	[345]
	Cyanothece spp.,	[346, 347]		*Cymbella cistula,*	[337]
	Mastigocladus laminosus,	[348, 349]		*Cylindrotheca spp.,*	[350, 351]
	Microcoleus vaginatus,	[352]		*Diadesmis gallica*,*	[10]
	Microcystis spp.,	[353]		*Haslea ostrearia,*	[354]
	Nostoc spp.,*	[161, 355, 356]		*Melossira nummuloides,*	[303]
	Oscillaria sp.,	[169]		*Navicula spp.,*	[10, 303, 357]
	Phormidium tenue,	[358]		*Nitzschia spp.,*	[304, 359, 360]
	Scytonema javanicum,	[361, 362]		*Phaeodactylum tricornutum,*	[363]
	Synechococcus sp.,	[364-369]		*Stauroneis sp.,*	[370]
	Synechocystis spp.	[371]		*Thalassiosira spp.*	[372, 373]

*Exopolymeric substances producer

1.5. Aim of this work

The aim of this work is the identification, characterization, cultivation and optimization of microalgae strains capable of producing EPS. As well, microalgae EPS has to be chemically characterized and their rheological properties elucidated.

To achieve this goal, the first step has to be to screen several microalgae strains to identify the EPS producing ones. Then, the growth rate and nutrient uptake must be evaluated to better understand the factors that influence growth and EPS production. Also, the microalgae EPS monomer composition has to be elucidated using the HT-PMP method and enzymatic tests previously developed and standardized for microbial EPS and microalgae carbohydrates [31, 374, 375].

Subsequently, because of the low EPS yields generated by microalgae, the bioprocess must be optimized in order to increase EPS production. The optimization procedure should focus on two aspects: the maximization of microalgae growth and increasing EPS yields. A suitable methodology has to be developed to boost the optimization process, varying multiple medium components and parameters at the same time, to enhance microalgae growth and increase EPS yields.

Microalgae EPS have to be thoroughly investigated focusing in two aspects. Firstly, the carbohydrate monomer composition and non-carbohydrate decorations have to be elucidated. Then, EPS rheological properties must be elucidated [181, 184]. Both the chemical composition and rheological behavior must be taken into account to understand the EPS particular properties and putative applications.

Also, the production of other biogenic compound beside EPS should be explored, in order to propose a single bioprocess from which several compounds could be generated at the same time. The most suitable cultivation and harvesting procedure need to be elucidated to propose a feasible biorefinery concept in a near future [33].

If successful this project will identify and characterize microalgae strains capable of producing EPS. In addition, an optimal bioprocess (cultivation medium and abiotic factor EPS inducers) should be proposed in order to obtain sufficient EPS for further investigation. Then, microalgae EPS chemical and rheological characteristic will be elucidated. Finally, a viable harvesting procedure will be proposed to produce, in addition to microalgae EPS other valuable biogenic compounds from microalgae biomass.

2. Materials

2.1. Consumables

Consumables	Manufacturer	Catalog Number
96 microtiter plate F-Bottom	Greiner Bio-One	651201
96 well microtiter plate V-Bottom	Nunc	249944
96 well silicon cap mat	Whatmann	7704-0125
96-well PCR plate	Brand	781350
Aluminum sealing film	Axygen	PCR-AS-200
Disposable cuvettes	VWR	634-0676
Falcon tube 15 mL	Sarstedt	62.554.502
Falcon tube 50 mL	Sarstedt	62.547.254
Filter plate 0.2 µm Supor	Pall Corporation	FZ 9836
Pipette tips 10 µL	Brand	732024
Pipette tips 200 µL	Brand	732028
Pipette tips 1000 µL	Brand	732032
Pipette tips 5000 µL	Brand	112
Pipette tips 10000 µL	Brand	2394
Syringes 50 mL	Dispomed	22050
Syringes 3 mL	Braun	4606024V
Test tube 1.5 mL	Sarstedt	72.690
Test tube 2 mL	Sarstedt	72.691
Transparent breathable film	DiversifiedBiotech	BEM-1
Filter 0.2 µm PTFF Midiart® 2000	Sartorious stedium	17805
Sylicon tube ID 6mm, OD 9mm	Roth	9572.1
PCR 8 tube strips	VWR	732-1517
PCR 8 cap strips	VWR	732-1518
Test tube 1.5 mL safe lock	Sarstedt	72.706
Test tube 2 mL safe lock	Sarstedt	72.695
Silicone sealing ring, GL45	Roth	L994.1
Screw caps with hole made of PBT (red) GL45 with 34mm hole	Roth	LL52.1

2.2. Devices

Name of devices	Manufacturer	Model
Autoclave	Thermo Scientific	Varioklav 135S
Agarose electrophoresis apparatus	Bio-Rad Laboratories GmbH	Power Pac Base
Bench Centrifuge	VWR	Galaxy MiniStar
Bench Centrifuge	Thermo Scientific	Heraus Frisco 21
Block heater	Süd-Laborbedarf GmbH	SLG digital dry bath
Bench Centrifuge	Thermo Scientific	Heraus Pico 17
Centrifuge	Thermo Fischer Scientific	Sorvall RC-6+

Name of devices	Manufacturer	Model
Rotors	Thermo Fischer Scientific	F9-4x1000Y
Rotors	Thermo Fischer Scientific	F10S-6x500Y
Centrifuge	Thermo Fischer Scientific	SS-34
Rotors	Thermo Fischer Scientific	Sorvall Lynx 6000
Rotors	Thermo Fischer Scientific	F9-6x1000
Rotors	Thermo Fischer Scientific	LEX F12-6x500
Rotors	Thermo Fischer Scientific	LEX A27-8x50
Centrifuge	Hettich Lab Technology	Rotanta 460R
Clean Bench	Thermo Fischer Scientific	MSC Advantage
Clean Bench	Heraus Instruments	Hera Safe
Crossflow system	Sartorius AG	Hydrosart®
Ultrafiltration cassette	Sartorius AG	Hydrosart®
Drying oven	Thermo Scientific	Function Line T12
Electric Scale	Sartorius AG	TE1502S
Electric Scale	Sartorius AG	TE6101
Electric Scale	Ohaus Europe GmbH	Pioneer
Electric Scale	Shimadzu Corporation	AW320
Freezer -20°C	Liebherr	Liebherr Compact
Freezer -80°C	Thermo Scientific	Forma 900 series
Gel documentation	Intas	GelDock
Incubator	Infors AG	HAT Minitron
Incubator	Thermo Scientific	Heraus B12
Incubator	Thermo Scientific	Heraus Kelvitron kp
Incubator	Eppendorf AG	New Brunswick™ Innova® 44/44R
LED white warm	MÜLLER licht	E-7
Magnetic stirrer	Heidolph Instruments GmbH	MR 3001 K
Magnetic stirrer	VWR	VMS C7
Mechanical mini timer	IKA® Werkc	RO 15 Power
Mechanical mini timer	Intertek /GS	25010A
Micropipette	Brand	10000 µL
Micropipette	Brand	5000 µL
Micropipette	Brand	1000 µL
Micropipette	Brand	200 µL
Micropipette	Brand	100 µL
Micropipette	Brand	10 µL
Microplate Shaker	Edmund Bühler GmbH	TH 15
Microscope	Carl Zeiss	Axio Observer Z1
Microwave	ECG	MH 25 ED

Name of devices	Manufacturer	Model
Mixer mill	Retsch®	MM400
Multichannel pipette	Eppendorf	1200 mL Resenal PRO
Multichannel pipette	Brand	Transferpette S12
Overhead stirrer	Heidolph Instruments GmbH & Co. KG	RZR2051
Parallel photo-bioreactor fermenter	DASGIP/Eppendorf	
Exhaust analysis	DASGIP/Eppendorf	GA 4
Gas mixing	DASGIP/Eppendorf	MX 4/4
pH, OD and Redox	DASGIP/Eppendorf	PH4 PO4 RO4
Photobioreactor Illumination	DASGIP/Eppendorf	PBR4
Temperature and Agitation control	DASGIP/Eppendorf	TC 4 SC 4
Vessel	DASGIP/Eppendorf	
Peristaltic pump	Cole-Parmer®	Masterflux® LSTM 7518-00
PCR cycler	Bio-Rad Laboratories GmbH	MJ Mini™ Personal
pH meter	Mettler-Toledo GmbH	FiveEasy
pH meter	Mettler-Toledo GmbH	FiveGo
Nitrate electrode	PRONOVA Analysentechnik GmbH & Co. KG	Nitrate electrode
pH meter electrode	Mettler-Toledo GmbH	LE438
pH meter electrode	Mettler-Toledo GmbH	InLab® Micro Pro-ISM
Refrigerator		
Rheometer	Anton Paar	MCR300
Cone plate geometry	Anton Paar	CPso-1
Sand bad		
Shaker	Thermo Fischer Scientific	MaxQ 2000
Special Clamping device	Broder Rühmann	In-house develop
Spectrophotometer	Thermo Scientific	Multiskan Spectrum
Spectrophotometer	Thermo Scientific	Varioskan
Spectrophotometer	Implen Nanophotometer	P-class
Timer	Oregon scientific	TR118
Transilluminator	Clara Chemical Research	DR 46B
Ultrapure water system	ELGA LabWater	PURELAB classic
Ultrapure water system	Merck Millipore	Q-POD MiliQ
UHPLC	Dionex	Ultimate 3000RS
Degasser	Dionex	SRD 3400
Pump module	Dionex	HPG 3400RS
Auto-sampler	Dionex	WPS 3000TRS
Column compartment	Dionex	TCC 3000RS
Diode array detector	Dionex	DAD 3000RS

Name of devices	Manufacturer	Model
High-capacity ion trap	Bruker Daltonics	HCT
Vacuum drying chamber	Binder	VD 53
Vortex	Scientific Industries Inc.	Vortex Genie 2
Water bath	Huber	CC1 Compatible
Water bath	Huber	MPC

2.3. Enzymes

Name of the Enzymes	Manufacturer	Catalog Number
Glucose Oxidase	Sigma Aldrich	G2133
Adenosine triphosphate sulfurylase	CBR TUM CS	
Adenylyl-sulfate kinase	CBR TUM CS	
Horseradish peroxidase	Sigma Aldrich	P6782
Pyruvate oxidase	Sigma	P4591
Pyruvate phosphate dikinase	CBR TUM CS	
Taq polymers NEB (Kit)	NEB	M0267L

2.4. Glassware

Name of the Glassware	Manufacturer	Catalog Number
Conical flask 250 mL	Duran	1268241
Conical flask 100 mL	Duran	1267659
Conical flask 10 mL	Lenz	3021413TH
Volumetric flask 2000 mL	Brand	9.05
Volumetric flask 1000 mL	Wtleg	12.08
Volumetric flask 50 mL	Wtleg	10.08
Volumetric flask 25 mL	Wtleg	3.09
Media Bottles 1000 mL	VWR	2151595
Media Bottles 500 mL	VWR	2151594
Media Bottles 250 mL	VWR	2151593
Media Bottles 100 mL	VWR	2151592
Thomas chamber	Glaswarenfabrik Karl Hecht GmbH & Co KG	40440701

2.5. Kits

Name of the Kits	Manufacturer	Catalog Number
NucleoSpin® Gel and PCR Clean-up Kit	Machery and Nagel	740609250
Phosphate-test	Merck	1148420001

2.6. Reagents

Mane of Reagents	Manufacturer	Catalog Number
1-Phenyl-3-methyl-5pyrazolone (PMP)	Aldrich	M70800
2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)	Sigma-Aldrich	A1888-2G
2-deoxy-D-glucose	Alfa Aesar	A11990
2-deoxy-D-ribose	Roth	CN96
2-propanol	Chemsolute	1157.9025
2-Log DNA ladder (0.1–10.0 kb)	NEB	N0469L
4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES)	Carl-Roth	HN78
Acetic acid glacial	Carl-Roth	6755
Acetronitrile LC-MS grade	VWR Chemicals	83640
Adenosine triphosphate (ATP)	Carl-Roth	HN35.4
Agar Kobe	Carl-Roth	5210
Agarose	Serva	11404
Barium chloride dihydrate	Sigma-Aldrich	B0750
Boric acid	Sigma	B7.901
Bovine serum albumin (BSA)	Ammresco	332
Calcium chloride dihydrate	Carl-Roth	52395
κ-carragenan	Roth	3059.1
κ-carragenan	Marcel cooperation	5111I033-B
Chloroform Isoamyl alcohol ROTI C/I (24:1)	Roth	X984
Citric Acid	Carl-Roth	X863.2
Cobalt (II) chloride hexahydrate	Alfa Aesar	A16346
D-cellobiose	Fluka	22150
Deoxynucleotide Triphosphates (dNTPs)	VWR	733-1363
D-galactosamine	Roth	7411
D-galactose	Serva	22020
D-galacturonic acid	Fluka	48280
D-gentiobiose	Roth	4123
D-glucosamine	Merck	346299
D-glucose	Serva	22720
D-glucuronic acid	Molekula	M24989192
D-lactose	Roth	6868
D-mannose	Serva	28460
DNA Stain clear G	Serva	39804
D-ribose	Sigma	R7500
D-xylose	Fluka	95731
Dimethyl sulfoxide (DMSO)	NEB	M0530L
Ethanol absolute	VWR Chemicals	20821.321
Ethylene diamine tetra acetic acid (EDTA)	Carl-Roth	8040
Ethylene diamine tetraacetic acid disodium ($EDTANa_2$)	Carl-Roth	8043

Mane of Reagents	Manufacturer	Catalog Number
Ferric ammonium citrate	Carl-Roth	CN77.1
Gel loading dye, Purple 6X	NEB	B70245
Gellan gum	Collstab®	GE111202
Guanine triphosphate (ATP)	Carl-Roth	HN53.1
Hydrochloric acid	Merck Millipore	30721
Iron (II) sulfate heptahydrate	Sigma	F8633
L-arabinose	Roth	KK10
L-fucose	Roth	5118.1
L-rhamnose	Fluka	83650
Magnesium chloride hexahydrate	Roth	21891
Magnesium sulfate heptahydrate	Merck/VWR	1.05886
Manganese (II) chloride tetrahydrate	Merck	1.05927
Methanol LC-MS	VWR Chemicals	83638.32
N-(Carboxymethylaminocarbonyl)-4, 4'-bis(dimethylamino)diphenylamine Sodium Salt (DA-64)	Wako Chemicals GmbH	13287
N-acetyl-D-galactosamine	Roth	8993
N-acetyl-D-glucosamine	Sigma	A2795
Polyethylene glycol (PEG) 6000	Serva	39778
Phenol red	Alfa Aesar	16294
Phenol ROTI	Carl-Roth	38
Phosphoenolpyruvate acid	Alfa Aesar	B20358
Potassium hydroxide	Carl-Roth	6751
Potassium phosphate dibasic	Carl-Roth	3904
Potassium phosphate monobasic	Carl-Roth	P749
Roti® Quant	Roth	K0513
Sodium bicarbonate	Sigma-Aldrich	P030
Sodium carbonate	Carl-Roth	8563
Sodium chloride	Carl-Roth	P029
Sodium hydroxide	Carl-Roth	6771
Sodium molybdate dihydrate	Sigma	27411
Sodium nitrate	Merck	1065351000
Sulfuric acid, 95-98%	Carl-Roth	4632
Thiamine pyrophosphate	Sigma-Aldrich	C8754
Trifluoroacetic acid	Serva	36910
Tris(hydroxymethyl)aminomethane - Hydrochloric acid (TRIS-HCl)	Roth	9090
Xanthan gum	Sigma	61253
Zinc sulfate heptahydrate	Merck	1.0888803

2.7. Softwares

Product	Manufacturer	Application
Basil local Alignment Search Tool (BLAST)	Nacional Center for Biotechnology Information	DNA sequence alignment

Product	Manufacturer	Application
Chromas lite v2.6.2	Technelysium Pty Ltd	Chromatogram viewer
Chromeleon	Dionex	Data Analysis UHPLC
Data Analysis	Bruker	Data Analysis MS
EndNote X7	Clarivate Analytics	Literature citing
GraphPad Prism v6.01	GraphPad Software Inc.	Graph editor
HyStar	Bruker	UHPLC-MS System control
QuantAnalysis	Bruker	Quantification of MS-data
MS Office 2016	Microsoft Corporation	Text processing (Word) Data analysis (Excel)
Minitab v17	Minitab	DOE analysis
Rheoplus/32 v3.61	Anton Paar GmbH	Data analysis rheometry

2.8. Strains

Strains	Source	Catalog Number
Chorella vulgaris	Isolate from Peru water sample	
Coccomyxa polymorpha	Isolate from Peru water sample	
Coelastrum sp.	Isolate from Peru water sample	
Cyanothece sp.	Isolate from Portuguese sample	
Haematococcus sp.	Isolate from Peru water sample	
Isochrysis galbana	The Culture Collection of Algae at Göttingen University (SAG)	SAG 13.92
Nannochloropsis oculata	SAG	SAG 38.85
Nannochloropsis salina	SAG	SAG 40.85
Nostoc commune	SAG	SAG 1453-3
Nostoc muscorum	SAG	SAG 1453-12b
Porphyridium purpureum	Biogenic Polymers (BGP) TUM CS	SAG 1380-1
Porphyridium sordidum	BGP TUM CS	SAG 114.7
Scenedesmus sp.	Isolate from Peru water sample	
Synechococcus sp.	BGP TUM CS	
Synechocystis PCC 6803	Isolate from Portuguese sample	

3. Methods

3.1. Preparation of media and buffers

Cultivation media and buffers were prepared as it is described in the following lines unless it is stated for any specific cases. All cultivation media components were sterilized at 121 °C for 20 min or filtrated with 0.2 µm sterile cellulose acetate filters. The details involving the preparation of the different cultivation media are described in the following sections. The chemical composition of each media used in this investigation are presented in Result 4.2, Annex 1; Result 4.3, Fig. 3 and Appendix 1.

3.1.1. Preparation of general media

For general media (BBM, CHU, BG-11), the macroelements were mixed before been autoclaved. The Iron and microelements solutions were filtrated to avoid salt precipitations during the autoclaving process. 1000x solutions of iron-EDTANa_2 and microelements solutions were dissolved and filtrated with a 0.2 µm sterile cellulose acetate filters. The macroelements and microelements solution were mixed at RT under sterile conditions.

3.1.2. Preparation of High Salinity Media (HSM)

Macroelements of HSM media (ASN III, Pm, ASW, IA, IB, IC, I1, I2, I3) precipitated when they were autoclaved. To avoid this phenomenon, macroelements solutions were sterilized separately. Each media has different composition, however, in general the media compounds were dissolved in 85% of the final volume except the sulfate and phosphate source. A 100x sulfate and a 20x phosphate solution were dissolved separately. pH was adjusted before completing the final volume with ultra-pure water to be then autoclaved. Microelements and iron-EDTANa_2 solutions were sterilized as described in the general media (filtration). After been autoclaved sulfate and phosphate were mixed with the other macroelements, microelements and the iron-EDTANa_2 solutions at room temperature (RT) in sterile conditions.

3.1.3. Ammonium-acetate pH 5.6

Acetic acid (MS-grade) 572 µL were mixed with ultrapure water with 1.9 L in a 2 L volumetric flask. The pH was adjusted to 5.6 with 32% ammonium solution, before completing the 2 L volume. The solution was filtered using the 0.2 µm cellulose filter membrane using a vacuum pump. This solution was suitable to be used for maximum 48 hours.

3.1.4. Eluent A

The ammonium-acetate pH 5.6 was mixed (1,700 mL) with acetonitrile (300 mL). The Eluent A should not be used after 48 hours of its preparation.

3.1.5. DNA extraction buffer

Compounds were dissolved in 250 mL of ultrapure water, reaching a final concentration of 200 mM for TRIS-HCl, 250 mM for KCl and 25 mM EDTA. The pH was fixed at 7.5 before completing the final volume.

3.2. Molecular biology techniques

3.2.1. Genomic DNA extraction

2 mL of microalgae cultivation broth were centrifuged at 6,000 ×g for 4 min in a 2 mL reaction tube. The supernatants were separated from the biomass pellets, which were re-suspended in 1.5 mL of lysis buffer, 4 ceramic beads were added to the test tube. Samples were frozen in liquid nitrogen and then milled at 30 Hz for 2 min in a mixer mill (Retsch®). Microalgae suspension from each test tube were separated from the beads to be again frozen in liquid nitrogen and then thawed at 60°C for 3 min in a safe lock reaction tube, this process was repeated three times to enhance cell disruption. 0.5 µL of phenol-chloroform-isoamyl alcohol (25:24:1) were added and vortexed for 1 min. The samples were centrifuged at 12,000 ×g for 10 min to separate the samples in layers. 1.3 mL of the top layer of each test tube was transferred to a new test tube. 0.5 mL of chloroform-isoamyl alcohol (24:1) was added to the top layer and vortexed for 1 min. The samples were centrifuged ones again at 12,000 ×g for 10 min. 0.5 mL of the top layer were aliquoted into new safe lock test tube to be mixed by inversion 5 times with 1 mL of 2-propanol (previously filtrated with a 0.2 µm cellulose filter) and stored at –20 °C. To enhance DNA precipitation, the alcoholic mixture was stored at –20 °C overnight. Samples were centrifuged: 12,000 ×g for 10 min at 4 °C to pellet the DNA. The supernatant was separated and 0.5 mL ethanol (–20 °C) were added to the DNA pellet. The DNA samples were pelleted by centrifuging (12,000 ×g for 10 min at 4 °C) the samples to eliminate the alcoholic fraction. The ethanol traces left was eliminated by drying the samples for 24 hours at RT. The DNA pellets were re-suspended in 50 µL of autoclaved ultrapure water.

3.2.2. 18S and 28S rRNA amplification

The genera were identified by amplifying 18S and 28S ribosomal sequence in this investigation. TAB NEB polymerase from New England BioLabs® Inc., was used to amplify the target gene; in a final volume of 25

µL. Each sample contained 0.4 mM of each primer, 0.2 mM of dNTPS, 0.4 mM of DMSO (NEB), 2.5 U of TAQ (NEB) and 1x Thermopol® buffer (NEB). The PCR programs used is presented in Table 3.1.

Table 3.1.
Detail of the PCR programs used in this study. The programs were used to amplify the ribosomal sequence for the identification of the microalgae strain.

Stage	18S rRNA			28S rRNA		
	Temperature (°C)	Time (s)	Number of cycles	Temperature (°C)	Time (s)	Number of cycles
Initial denaturation	95	300	1	95	240	1
Denaturation	95	30		95	30	
Annealing	56	60	35	52.5	20	30
Extension	68	60		68	90	
Final extension	68	300	1	68	480	1
Storage	16	∞	1	16	∞	1
Forward (5´ to 3´)	GCGGTAATTCCAGCTCCAATAGC			AGCGGAGGAAAAGAAACTA		
Reverse (5´ to 3´)	GACCATACTCCCCCCGGAACC			TACTAGAAGGTTCGATTAGTC		
References	[37]			[376]		

3.2.3. Agarose gel electrophoresis

Genome samples and PCR products were analyzed via electrophoresis, using a 1% (w/v) agarose gel in 1x TAE buffer, prepared from a stock solution 50x TAE (2 M TRIS, 0.05 M EDTA, 57.1 mL of acetic acid in 1,000 mL of ultrapure water. The samples were mixed in a 5:1 ratio with loading dye (NEB). Electrophoresis was carried out for 35 min at 110 mV and 400 mA. The gel was evaluated using gel doc by UV light excitation of the DNA stain (Serva).

3.2.4. Purification of PCR product

The PCR products were cleaned with the NucleoSpin kit (Macherey). Clean PCR product concentrations were quantified using the Nanophotometer P-class (Implem). Purified PCR products concentration was adjusted following the specification required by Eurofins genomics to be sent for sequencing in a final volume of 17 µL.

3.2.5. Identification of the microalgae strain

The chromatograms provided by Eurofins were evaluated with Chromas lite program v2.6.2 and the sequences were blasted in the NCBI webpage to identify the microalgae genus. The results with the higher score were selected as probable genus of the microalgae in evaluation.

3.3. Establishment of microalgae cultivation systems

Microalgae were cultivated in different systems to perform the test described in this investigation.

3.3.1. Cultivation in 100- and 250- mL conical flask

Cultivation was performed at RT and 120 rpm with a 12/12 hours light/dark cycle under a white warm LED (MÜLLER Licht, 15W 1000lm, Germany).

3.3.2. Cultivation in 0.5 L cylinder system (In house design)

Samples were maintained in conical flasks for the conducted studies in modified ASW media. To evaluate growth behavior, both strains were cultivated in a cylinder system (Figure 3.1) using the following parameters. Illumination of the system was done by an external white fluorescent lamp (LUXLINE PLUS, F18W, Germany) and an additional blue fluorescent lamp (AQUA-GLO, 20W T8, Japan) in a 12/12 day-night cycle. Air was provided to the cylinder system at a fixed flow of 100 mL min^{-1} controlled by a flowmeter (Read-y for gas flow, Switzerland). The set-up of the entire system is presented in Result 4.2, Annex 2. The inoculum was cultivated in a 250 mL conical flask for 15 days at RT, 170 rpm and 12/12 hours light/dark cycle with a white LED light (1,000 lm, 15W, Germany).

Figure 3.1 0.5 L cylinder cultivation system. It allowed to cultivate both strains in order to evaluate their growth behavior, nutrient uptake and EPS characterization tests.

3.3.3. Cultivation in DASGIP® Bioblock system

The DASGIP® Bioblock system (Eppendorf, Germany) was equipped with three internal LED probes per reactor. The reactors were covered with opaque sheathings to avoid light interference between them. The irradiance increased following a cosine function, reaching a maximum of 1.87 µE s^{-1} at noon followed by a decrease to zero, emulating 12/12 hours' day/night cycle. The volume of the reactor was 1.5 L, the temperature was maintained at 25 °C, and the system was agitated at 150 rpm and aerated at 50 L h^{-1}. The running DASGIP system is shown in Appendix 2, Fig. A 1.

3.3.4. Cultivation in 4.5 L cylinder system (In house design)

Microalgae were cultivated in 4.5 L systems, Figure 3.2. The system ran at RT, with a white fluorescence LED 10 m (SimpLED, 300 LED, Paulmann), the air inlet was fixed at 2000 mL min^{-1} with a flowmeter (Ready for gas flow, Switzerland), in 4,500 mL of culturing volume.

Figure 3.2. 4.5 L cylinder cultivation system. It allowed to cultivate both strains in order produce EPS for both *Porphyridum* EPSs rheological characterization.

P. sordidum was cultivated in improved medium B (IB) and *P. purpureum* in artificial sea water (ASW) medium. The complete system is displayed in Result 4.4, Fig. S 1.

3.4. Evaluation of microalgae growth and morphology

Each strain was cultivated in biological triplicate in a final volume of 0.5 L. Daily; 2 mL were sampled and centrifuged at 27,000 ×g for 5 min, the free-cell media were stored at –20 °C for further analysis. Growth was evaluated by measuring the OD at 750nm, which was reported to be used for different microalgae strains as it allows the evaluation of the cell growth without the interference of the pigments that are naturally produced by microalgae [9, 377]. The specific growth rate (μ_{max}) and doubling time (t_d) were calculated for both microalgae [37]. But it must be taken into account, that these values are apparent, as factors that affect microalgae growth (photoperiod, light shadowing in high concentrated cultivation broth, etc.) were not considered for their calculation. Zeiss Axio Observer Z1 microscope was used to measure the diameter of 12 random microalgae cells and the t-test was used to evaluate the data.

3.5. Nutrient uptake evaluations

The nitrate and phosphate content were measured from the free cell supernatant after centrifuging the cultivation broth at 21,000 ×g for 5 min. The supernatant stored at –20°C for further analysis.

Nitrate (NO_3^-): Its uptake was determined by a NO_3^- electrode (PRONOVA Analysentechnik GmbH & Co. KG). Standards of sodium nitrate (1, 2, 4, 6, 8, 10 and 12 mM) were added to ASW medium without nitrate and the supernatant from these samples were measured in technical triplicate.

Phosphate (PO_4^{3-}): Its uptake was tested in technical triplicate by use of the colorimetric assay Phosphate-test PO_4^{3-} (Merck). 100 µL monopotassium phosphate standards solutions (0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM) were prepared in phosphate free ASW medium, and supernatants free of microalgae cells were mixed with 25 µL of the reactant and measured at 400 nm in 96 flat bottom microtiter plates (MTP).

3.6. EPS harvesting procedures

3.6.1. Centrifugation

The microalgae cells were pelleted by centrifugation at 4,000 ×g for 10 min at RT. The supernatant was separated from the cell pellets and mixed with two volumes of 2-propanol with an overhead stirrer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) to precipitate microalgae EPS. The EPS fibers were sieved to be separated them from the alcohol mixture. Then, the collected EPS was re-dissolved overnight in 250 mL of ultra-pure water, to be centrifuged at 75,000 ×g for 15 min to eliminate remaining contaminants. EPS was re-precipitated from the supernatant with 2 volumes of 2-propanol, to

and the EPS was collected by sieving the alcoholic mixture with an organdy cotton cloth and was dried at RT for 3 days.

3.6.2. Cross-flow filtration

The biomass and supernatant were separated by centrifuging 1 L of cultivation broth at 4,000 ×g, 20 °C for 30 min. The cell-free supernatant was diluted 1:5 with ultrapure water, and the EPS was concentrated using the Sartorius crossflow system with a 100 kDa ultrafiltration cassette (Hydrosart®, AG, Germany) until reaching a volume of 0.75 L. The salt free concentrated supernatant was precipitated with two volumes of 2-propanol and dried at 45 °C for 3 days in a vacuum oven.

3.7. EPS chemical composition evaluations

The microalgae EPS constituents were determined with the following procedure.

3.7.1. Sample preparation

A 0.1% EPS solution was prepared from each sample by dissolving in ultrapure water. The samples were stirred at 350 rpm, overnight and RT for analysis.

3.7.2. Carbohydrate fingerprint via HT-PMP

The carbohydrate monomers of the *P. sordidum* and *P. purpureum* EPSs were analyzed using the HT-PMP analysis [374]. In brief, a 0.1% solution of each EPS sample was dissolved in ultra-pure water. The evaluations were performed as technical triplicates. A 20 µL volume of aliquots was hydrolyzed with 20 µL of 4 M trifluoroacetic acid (TFA) at 121 °C for 90 min, centrifuged at 2,000 ×*g*, 2 min, 20 °C and neutralized with an appropriate volume of 3.2% ammonium hydroxide. Then, 25 µL of the neutralized EPS solution was transferred to a microtiter plate and mixed with 75 µL of a PMP reagent (0.1 M methanolic 1-phenyl-3-methyl-5-pyrazolone solution: 0.4% ammonium hydroxide solution 2:1), incubated at 70 °C for 99 min in a PCR cycler. A 20 µL volume of aliquots of the samples was mixed with 130 µL of 1:26 diluted 0.5 M acetic acid, and filtrated (0.2 µm Supor, Pall Corporation) into a microtiter plate. The settings used for the HPLC were the following: pure acetonitrile (eluent A) and 5 mM ammonium acetate buffer (pH 5.6) with 15% acetonitrile (eluent B); the Macherey-Nagel Gravity C18 column, 100 mm length, 2 mm i.d., 1.8 µm particle size, was used in this test at a fixed temperature of 50 °C and a flow rate of 0.6 mL min^{-1} coupled to a mass-spectrophotometer. The chromatograms were evaluated using the Chromeleon™, Bruker Hystar and Quant Analysis software.

3.7.3. Glucose assay

The glucose content was measured before and after the hydrolysis and neutralization steps using an enzymatic assay [378]. 1:10 dilutions of the hydrolyzed samples from PMP were used in this evaluation. Briefly, 50 µL of standard solutions (0, 2.5, 5, 10, 25, 50, 100, 250 and 500 µM) and samples were placed in 96-well plates (technical triplicate). Followed by the addition of 50 µL of master mix (40 mM potassium phosphate at pH 5.7, 1.5 mM *2.2-azinobis-(3 ethylbenzthiazoline)-6-sulfonic* acid, 4 U glucose oxidase and 0.2 U horseradish peroxidase) into the samples and standards. An incubation step was required at 400 rpm for 30 min at 30 °C to measure the samples at 418 nm and at 480 nm for background correction.

3.7.4. Pyruvate assay

This assay was performed using an enzymatic assay [378]. 100 µL of a 1:10 diluted samples (before and after hydrolysis and neutralization) and standards (0, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µM) were placed in a microtiter plate and mixed with 100 µL of master mix; 0.1 mM *N-(carboxymethylamino-carbonyl)-4.4'-bis(dimethylamino)-diphenylamine sodium* salt (DA-64), 0.1 mM thiamine pyrophosphate, 0.2 mM magnesium chloride, 40 mM potassium phosphate buffer at pH 5.7, 0.1 U pyruvate oxidase and 0.4 U horseradish peroxidase. Followed by incubation at 37 °C, 400 rpm for 30 min; and measured at 727 nm and 540 nm (correction wavelength) for the pyruvate quantification. All measurements were performed as technical triplicate.

3.7.5. Determination of the EPS sulfate content

The sulfate content was quantified and compared using the two methods described in the following lines. *Enzymatic assay***.** An enzymatic method with the following characteristics [375]. 50 µL of hydrolyzed/neutralized samples (1:20 dilution) and magnesium sulfate standards (2.5, 5, 10, 25, 50, 100, 150, 200 and 250 µM), positive control (κ -carrageenan) and negative control (gellan) were mixed with 50 µL of master mix (12.5 mM of *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES) buffer at pH 7.8, 1 mM guanosine triphosphate, 1 mM adenosine triphosphate, 3 mM magnesium chloride, 0.46 µM adenosine triphosphate sulfurylase and 5.3 µM adenylyl-sulfate kinase), incubated at RT for 45 min. Then, 100 µL of a second master mix (100 mM dipotassium phosphate at pH 6.5, 100 µM *N-(carboxymethylamino-carbonyl)-4.4'-bis(dimethylamino)-diphenylamine sodium* salt (DA-64), 50 µM thiamine pyrophosphate, 100 µM magnesium chloride, 500 µM phosphoenolpyruvate, 500 µM adenosine monophosphate, 0.05 U pyruvate phosphate dikinase, 0.05 U pyruvate oxidase and 0.2 U horseradish peroxidase) was added and incubated at 37 °C for 30 min; measured at 727 nm and 540 nm.

Turbidimetry assay. The second method used was a modified version of the turbidimetric method [379]. In brief, 200 µL of standards (0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mM), as well as 1:10 hydrolyzed samples were placed in measuring triplicate (96 well plates), 100 µL of a master mix at 300 rpm, which contained 2.4 mM barium chloride and 15 mM PEG 6000, to be measured at 600 nm. All evaluations were performed in technical triplicate.

3.7.6. Determination of the EPS protein content

0.1% EPS solutions were used to measure the protein content using the Roti®-Quant Kit (Carl Roth). For this, 50 µL of sample and 0, 20, 40, 60, 80 and 100 mg L^{-1} of BSA standards were placed in technical triplicates in 96-well MTP and mixed with 200 µL of the Bradford reagent at 100 rpm for 30 s and incubated at 37 °C for 5 min to be measured at 590 nm.

3.8. Wavelengths effect on the EPS production and microalgae growth

DASGIP® Bioblock system (Eppendorf, Germany) was used to evaluate the effect of different wavelengths on the growth and EPS production, using the cultivation settings described in Methods 3.3.3. To achieve the evaluation the reactors LED setup was the following: in reactor 1 blue light (430 nm) was tested; in reactor 2 green/yellow/orange light (572/625/640 nm) was used; in reactor 3 orange/red light (660/780 nm) was applied and in reactor 4 a combination of wavelengths (white light emulation) was tested. For 17 days, the strains were adapted to each light evaluation. 0.12 L of inoculum from the cylinder system was transferred into 1.2 L working volume of fresh ASW medium. At the end of the adaptation process, the biomass concentration was normalized in all reactors by adding an adequate volume of fresh medium until reaching similar optical density (OD) values. The growth behavior was evaluated for 7 days by taking daily samples to be measured at OD_{750nm}. At the end of the growth phase, the supernatant was separated from the biomass by centrifugation at 4,000 ×g for 10 min at 20 °C. The EPS was precipitated by mixing the supernatant with two volumes of 2-propanol. The precipitated EPSs were further purified by re-dissolving them in 140 mL of ultrapure water (RT, overnight) followed by centrifugation at 21,000 ×g for 15 min and 20 °C. A second alcohol precipitation was performed as described above, and then the EPS fibers were collected and dried in a vacuum oven at 45 °C for 48 h. All experiments were performed in biological triplicate. The growth behavior was evaluated for 7 days as described in Methods 3.4. At the end of the growth phase. The EPS and biomass were separated as described in Methods 3.6.1.

3.9. Optimization of microalgae cultivation medium

3.9.1. Selection of the most important medium components by Plackett-Burman (PB) design

The identification of the most significant medium components on the growth and EPS production were accomplished utilizing the PB design for both strains. In brief, PB design was used to identify the effect and significance of each ASW medium components. The effect of each compound is calculated by subtracting the summation of the positive responses (Σy^+) with the summation of the negative responses (Σy^-); that value is divided by the number evaluations used in the PB (N) and multiple by 2 (Appendix 2, Equation A 1) [380]. The significance of the components is calculated by squaring the effect and multiplying by N and dividing by 4 (Appendix 2, Equation A 2). The nine components of ASW medium were tested in two levels: presence (+) and absence (–). A summary of 13 runs used in the PB design is presented in Appendix 2, Table A.3. 1 L homogenous pre-culture from the cylinder system was split into 25 mL aliquots and centrifuged at 4,000 ×g for 10 min at RT. The cell pellets were re-suspended in a final volume of 75 mL in sterile conical flasks. Experiments were done in technical triplicate. Cultivation was performed for 7 days in 100 mL flask using the settings described in Methods 3.3.1. The biomass from each evaluation were separated from the supernatant as in Methods 3.6.1. The biomasses were dried at 105 °C for 3 days for gravimetrical analysis. As well the EPS were gravimetrically evaluated. Confirmation runs were performed in biological duplicates, using the cultivation parameters and harvesting technique above described.

3.9.2. Optimization of the cultivation medium

Cultivation medium for each strain was optimized using the Taguchi design. The Taguchi design, is a powerful design of experiment (DOE), which uses am orthogonal tabulated system that allow the estimation in an unbiased fashion of the optimal levels, using random combination of fixed levels in evaluating using the minimum amount of experimental runs [381, 382]. In addition, the Taguchi design provides the larger the better definition which allows a maximization of the outcome variable [381, 383, 384]. In this investigation the concentrations of the six most important medium compounds selected by the PB design for each strain were varied simultaneously. The selected compounds for *P. sordidum* were NaCl, $MgSO_4$, $CaCl_2$, $NaNO_3$, microelements and KH_2PO_4, while $EDTANa_2$, $FeSO_4$ and $NaHCO_3$ concentrations were maintained at the concentrations present in the original ASW medium. The compounds selected for *P. purpureum* were: NaCl, $MgSO_4$, $NaNO_3$, KH_2PO_4, $NaHCO_3$ and $CaCl_2$, while the microelements, $EDTANa_2$ and $FeSO_4$ concentrations were held constant. Five compounds were evaluated in three levels (g L^{-1}) and the last compound was evaluated in two levels in an orthogonal array (L_{18}). The

larger the better definition was used because the objective of the Taguchi design was to enhance the growth. The different runs generated by the Minitab v17 program are shown in Appendix 2, Table A 4. The evaluations (biological triplicate) were cultivated for 7 days (Methods 3.3.1) and the biomass was harvested as described Methods 3.9.1. A confirmation run for both rhodophyta strains was performed to compare the optimal medium against ASW medium for 40 days using the same cultivation parameters and EPS harvesting procedures as described in Methods 3.6.1.

3.10. Comparison of abiotic stress factors on EPS production by Taguchi design

Four abiotic factors were tested on EPS induction using the Taguchi design in three levels (L_9). The factors evaluated were the NaCl concentration (0, 13.5, 27 g L^{-1} for *P. sordidum* and 0, 27 and 50 g L^{-1} for *P. purpureum*), pH (3, 7 and 11), hours of light exposure (12, 18 and 24 hours) and temperature (20, 25 and 30 °C). The combination of factors was again generated by the Minitab v17 program and is summarized in Appendix 2, Table A 5. The larger the better definition was used as the objective was to increase in the EPS production. The factor combination was achieved by adding 25 mL of different buffers with fixed pH and NaCl concentrations to 50 mL of microalgae cultivation broth obtained from the cylinder system. Aluminum shades were used to vary the time, which cultures were exposed to light. The induction was performed for 5 days in biological triplicate using settings described in Methods 3.3.1. The EPS was separated from the biomass as described in Methods 3.6.1.

3.11. Two-stage cultivation system

The DASGIP® Bioblock system was used to compare two-stage and one-stage systems with the initial cultivation parameters used in this investigation (biological duplicate). For the one-stage cultivation, the parameters described in Methods 3.3.3 were used; the wavelengths combination (white light emulation) was used for both *Porphyridium* strains. The two-stage cultivation was performed as follows: the first stage focused on the microalgae growth using the same cultivation parameters that were used in the one-stage process for 15 days, followed by a second stage that focused on induction of EPS production for 5 days. The induction parameters for *P. sordidum* were the following: temperature decreased to 21 °C and light exposure increased to 24 hours. The EPS induction phase for *P. purpureum* was achieved by adding 180 mL of 20x improved medium 3 (I3) to decrease the pH to 6. All other parameters were the same as for the growth phase. The growth behavior was evaluated as described in Methods 3.4. The EPS was obtained as described in Methods 3.6.2.

3.12. Rheological evaluations

The EPS for this evaluation was obtained using the 4.5 L cultivation system (Methods 3.3.4) and the EPS was obtained using with the procedure described in Methods 3.6.2. The rheological measurements were performed using 830 µL of EPS solutions in the air-bearing MCR300 controlled stress rheometer (Anton Paar); using a cone plate geometry (50 mm diameter, 1◦cone angle, 0.05 mm gap) the samples were equilibrated at a fixed temperature of 20±0.1 °C for 5 min. The data were collected and analyzed with Rheoplus V.3.61 software (Anton Paar). Five moduli were tested in technical triplicate to determine rheological properties, which are described in the following sections.

3.12.1. Sample preparation

Solutions (1% w/v) of *P. sordidum* and *P. purpureum* EPS (60 mL) were prepared by stirring at 500 rpm overnight in ultrapure water in 100 mL conical flask. 20 mL of free salt polymers, separated to be used for rheological evaluation. 0.4 mL of 4.3 M solution NaCl and 0.8 mL of 2.1 M $CaCl_2$ were mixed with the 1% EPS solution reaching a final volume of 20 mL and a final salt concentration of 85 mM. Additionally, microalgae EPS samples were dissolved in their respective cultivation media at a 1 % (w/v) concentration. The flasks were hermetically sealed to avoid water loss during the dissolution process. To evaluate the effect of pH and temperature on *P. purpureum* EPS several batches were collected and mixed in 9 mL of ultra-pure water, overnight at 500 rpm reaching a final concentration of 0.75 % w/v. The following day 1.8 mL were aliquoted; 0.2 mL of 1M HCl was added to one aliquot, the same volume of 1M NaOH was added to a second aliquot and the last three aliquots were mixed with 0.2 mL of ultra-pure water. The samples were mixed overnight at 500 rpm. One of the three samples dissolved in water was used as control. One was autoclaved as described in Methods 3.1 and the other was placed overnight at –80°C.
Rheological measurements were conducted with an air-bearing MCR300 stress-controlled rheometer (Anton Paar, Germany), using a cone-and-plate geometry (50 mm diameter, 1° cone angle, 0.05 mm gap). The data were collected and analyzed with Rheoplus V.3.61 software (Anton Paar, Germany). The samples (830 µL) were equilibrated at a fixed temperature of 20 ± 0.1°C for 5 min before measuring.

3.12.2. Rheological measurements

Viscosity curves: They were analyzed during a logarithmic shear rate ramp ($\dot{\gamma}$, 0.001 – 1000 s^{-1}) with 25 points with a gradually decreasing measurement time from 100 to 5 s per data point.

Amplitude sweeps: They were measured logarithmically by an increased shear stress amplitude from 10^{-1} to 10^{2} Pa at a fixed frequency of 1 Hz. Amplitude sweeps were used to determine the linear viscoelastic (LVE) region.

Frequency sweeps: They were measured at constant shear stress amplitude within the LVE from 10^{-2} to 10^{1} Hz.

Temperature sweeps: They were conducted within the LVE region at a frequency of 1 Hz. A discrete temperature ramp from 20 °C to 75 °C at a heating ramp of 4 °C min^{-1}. To prevent evaporation, the edge of the sample was covered with low viscosity paraffin oil (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

Thixotropic behavior: Samples was evaluated in a three-stage oscillatory shear test. During the first stage, the sample was measured within the previously determined LVE region followed by a high oscillatory shear of 10^{2} Pa. The recovery of the structure was then measured over 10 min within the LVE region.

3.13. Phycobiliproteins extraction and quantification

P. sordidum and *P. purpureum* biomasses were obtained from the 0,5 L cylinder system in biological duplicate (Methods, 3.3.2), to test the phycobiliproteins as follows. The biomasses were re-suspended in 10 % of the original volume (50 mL) in ultrapure water and mixed for 1 h at 4 °C and 500 rpm. This was done to eliminate contaminants that could be attached from the biomass. The biomass was pellet at RT, 4,000 ×g for 30 min. The *P. sordidum* biomass was resuspended in 50 mL of a 50 g L^{-1} of NaCl solution. Contrary *P. purpureum* biomass was re-suspend in 100 mL of buffer phosphate 20 mM fixed at pH 7 as it was previously described for this strain [135, 385]. The suspension was mixed overnight at 4 °C and 500 rpm. And supernatant was separated from the biomass by centrifuging the suspension at 4 °C 4,000 ×g for 30 min.

The supernatant (Phycobiliprotein extract) was collected and stored at 4°C for further analysis. 1 mL of the phycobiliprotein extract was measured in technical triplicate at OD_{650nm}, OD_{620nm}, and OD_{565nm} to determine the concentration of phycocyanin (R-PC), allophycocyanin (APC) and phycoerythrin (B-PE) the equations were obtained from previous investigations [135, 385, 386]. The equations are listed in Appendix 3. The evaluation was performed in 1 mL disposable cuvettes.

Phycobiliproteins crude extract fluorescence spectrum was evaluated from using the Varioskan. The emission was measured from 409 to 700 nm and the excitation from 250 to 615 nm. The maximum value from each evaluation was selected and use to divide all the measured values to normalize the evaluation.

4. Results

4.1. Selection of EPS producing microalgae strains

The first step performed in this investigation was the selection of microalgae capable of producing EPS. For that, after the cultivation; free cell supernatant was used to test EPS production. Although, there are several methods to determine EPS producing microbes; alcoholic precipitation was selected as the most suitable technique for microalgae [387]. Other techniques required larger volumes of cell free medium to identify microalgae EPS producers. Alcoholic precipitation only needed 50 mL supernatant to perform the test. Otherwise, methods that use less volume for the identification, did not detected EPS due to the low concentration produced by microalgae. The only drawback of this method was the detection of false positives (salts co-precipitation from the cultivation medium). This problem was solved by sieving the alcoholic mixture (1 volume of free cell supernatant with 2 volumes of 2-propanol) through an organdy cotton cloth, which collect EPS fibers while the non-cohesive salts precipitants passed through it. From the pool of microalgae tested (Material, 2.7), three strains were identified as EPS producers, *Cyanothece sp.* (cyanobacteria); *P. purpureum and P. sordidum* (both rhodophytas). The first two strains were already characterized EPS producers; while the third was briefly described as EPS producer, so it was selected for this research [151, 332, 355]. Also, *P. purpureum*, was selected as benchmark for *P. sordidum* evaluation. The next step was the molecular confirmation of the rhodophyta strain genus. This procedure was particular challenging as the 18S rRNA primers were selective to identify eukaryotic microalgae strains but not specific for rhodophyta strains [37]. This was solved by lowering the annealing temperature from 56 to 54 °C and adding DMSO into the PCR master mix. After adjusting the PCR settings, an 18S rRNA amplicon (around 500 bp) was obtained and sequenced, corroborating the genus of *P. purpureum*. Contrary, although 18S rRNA region was amplified in the *P. sordidum* DNA samples; the sequencing and confirmation of the genus was only achieved using the reverse 18S rRNA primer. Because the amplicon obtained with the 18S rRNA primers were rather small, a second set of primers targeting the 28S rRNA (700 bp amplicon) were also utilized. The 28S rRNA primer target D1-D2 region, which is reported to detect eukaryote organism including microalgae [376, 388]. Unfortunately, no amplifications were generated using those primers, possibly because the D1-D2 conserved region is not part of the rhodophyta genus. This suggests that rhodophyta are genetically different from other eukaryotic microalgae strains that were identified by both set of primers like *Chlorella spp., Scenedesmus spp., Pavlova spp.* and *Chaetoceros spp.* [37, 389]. After corroborating the genus of both rhodophyta solely based on the 18S rRNA, they were cultivated in controlled environments to perform further investigation.

4.2. Characterization and comparison of *Porphyridium sordidum* and *Porphyridium purpureum*

4.2.1. Characterization and comparison of *Porphyridium sordidum* and *Porphyridium purpureum* concerning growth characteristics and polysaccharide production

In this work *P. sordidum* was characterized in detail for the first time and compared to *P. purpureum*, an already known EPS producer. Several aspects were evaluated to detect their effect on growth and EPS production. Firstly, both *Porphyridium* strains were phenotypically compared; identifying that *P. sordidum* had a slightly larger cell size than *P. purpureum*. Furthermore, for *P. sordidum* olive-green and *P. purpureum* red biomass color, typical for *Porphyridium* phyla (rhodophyta) were observed.

After recognizing phenotypical differences, the growth behavior was evaluated, cultivating both strains in a bubbling cylinder system. Microalgae growth was measured indirectly using the optical density (OD_{750nm}) for 30 days. 750 nm was selected because *Porphyridium* strain pigments did not interfere in the growth measurement evaluation. In addition, the apparent specific growth rate (μ_{max}) and doubling time (t_d) were calculated in order to compare both strains in the cultivation system. Furthermore, nitrate and phosphate uptake were daily measured. All together it was determined that the cultivation setting used in this investigation favor *P. purpureum* growth in contrast to *P. sordidum*.

At the end of the cultivation-stage, both EPSs were harvested and gravimetrically measured. Afterwards, the chemical compositions of *Porphyridium* EPS were elucidated. Both EPSs contained xylose, galactose, glucose and glucuronic acid. Furthermore, HT-PMP analysis allowed to identify methyl carbohydrates in both EPS samples: *P. sordidum* EPS contain a methyl pentose and methyl hexose and *P. purpureum* EPS had a methyl hexose uronic acid dimer. In addition, sulfate content (non-carbohydrate fraction) was quantified in both EPS. Furthermore, the characterization was completed conducting a preliminary rheological evaluation (flow curve and amplitude sweep), identifying a typical thinning behavior of *Porphyridium* EPSs; similar to the ones measured in other carbohydrate polymers.

This investigation allowed to elucidate the parameters that influence microalgae growth and EPS production in *P. sordidum* and *P. purpureum* which were further investigated in subsequent tests.

The author designed and performed the experiments, analyzed and interpreted the data. Broder Rühmann analyzed and interpreted the data. Jochen Schmid designed the study, analyzed and interpreted the data. Volker Sieber designed the study. All authors contributed in the manuscript preparation for publication.

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Characterization and comparison of *Porphyridium sordidum* and *Porphyridium purpureum* concerning growth characteristics and polysaccharide production

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ABSTRACT

Microalgae as photosynthetic microorganisms are the source of valuable compounds such as proteins, carotenoids, lipids and carbohydrate polymers. Among the different phyla, *Porphyridium* as part of the red microalgae (Rhodophyta) are of high importance as producers of sulfated polysaccharides. As the name suggests these microalgae are typically red; however, *Porphyridium sordidum* has an olive-green color and was recently described as a putative exopolysaccharide producer. Exopolysaccharides (EPS) are sugars polymers excrete to the culture medium. In this study *P. sordidum* is evaluated for the first time in detail as newly described EPS producer in direct comparison to the already characterized EPS producer *Porphyridium purpureum*. The evaluation was performed on several aspects, such as morphological differences between the two strains followed by the comparison of the growth behavior, nitrate and phosphate uptake during cultivation of both strains, and a detailed analysis of the EPS. *P. purpureum* consumed 51 ± 4.6% of nitrate and 95 ± 2.1% of phosphate compared to *P. sordidum* which utilized 17 ± 0.1% of nitrate and 68 ± 0.1% of phosphate at day 30 of cultivation. The produced EPS were analyzed and revealed the major constitutional carbohydrate monomers of the EPS to be xylose, galactose, glucose and glucuronic acid. In addition, other substituents that decorate the EPS were identified as sulfate and methyl groups. Finally, rheological measurements (viscosity curves and amplitude sweeps) were performed to analyze the physicochemical properties.

1. Introduction

Among the different products generated by microalgae, the polysaccharides represent important bio-macromolecules. Basically, there are two types of polysaccharides that are produced by microalgae, the storage polysaccharides in the cytoplasm and the secreted ones, which can be found in the culture media, also known as exopolysaccharides [1,2]. EPSs are produced by various microbes, with the xanthan gum producing bacteria *Xanthomonas campestris* as the best known commercial representative [3]. Other bacteria described to produce EPS are *Azotobacter vinelandii* (alginate), *Sphingomonas paucimobilis* (gellan), and *Bacillus subtilis* (levan) [4–6]. Additionally, some fungi have been described as EPS producer, such as *Aureobasidium pullulans* (pullulan) and *Sclerotium glutanicum* (scleroglucan) to name just two [4,7]. These EPSs have multiple applications in the pharmaceutical, cosmetic and food industries because of their rheological and health promoting properties [8,9]. Unlike most bacterial and fungal EPSs, microalgae EPSs can bear specific functional substituents like methyl (CH_3) and sulfate (SO_4^{-2}) groups. These substituents confer unique properties to the EPS for broader applications as antiviral, anticoagulation and bioremediation agents [10–12].

Currently, several cyanobacteria (prokaryotic algae) are known as EPS producers. The most important ones are *Nostoc, Phormidium, Arthrospira and Cyanothece* genus [13,14]. On the other hand, several phyla of eukaryotic microalgae are also reported to produce EPSs, such as Chlorophyta (green microalgae), Bacillariophyta (diatoms), Cryptophyta, Miozoa (dinoflagellates) and Rhodophyta (red microalgae) [2,13,15]. Several genuses from the Rhodophyta such as *Rhodella, Flintiella, Rhodosorus* and *Porphyridium* have been described as EPS producers [16–20].

In this work, *Porphyridium sordidum* was characterized in detail as an EPS producer for the first time. Although, this strain is a

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Rhodophyta, it has a distinctive olive-green color [21–24]. In addition, *P. sordidum* was compared to the better characterized EPS producing Rhodophyta, *P. purpureum*, by analyzing growth behavior, EPS productivity and composition [25].

2. Material and methods

2.1. Chemical and reagents

All reagents were of analytical or microbiological grade and were purchased from Carl Roth GmbH (Karlsruhe, Germany) or Merck KGaA (Darmstadt, Germany) unless stated otherwise. All the experiments were performed with ultra-pure water obtained from a PURELAB system.

2.2. Microalgae strains and culture conditions

The two microalgae used in this work, *Porphyridium sordidum* (SAG 114.7) and *Porphyridium purpureum* (SAG 1380-1a) were initially cultivated in different media such as Bold's Basal Medium (BBM), BG-11, modified Provasoli (Pm) and modified Artificial seawater (ASW) medium, all the media compositions are given in the supplementary data, Annex 1. The media were autoclaved at 121 °C for 20 min.

Samples were maintained in conical flasks for the conducted studies in modified ASW media. To evaluate growth behavior, both strains were cultured in a cylinder system designed in our group using the following parameters. Illumination of the system was done by an external white fluorescent lamp (LUXLINE PLUS, F18W, Germany) and an additional blue fluorescent lamp (AQUA-GLO, 20 W T8, Japan) in a 12/12 day-night cycle. Air was provided to the cylinder system at a fixed flow of 100 mL min^{-1} controlled by a flowmeter (Read-y for gas flow, Switzerland). The set-up of the entire system is displayed in the supplementary data, Annex 2. The inoculum was cultured in a 250 mL conical flask for 15 days at room temperature (RT), 170 rpm and 12/12 h light/dark cycle with a white LED light (1000 lm, 15 W, Germany).

Each strain was cultured in biological triplicate in a final volume of 0.5 L, daily; 2 mL samples were taken and centrifuged at 27,000 x*g* for 5 min, the free-cell media were stored at −20 °C for further analysis. The growth was evaluated by measuring the OD at 750 nm, which was reported to be used for different microalgae strains as it allows the evaluation of the cell growth without the interference of the pigments that are naturally produced by microalgae in *Griffiths* et al., *2011* and *Gaignard* et al., *2019* [26,27]. The specific growth rate (μ_{max}) Eq. (1) and doubling time (t_d) Eq. (2) were calculated for the microalgae used in this study (supplementary data, Annex 3) [28]. But it must be taken into account, that these values are apparent, as factors that affect microalgae growth (photoperiod, light shadowing in high concentrated culture, etc.), were not considered for their calculation.

The Zeiss Axio Observer Z1 microscope was used to measure the diameter of 12 random microalgae cells and the *t*-test was used to evaluate the data.

2.3. Evaluation of nitrate and phosphate consumption

The nitrate (NO_3^-) uptake was determined by a NO_3^- electrode (PRONOVA Analysentechnik GmbH & Co. KG). Standards of sodium nitrate (1, 2, 4, 6, 8, 10 and 12 mM) were added to ASW medium without nitrate and the supernatant from these samples were measured in technical triplicate.

The phosphate (PO_4^{3-}) uptake was tested in technical triplicate by use of the colorimetric assay Phosphate-test PO_4^{3-} (Merck). 100 µL monopotassium phosphate standards solutions (0.01, 0.02, 0.04, 0.08, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mM) were prepared in phosphate free ASW medium, and supernatants free of microalgae cells were mixed with 25 µL of the reactant and measured at 400 nm in 96 flat bottom microtiter plates (MTP).

2.4. Harvesting of exopolysaccharides (EPS)

At the end of the culturing process, EPS was harvested by alcohol precipitation. In brief, the microalgae cells were pelleted by centrifugation at 4000 x*g* for 10 min at RT. The supernatants (370 mL) were separated from the cell pellets and mixed by use of an overhead stirrer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) with two volumes of 2-propanol to precipitate the EPS. Finally, the EPS fibers were sieved to be separated from the alcohol mixture. Then, the collected EPS was re-dissolved overnight in 250 mL of ultra-pure water, centrifuged at 75,000 x*g* for 15 min to eliminate remaining contaminants, and then re-precipitated with 2 volumes of 2-propanol, to be collected by sieving the EPS with an organdy cotton cloth and dried at RT for 3 days to evaporate the 2-propanol.

2.5. Determination of the EPS monomer composition

The carbohydrate monomers of the *P. sordidum* and *P. purpureum* EPSs were analyzed as described by *Rühmann* et al., *2014* [29]. In brief, a 0.1% solution of each EPS sample was prepared in ultra-pure water. The evaluations were performed as technical triplicates. A 20 µL volume of aliquots was hydrolyzed with 20 µL of 4 M trifluoroacetic acid (TFA) at 121 °C for 90 min, centrifuged at 2000 ×*g*, 2 min, 20 °C and neutralized with an appropriate volume of 3.2% ammonium hydroxide. Then, 25 µL of the neutralized EPS solution was transferred to a microtiter plate and mixed with 75 µL of a PMP reagent (0.1 M methanolic 1-phenyl-3-methyl-5-pyrazolone solution: 0.4% ammonium hydroxide solution 2:1), incubated at 70 °C for 99 min in a PCR cycler. A 20 µL volume of aliquots of the samples was mixed with 130 µL of 1:26 diluted 0.5 M acetic acid, and filtrated (0.2 µm Supor, Pall Corporation) into a microtiter plate. The settings used for the HPLC were the following: pure acetonitrile (eluent A) and 5 mM ammonium acetate buffer (pH 5.6) with 15% acetonitrile (eluent B); the Macherey-Nagel Gravity C18 column, 100 mm length, 2 mm i.d., 1.8 µm particle size, was used in this test at a fixed temperature of 50 °C and a flow rate of 0.6 mL min^{-1} coupled to a mass-spectrophotometer. The chromatograms were evaluated using the Chromeleon™, Bruker Hystar and Quant Analysis software.

2.6. Glucose assay

The glucose content was measured before and after the hydrolysis and neutralization steps using the method described by *Rühmann* et al., *2016* [30]. 1:10 dilutions of the hydrolyzed samples from PMP were used in this evaluation. Briefly, 50 µL of standard solutions (0, 2.5, 5, 10, 25, 50, 100, 250 and 500 µM) and samples were placed in 96-well plates (technical triplicate). Followed by the addition of 50 µL of master mix (40 mM potassium phosphate at pH 5.7, 1.5 mM *2.2-azinobis-(3 ethylbenzthiazoline)-6-sulfonic* acid, 4 U glucose oxidase and 0.2 U horseradish peroxidase) into the samples and standards. An incubation step was required at 400 rpm for 30 min at 30 °C to measure the samples at 418 nm and at 480 nm for background correction.

2.7. Pyruvate assay

This assay was performed according to *Rühmann* et al., *2016* [30]. 100 µL of a 1:10 diluted samples (before and after hydrolysis and neutralization) and standards (0, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µM) were placed in a microtiter plate and mixed with 100 µL of master mix; 0.1 mM *N-(carboxymethylamino-carbonyl)-4.4′-bis(dimethylamino)-diphenylamine sodium* salt (DA-64), 0.1 mM thiamine pyrophosphate, 0.2 mM magnesium chloride, 40 mM potassium phosphate buffer at pH 5.7, 0.1 U pyruvate oxidase and 0.4 U horseradish peroxidase. Followed by incubation at 37 °C, 400 rpm for 30 min; and measured at 727 nm and 540 nm (correction wavelength) for the pyruvate quantification. All measurements were performed as technical triplicate.

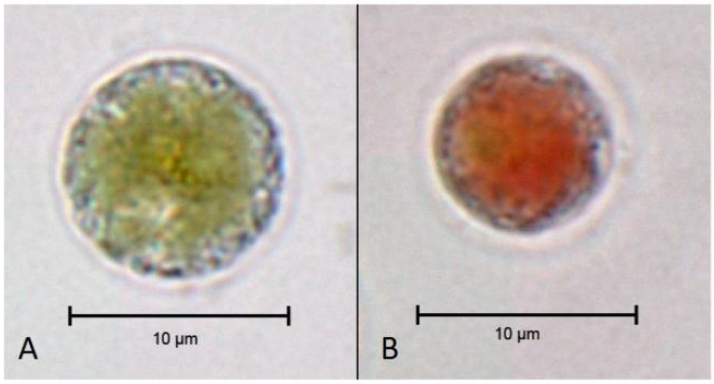

Fig. 1. Microscopic pictures were taken with the Zeiss Axio Observer Z1 with the ×100 objective on samples at day 20 of the culturing process. (A) *Porphyridium sordidum* showing olive-green color and a diameter of around 10 µm (B) *Porphyridium purpureum* with the typical red color of Rhodophyta. Compared to *P. sordidum* it has a smaller diameter (around 8 µm). The scales denote 10 µm for both strains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

2.8. Determination of the EPS sulfate content

The sulfate content was quantified and compared using the two methods described in the following lines. An enzymatic method described by *Ortiz-Tena* et al., *2018* [31]. In which, 50 µL of hydrolyzed/neutralized samples (1:20 dilution), magnesium sulfate standards (2.5, 5, 10, 25, 50, 100, 150, 200 and 250 µM), positive control (carrageenan κ) and negative control (gellan) were mixed with 50 µL of master mix (12.5 mM of *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES) buffer at pH 7.8, 1 mM guanosine triphosphate, 1 mM adenosine triphosphate, 3 mM magnesium chloride, 0.46 µM adenosine triphosphate sulfurylase and 5.3 µM adenylyl-sulfate kinase), incubated at RT for 45 min. Then, 100 µL of a second master mix (100 mM dipotassium phosphate at pH 6.5, 100 µM *N-(carboxymethylamino-carbonyl)-4.4′-bis (dimethylamino)-diphenylamine sodium* salt (DA-64), 50 µM thiamine pyrophosphate, 100 µM magnesium chloride, 500 µM phosphoenolpyruvate, 500 µM adenosine monophosphate, 0.05 U pyruvate phosphate dikinase, 0.05 U pyruvate oxidase and 0.2 U horseradish peroxidase) was added and incubated at 37 °C for 30 min; measured at 727 nm and 540 nm. The second method used was a modified version of the turbidimetric method described by *Lundquis* et al., *1980* [32]. In brief, 200 µL of standards (0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, 9 and 10 mM), as well as 1:10 hydrolyzed samples were placed in measuring triplicate (96 well plates), 100 µL of a master mix at 300 rpm, which contained 2.4 mM barium chloride and 15 mM PEG 6000, to be measured at 600 nm. All evaluations were performed in technical triplicate.

2.9. Determination of protein content

EPSs solutions of 0.1% were used to measure the protein content of the samples by use of the Roti®-Quant Kit (Carl Roth). For this, 50 µL of sample and 0, 20, 40, 60, 80 and 100 mg L^{-1} of BSA standards were placed in technical triplicates in 96-well MTP and mixed with 200 µL of the Bradford reagent at 100 rpm for 30 s and incubated at 37 °C for 5 min to be measured at 590 nm.

2.10. Rheological measurements

1% solutions from *P. sordidum* and *P purpureum* EPS were dissolved in ultra-pure water overnight for rheological characterization. In short, the measurements were performed in an air-bearing MCR300 controlled-stress rheometer, using cone plate geometry (50 mm diameter, 1 °cone angle, 0.05 mm gap) at a constant temperature of 20 °C.

Two rheological measurements were performed as technical triplicate, the viscosity curves which were obtained during a logarithmic shear-rate ramp ($\dot{\gamma}$ = 0.1–1000 s^{-1}), and the shear stress amplitude sweep (logarithmic ramp from 0.1–100 Pa) at a constant frequency of 1 Hz by recording measurements at 20 points per decade (log scale). Data were collected and analyzed with Rheoplus V.3.61 software.

3. Result and discussion

3.1. Evaluation of the most suitable media composition and morphological characterization

Different prevalent media were tested to identify the most suitable one for both *Porphyridium* strains due to the different sources of where this genus can be found (fresh and salty water) [33]. The use of BBM and BG-11 resulted in poor growth of *P. purpureum*. The modified Pm and ASW medium presented cell growth for *P. purpureum*, but a higher growth was detected in the modified ASW medium (data not shown). Therefore, this medium was chosen to perform the following experiments. Even more, the ASW medium was previously reported to be used for culturing *P. purpureum* [34,35]. For *P. sordidum*, the only medium that could sustain cell growth was the modified ASW, although it was reported that *P. sordidum* was isolated from the botanical greenhouse samples and not from a marine water environment [21,22]. The modifications performed on the ASW media for this study are the following. The nitrate source was changed from potassium (used in previous investigations) to the sodium salt at the same concentration of 9.88 mM because sodium nitrate is most commonly used in other microalgae media [36]. Additionally, TRIS-HCl was excluded from the ASW medium, given that it has no nutritional effect and other ASW media reportedly do not have this compound in their composition [35]. Finally, the microelement solution was prepared separately from the other compounds from the media (supplementary data, Annex 1.1).

The morphological evaluations of both Rhodophyta strains were performed with the Zeiss Axio Observer Z1 with the ×100 objective (Fig. 1). The phenotypical characteristic as reported, the red color of the *P. purpureum* and the olive-green color of the *P. sordidum* could be confirmed [21,22]. The morphological evaluation allowed the comparison of the mean cell-size, which could affect growth rate and EPS production. *P. sordidum* cells have a larger diameter (9.8 ± 0.8 µm) compared to the *P. purpureum* (7.9 ± 0.7 µm) cells, therefore *P. sordidum* has a lower doubling time because of its bigger dimension compared to *P. purpureum*. This effect was previously described in other microalgae (for example diatoms), showing that larger cell size had a lower growth rate when they were compared to other smaller cells [37]. The quantification of the microalgae diameter is presented in the supplementary data, Annex 4. A summary of the morphological characteristics, such as diameters, calculated surface and volumes are provided in Table 1. The growth parameters, which were calculated in this investigation, are applicable to this particular culture system.

Table 1
The diameter of the two Rhodophyta strains was calculated by measuring random cells ($n = 12$), and the surface and volume were calculated. A summary of the calculated growth parameters (t_d and μ_{max}), the final values of the OD_{750nm} at the end of 30 day culture in the modified ASW medium. The growth parameters are approximation as their calculation was done with OD_{750nm}. The data present denote the average and standard deviation as variability ($n = 3$).

Characteristics	Strain	
	P. sordidum	*P. purpureum*
Diameter [μm]	9.8 ± 0.8	7.9 ± 0.7
Surface [μm^2]	306 ± 55	197 ± 33
Volume [$\mu m]^3$	509 ± 142	264 ± 64
μ_{max}, [day^{-1}]	0.17 ± 0.01	0.22 ± 0.01
t_d, days	4.1 ± 0.1	3.2 ± 0.2
OD_{750nm}	0.47 ± 0.04	3.2 ± 0.5

3.2. Comparison of the growth behavior of P. sordidum to P. purpureum

The two Rhodophyta strains were compared in biological triplicate to evaluate their growth behavior in cylinder reactors. Two types of light (white and blue light) were used for the culturing process, since it is reported that blue light enhances cell growth as well as EPS production in different microalgae strains and especially in the *Porphyridium* genus [38,39]. Daily samples were taken and the optical density (OD) at 750 nm was used to evaluate the growth rate. In a preliminary work, other wavelengths (OD_{440nm} and OD_{680nm}) which were reported to be used to analyze microalgae growth, were tested and compared to OD_{750nm} [40–42]. It was corroborate that the OD_{750nm} was the most suitable wavelength to measure cell growth in microalgae in addition it was previously reported to be used for *P. purpureum* [34].

Both *Porphyridium* strains grew best in modified ASW medium, with *P. purpureum* showing significantly faster growth than *P. sordidum* (Table 1). This might be explained by the different origin of the strains, since *P. purpureum* was isolated from salty water and grew well in enriched media, such as the modified ASW medium [43]. The use of the OD_{750nm} allowed an indirect comparison between the growth of both strains (Fig. 2). The difference in the OD_{750nm} detected between the *P. sordidum* and *P. purpureum* were mediated by the different cell size. This behavior was observed in the standard curve shown in supplementary data, Annex 5.2.

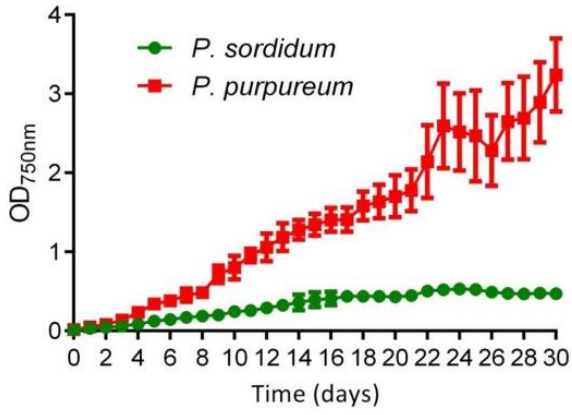

Fig. 2. Comparison of the growth curves ($n = 3$) of *P. sordidum* (green) and *P. purpureum* (red), measured at OD_{750nm}. The main difference of the measurements is the lower response of *P. sordidum* compared to *P. purpureum* at the wavelength of 750 nm. The graph is based on the average including the standard deviation as variability ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Moreover, the approximated doubling time (t_d) of *P. purpureum* (3.2 ± 0.2 days) was shorter than that of *P. sordidum* (4.1 ± 0.1 days). Despite the difference in the OD_{750nm} response, the calculated doubling time of both strains differ only by one day. The bigger *P. sordidum* had a lower response compared to the smaller *P. purpureum* in the entire visible spectra (supplementary data, Annex 5).

3.3. Comparison of nutrient uptake for P. sordidum and P. purpureum

The nutrients uptake was analyzed for a detailed evaluation of growth characteristics. Nitrate was quantified as it is a key nutrient for cell proliferation and protein generation of microalgae [44]. It is known that low concentrations of nitrate favor the shift to the stationary phase, thus inducing EPS production as response to starvation [45,46]. For *P. purpureum,* the nitrate uptake was 0.22 ± 0.0 mM day^{-1} in contrast to *P. sordidum* which only consumed 0.06 ± 0.0 mM day^{-1}. After 30 days of cultivation *P. purpureum* metabolized (68 ± 0.1%) and *P. sordidum* (17 ± 0.1%) of the total nitrate. Thus, it can be concluded that the nitrate uptake affects growth of *P. purpureum* much more than for *P. sordidum* due to its higher uptake as is shown in Fig. 3A.

As second nutrient phosphate consumption was evaluated, for which it is known that its depletion is strongly related to a decrease of microalgae cell generation [44]. In this respect, it was observed that in the first 10 days phosphate was consumed by *P. purpureum,* reaching 0.04 ± 0.00 mM day^{-1}. At the end of the cultivation process (day 30) 95 ± 2.1% of phosphate was consumed (Fig. 3B). *P. sordidum* consumed 0.02 ± 0.00 mM day^{-1} of phosphate in the first 10 days. After that, no significant phosphate uptake was observed until the end of the cultivation process (51 ± 4.6%). Thus, it could be concluded that phosphate greatly triggers cell growth as it was better metabolized than the nitrate.

3.4. Gravimetrical determination of EPS titers

The first precipitations of *P. purpureum* as well as *P sordidum* supernatants resulted in gravimetrically determined EPS titers of 0.69 ± 0.04 g L^{-1} and 0.51 ± 0.02 g L^{-1} respectively. The subsequent analysis of the monomer composition showed that only a very low recovery of 14% could be realized, indicating a quite low purity of the EPS, contaminated with e.g. cell debris, proteins and salts. By those observations, an optimized purification protocol was established, resulting in a carbohydrate recovery of 44% for *P. purpureum* and 42% for *P. sordidum,* which corresponds to recoveries described for other microbial EPSs quantified by the HT-PMP method [47–49].

The titers of the purified EPSs were thus determined to be 0.24 ± 0.03 g L^{-1} for the *P. purpureum,* and 0.16 ± 0.01 g L^{-1} of EPS for the *P. sordidum*. The final titers and the volumetric productivity are summarized in Table 2. The volumetric productivity of *P. purpureum* (8.1 ± 1.1 mg L^{-1} day^{-1}) seems to be higher compared to *P. sordidum* (5.4 ± 0.3 mg L^{-1} day^{-1}),

Low N/P ratios (below 4.9) seem to induce EPS production, while high N/P ratios (35–50) are described to induce cell growth, which, depending on the strain, can also be associated with high EPS production. The N/P ratio of around 16 is considered an equilibrated medium, where N and P will be depleted simultaneously until ideally both are limiting at the same time, leading to high cell growth as well as high EPS production [50,51]. During the cultivation process due to the lower nitrate consumption compared the phosphate uptake, the N/P ratio increased in both strains, being more drastic in *P. sordidum* than in *P. purpureum.* This behavior opens the possibility to modify the culture medium to enhance the growth and EPS production in future investigations, as it was shown for other *Porphyridium* strains [50,51]. Nevertheless, individual evaluation of nutrients consumption showed, that the phosphate consumption was higher compared to nitrate, and this might have a higher impact on the EPS production for both

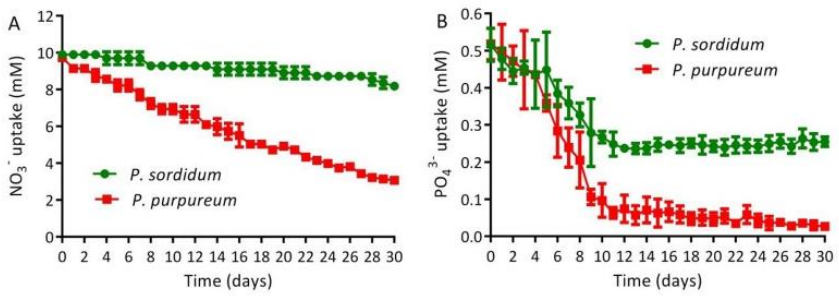

Fig. 3. Comparison of nutrients uptake ($n = 3$), nitrate (A) and phosphate (B) for both Rhodophyta strains; *P. purpureum* (red) and *P. sordidum* (green) over a 30 days cultivation process. *P. purpureum* consumed more nitrate and phosphate than *P. sordidum*, during the cultivation process. The graph is based on the average including standard deviation as variability ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Table 2
EPS titers obtained after the 30 days culture of both strains based on the optimized purification protocol. The volumetric productivity of EPS is given based on the calculations. Values are based on the average with the standard deviation as variability ($n = 3$).

Strain	EPS[a] [g L^{-1}]	Volumetric productivity[b] [mg L^{-1} day^{-1}]
P. sordidum	0.16 ± 0.01	5.4 ± 0.3
P. purpureum	0.24 ± 0.03	8.1 ± 1.1

[a] Concentration: EPS weight by volume.
[b] Volumetric productivity: EPS weight by culture time and volume.

Porphyridium strains. This behavior has also been observed in some cyanobacteria and diatoms [52–54].

3.5. Evaluation of the EPS chemical composition

The EPSs produced by *P. sordidum* were analyzed to consist of the following carbohydrate monomers expressed in mass percentage: 44 ± 2.2% (196 ± 11 mg L^{-1}) xylose, 31 ± 2.2% (136 ± 5.5 mg L^{-1}) galactose, 21 ± 0.83% (93 ± 3.4 mg L^{-1}) glucose, and 4.1 ± 0.03% (18 ± 0.26 mg L^{-1}) glucuronic acid. Moreover, the presence of uronic acids was corroborated by identifying an uronic acid hexose dimer peak ($m/z = 687$). The uronic acid hexose dimer was detected at 4.1 min; the fragmentation pattern is presented in the supplementary data, Annex 6.1. Also, another uronic acid hexose dimer was detected at 3.8 min, but the concentration was too low to obtain a fragmentation peak. These uronic acid hexose dimers can be explained in two ways. Since two hexoses were detected in the *P. sordidum* EPS, each one can generate a dimer with the glucuronic acid, thus two different dimers were detected. The monomer composition of the *P. sordidum* EPS is shown in Fig. 4A. Recently, *Nikolova* et al., *2019* provided a brief description of *P. sordidum* EPS, which was composed of xylose, galactose, glucose, glucuronic acid, mannose and rhamnose [55]. The more noticeable difference between the compositions of both EPSs is the presence of mannose in the composition of the EPS recently reported in contrast with the *P. sordidum* EPS in this study. On the other hand, the monomer composition of the EPS from *P. purpureum* was previously described by *Roussel* et al., *2015,* wherein the molar ratios calculated were: xylose 26.3%; galactose 44.2%; glucose 26.3%; glucuronic acid 2.3% and fucose 1.0% [25].

In contrast, a different composition was identified for the *P. purpureum* EPS in this study which is the following: 46 ± 1.4% (207 ± 9.1 mg L^{-1}) xylose, 30% ± 0.5 (132 ± 3.3 mg L^{-1}) galactose, 20 ± 0.7% (92 ± 2.2 mg L^{-1}) of glucose and 3.8 ± 0.22% (17 ± 0.86 mg L^{-1}) glucuronic acid. Fucose was not detected in the polymer. In addition, two uronic acid hexose dimers (at 3.8 and 4.1 min) were identified, which are also present in the polysaccharide produced by *P. sordidum*. The monomers detected in the hydrolysates of the *P. purpureum* EPS are shown in the following chromatogram (Fig. 4B). The polymeric nature of microalgae EPSs were underpinned by the glucose assay, because monomeric glucose was only detected in the hydrolyzed samples, indicating that no monomeric glucose originates from the culture medium. The polymeric structure of the EPSs in both strains was corroborated by the glucose assay (supplementary data, Annex 7).

Apart from the carbohydrate structure, the microalgae EPSs contained other molecules as decoration [13]. In this sense, various methodologies were used to detect those substituents. Pyruvate was the first substituent to be analyzed in the microalgae EPS, because several microbial and some microalgae EPS are to have it in their structure [56,57]. A very sensitive enzymatic assay that uses pyruvate oxidase (POX) was applied [30]. It revealed that the EPSs from both *Porphyridium* strains did not contain any pyruvate. The protein content of the EPS samples, as quantified with the Bradford assay, showed a protein content of 8.3 ± 0.6 mg L^{-1} for the *P. sordidum* EPS, which was 0.84 ± 0.01% of the total EPS weight and 7.5 ± 1.5 mg L^{-1} for the *P. purpureum* EPS which was 0.77 ± 0.28% of the total EPS sample. Thus, both EPSs contain a small amount of protein, which could be considered contamination.

The main difference in substituents between the EPSs was the sulfate content, which is described to be a prominent substituent in microalgae EPS [13,58]. For the EPS of *P. sordidum,* a content of 179 ± 38 mg L^{-1} was detected, which correlates to 18 ± 4.2% of the total EPS content and for the EPS of *P. purpureum,* 122 ± 29 mg L^{-1} were detected, which represent 13 ± 3.2% of the total weight. The sulfate content was validated by obtaining similar results by the two methods used in this study (supplementary data, Annex 7). The measured 18% of sulfate for the *P. sordidum* EPS, is higher than the sulfate content reported for other microalgae EPSs (6% to 10%) [58]. Furthermore, a higher concentration of sulfate (around 13%) was detected in this study compared to the 9.1% reported for *P. purpureum* EPS by *Roussel* et al., *2015* [25]. The complete chemical compositions of the EPSs are summarized in Table 3.

However, the most prominent difference between the *P. sordidum* and *P. purpureum* EPS, evidenced in the mass spectra chromatogram (Fig. 4) and in the mass fragmentation pattern (supplementary data, Annex 6), were the methylation patterns for the *P. sordidum* EPS, the presence of a methyl-pentose and a methyl-hexose was detected at 8.8 min of retention time in the chromatogram of the *P. sordidum* EPS (Fig. 4A). The methylated carbohydrates were identified by the particular fragmentation pattern of m/z 525 (hexose m/z 511 + methyl m/z 14) and m/z 495 (pentose m/z 495 + methyl m/z 14) (supplementary data, Annex 6A). Quantification of these methylated carbohydrates was not possible due to the lack of specific standards. Methylated carbohydrates are not very common in microbial polysaccharides, but they are described to be present in some polysaccharides of microalgae and specifically in the polysaccharides of the *Porphyridium sp.* [59,60]. These methylated carbohydrates are of high interest, because they can confer novel properties to the polysaccharide. Methyl carbohydrates have never been described for *P. sordidum* EPS [55].For *P. purpureum* no methyl pentose or methyl hexose was detected. However, an uronic

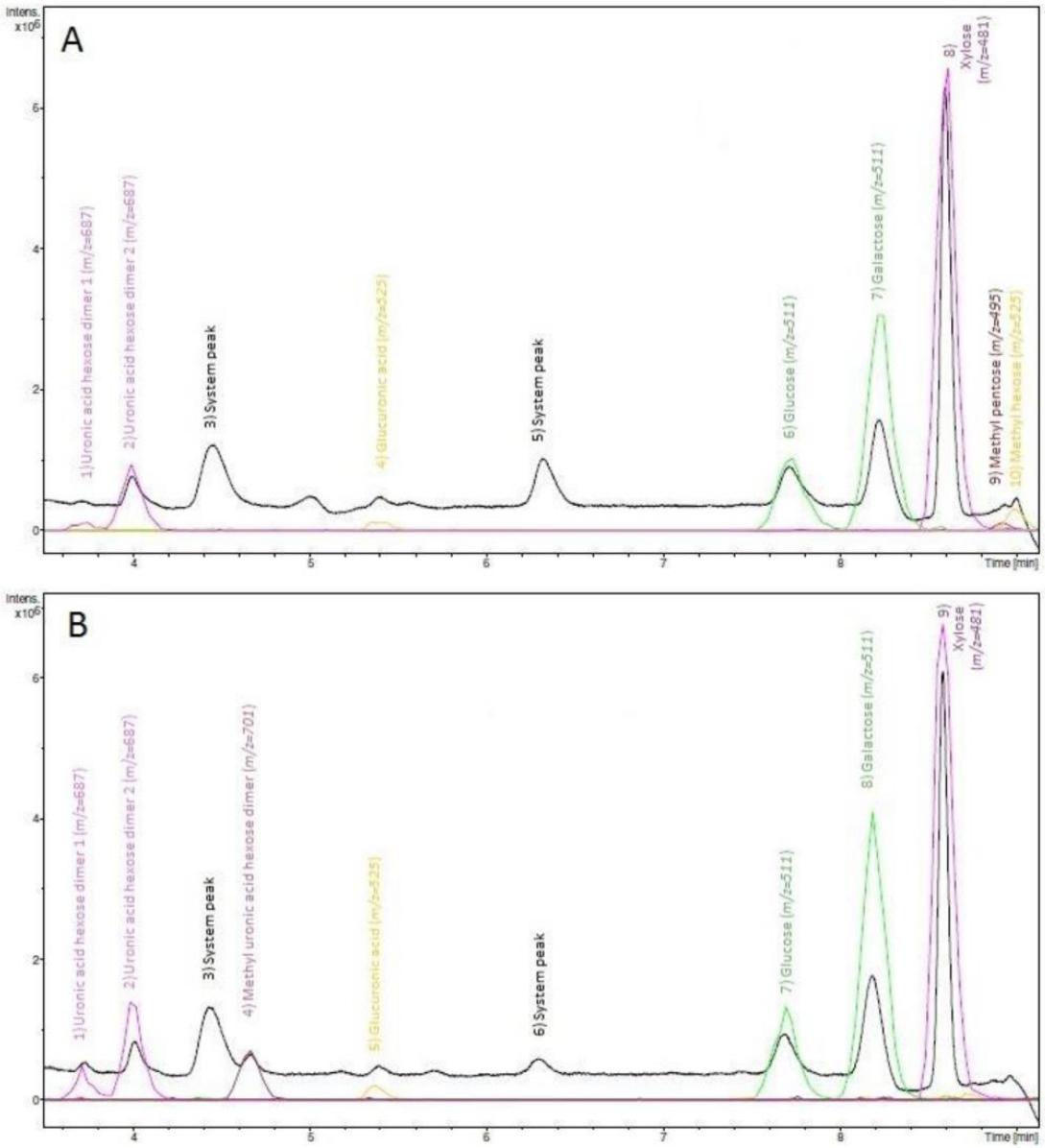

Fig. 4. Overlay of UV 245 nm (black) and MS extracted ion (color) chromatograms (3–9 min), for *P. sordidum* (A) and *P. purpureum* (B); the EIC colors are the following: pink *m/z* 687, purple *m/z* 701, yellow *m/z* 525, green *m/z* 511, magenta *m/z* 481, red *m/z* 525 and yellow *m/z* 495. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

acid hexose dimer was eluted at 4.8 min with an $m/z = 701$ (uronic acid hexose dimer $m/z = 687$ + methyl $m/z = 14$), which an offset to the other previously mentioned dimers due to a hydrophobic group, such as methyl group is part of this dimer. However, the position of the methyl group and the dimer could not be identified due to the lack of an appropriate standard. The methyl group could be linked to the hexose as it was previously reported in other microalgae EPS or the methyl group could be linked to the uronic acid [13,59,61]. Remarking that this is the first time that a methyl pattern was detected in the *P. purpureum EPS,* whose monomer composition was described before [13,25].

The chemical compositions determined for the *P. purpureum* and *P. sordidum* EPSs in this study were different from the ones previously reported [25,55]. This could be explained by differences of the cultivation media, cultivation process and analytics used to unravel the chemical composition of the EPS. This phenomenon has been reported for various EPS producing microalgae and cyanobacteria [13,59,61–69]. The amount of harvested EPS for both Rhodophytas decreased dramatically due to the applied intensified down-stream processing (2 x precipitations with 2-propanol, 2 x centrifugation and sieving steps). This decreased the overall EPS yields, but at the same time increased the content of the carbohydrate fraction, due to the

Table 3
Summary of the monomer composition and decorations as analyzed for both Rhodophyta EPSs (mg L^{-1}). The table denotes the average and standard deviation as variability ($n = 3$).

	Components	*P. sordidum*	*P. purpureum*
Quantifiable	Glucose	93 ± 3.4	92 ± 2.2
	Galactose	135 ± 5.5	132 ± 3.3
	Xylose	196 ± 11	207 ± 9.1
	Glucuronic acid	18 ± 0.26	17 ± 0.86
	Protein	8.3 ± 0.6	7.5 ± 2.8
	Sulfate	179 ± 38	122 ± 0.29
	Sample concentration	972 ± 15	979 ± 17
	% Recovery	65 ± 4.9%	59 ± 4.4%
Unquantifiable	Pyruvate	–	–
	Methyl pentose	+	–
	Methyl hexose	+	–
	Methyl uronic acid hexose dimer	–	+

elimination of salts and other co-precipitates.

3.6. Rheological characterization of the microalgae EPS

To analyze the physico-chemical properties of both EPSs and the putative influence of the different decorations with substituents, basic rheological measurements were performed. The first evaluation was based on the determination of viscosity curves by increasing the shear rates to detect the change in viscosity. Other EPSs such as xanthan, *Porphyridium* sp. EPS and *Porphyridium cruentum* EPS showed a shear thinning behavior as indicated by lowered viscosity at increased shear rates [17,49,70]. This behavior is explained by a decrease in the interactions between the single EPS fibers due to increased shear rates, thus decreasing the viscosity. The viscosity curves for both EPSs were very similar, which might be due to the similar basic chemical compositions (Fig. 5A). The flow curve data fit the power-law ($\eta = K\dot{\gamma}^{n-1}$), obtaining the consistency (n) and flow index (K), as presented in Table 4.

The second measurement was based on amplitude sweeps to evaluate the viscoelastic behavior by determination of the storage (G') and the loss modulus (G''). As the shear stress increased, both moduli maintained until they reached the yield stress where the values of the moduli dramatically changed. In this evaluation a difference between the *P. sordidum* EPS and *P. purpureum* EPS was observed. The yield stress of the *P. sordidum* EPS was reached at a lower shear rate compared to the *P. purpureum* EPS. Both amplitude sweeps are presented in Fig. 5B. The rheological evaluation of *Porphyridium* sp. and *P. cruentum* EPSs was reported in previous investigations. Although, each study had a different focus; the flow curve was determined, showing a similar shear thinning behavior to the EPS produced by *P. sordidum* and *P. purpureum* [17,70,71].

Table 4
Consistency and flow index were obtained by fitting the viscosity curve data of both EPSs into the power-law. The R^2 was calculated to corroborate the corrected fitting of the three biological replicate used in this evaluation. The table denotes the average and standard deviation as variability ($n = 3$).

Strain	Consistency Index (K)	Flow index (n)	R^2
P. sordidum EPS	8.45 ± 0.31	0.08 ± 0.01	0.9998
P. purpureum EPS	8.62 ± 1.03	0.08 ± 0.00	0.9998

4. Conclusions

P. sordidum, as relative new EPS producer, was compared to the already described EPS producer *P. purpureum* in detail. The phenotypical differences were mainly the bigger sized cells and the olive-green color of *P. sordidum*, compared to the red colored and smaller sized cells of *P. purpureum*. Uptake of nitrate and phosphate was higher for *P. purpureum*, which led to better growth, corroborating that an enriched medium (modified ASW) enhanced the growth of *P. purpureum* in contrast to *P. sordidum*. The better uptake of nutrients by *P. purpureum* lead to higher EPS titers compared to *P. sordidum*. The carbohydrate fraction was similar in both EPS, being xylose the most abundant carbohydrate, follow by the galactose, glucose and glucuronic acid in that order. The most noticeable differences in the chemical structures were observed in the EPS decorations such as the sulfate content, which was higher in the *P. sordidum* EPS compared to *P. purpureum* EPS. Furthermore, the main difference was the methylation pattern on the EPSs. In the *P. purpureum* EPS, a methylation was identified in a hexose glucuronic acid dimer for the first time, in contrast the *P. sordidum* EPS, which had a methyl pentose and methyl hexose in its structure. Due to the similarities in the chemical compositions, the rheological properties were quite similar, only the amplitude sweeps showed noticeable differences, possibly because of the distinct sulfate content and methyl pattern of the carbohydrate polymers. A deeper physico-chemical characterization can be achieved by applying further rheological evaluations. Productivity might be enhanced via optimized cultivation conditions. For this, intensified optimization of the media composition and a scale up of the cultivation system can be investigated; next steps are to enhance the EPS production and in that way continue in depth investigation of both EPSs.

Author contributions

EVMC performed the experiments. JS VS and EVMC designed the study. EVMC, JS and BR analyzed and interpreted the data. EVMC JS and BR wrote the manuscript. All authors revised it critically for scientifically as well as technically soundness. All authors approve of the final version to be submitted.

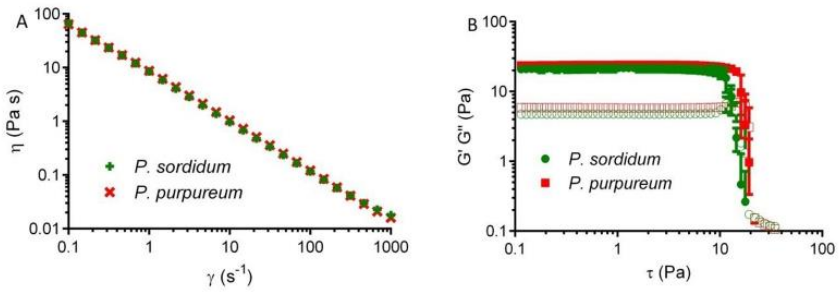

Fig. 5. Both rheological evaluations ($n = 3$) were performed at 20 °C with a 1% solution of each of the EPSs. (A) Viscosity curves, for the EPS of *P. sordidum* + and *P. purpureum* x. (B) Amplitude sweeps, with storage modulus G' (● *P. sordidum* and ■ *P. purpureum*) and loss modulus G'' (○ *P. sordidum* and □ *P. purpureum*). The graph is based on the average including the standard deviation as variability ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

Informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2020.101931.

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Algal Research

Characterization and comparison of *Porphyridium sordidum* and *Porphyridium purpureum* concerning growth characteristics and polysaccharide production

Appendix A:

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Annex 1. Composition of the different media used in the investigation.

Annex 1.1.

Modified artificial seawater (ASW)

Macroelements			Microelements		
Components	Concentration		Components	Concentration	
	g L^{-1}	mM		g L^{-1}	mM
NaCl	27	462.01	$MnCl_2$ x $4H_2O$	1.81	6.29
$MgSO_4$ x $7H_2O$	6.3	25.56	Na_2MoO_4 x $2H_2O$	0.39	1.36
$MgCl_2$ x 6H2O	5.6	277	$ZnSO_4$ x $7H_2O$	0.22	0.77
$CaCl_2$ x $2H_2O$	1.13	7.69	$CuSO_4$ x $5H_2O$	0.08	0.27
$NaNO_3$	0.84	9.88	H_3BO_3	2.86	9.95
KH_2PO_4	0.07	0.51	$CoCl_2$	0.05	0.17
$NaHCO_3$	0.04	0.48			
$EDTANa_2$	0.08	0.24			
$FeSO_4$ x $7H_2O$	0.01	0.03			
Microelements	1 mL				

Original ASW composition in *You et al, 2004* [1].

Annex 1.2.

Modified Pm

Macroelements		
Components	Concentration	
	g L^{-1}	mM
K_2HPO_4	0.90	5.17
$NaNO_3$	1.70	20.00
NaCl	15	256.67
$CaCl_2 \times 2H_2O$	16	10.54
$MgSO_4 \times 7H_2O$	7.20	59.82
$EDTANa_2$	0.080	214.92
$FeSO_4 \times 7H_2O$	0.010	11.2
Microelements	1 mL	

Original Pm composition in Soanen *et al, 2016* [2].
*Microelements used from the ASW.

Annex 1.3.

Bold's basal medium (BBM)

Macroelements			Microelements			Solution 1		
Components	Concentration		Components	Concentration		Components	Concentration	
	g L^{-1}	mM		g L^{-1}	M		g L^{-1}	M
KH_2PO_4	0.18	1.29	$ZnSO_4 \times 7H_2O$	8.82	30.67	$EDTANa_2$	50	134.32
$CaCl_2 \times 2H_2O$	0.025	0.17	$MnCl_2 \times 4H_2O$	1.44	7.28	KOH	3.1	55.25
$MgSO_4 \times 7H_2O$	0.075	0.62	$CuSO_4 \times 5H_2O$	1.57	9.84	Solution 2		
$NaNO_3$	0.25	2.94	$Co(NO_3)_2 \times 6H_2O$	0.49	2.68	Components	Concentration	
K_2HPO_4	0.075	0.43	$Na_2MoO_4 \times 2H_2O$	0.39	1.89		g L^{-1}	M
NaCl	0.025	0.43				$FeSO_4 \times 7H_2O$	4.98	329.15
H_3BO_3	0.011	0.18				H_2SO_4 (95-98%)	1 mL	
Microelements	1 mL							
Solution 1	1 mL							
Solution 2	1 mL							

Original BBM composition in *Barsanti et al, 2011* [3].

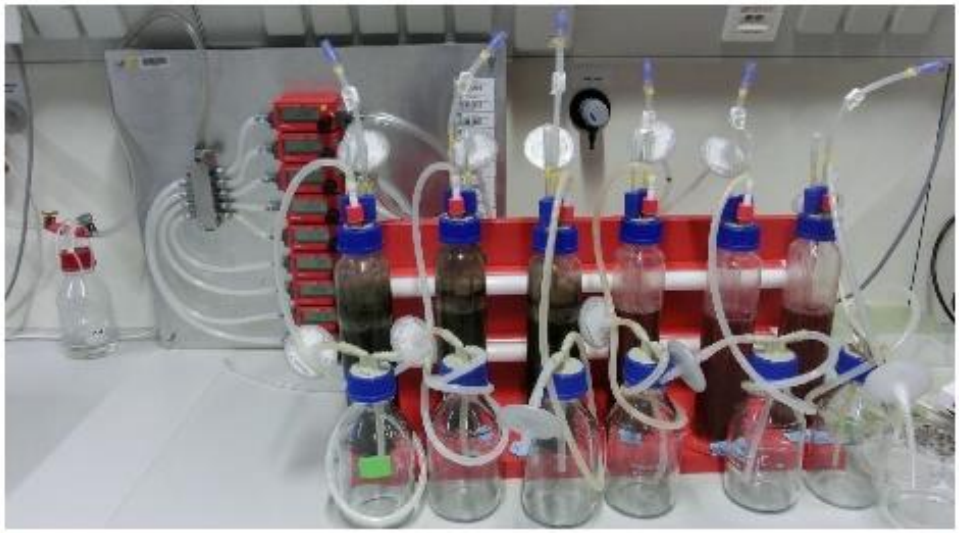
Annex 1.4.

BG-11 medium

Macroelements		
Components	Concentration	
	$g\ L^{-1}$	mM
K_2HPO_4	0.040	0.23
$MgSO_4 \times 7H_2O$	0.075	0.62
$CaCl_2 \times 2H_2O$	0.036	0.24
$EDTANa_2$	0.001	2.69
$NaNO_3$	1.50	17.65
Citric Acid	0.006	0.03
Ferric Ammonium Citrate	0.006	0.02
Na_2CO_3	0.020	0.19
Microelements	1 mL	

Original BG-11 composition in *Barsanti et al, 2011* [3].
*Microelements used from the ASW.

Annex 2. Cylinder system used for the investigations



Settings: Incubation at room temperature (RT), white fluorescence lamp (LUXLINE PLUS, F18W, Germany) and blue fluorescence lamp (AQUA-GLO, 20W T8, Japan), air flowmeter 100 mL min^{-1} (Read-y for gas flow, Switzerland), volume 500 mL.

Annex 3. Placket-Burman design formulas used evaluations obtained from *Lim et al, 2012* [4].

Equation A.1. Specific growth rate

$$\mu_{max} = \frac{\ln \frac{x_2}{x_1}}{t_2 - t_1}$$

Equation A.2. Doubling time

$$t_d = \frac{\ln 2}{\mu_{max}}$$

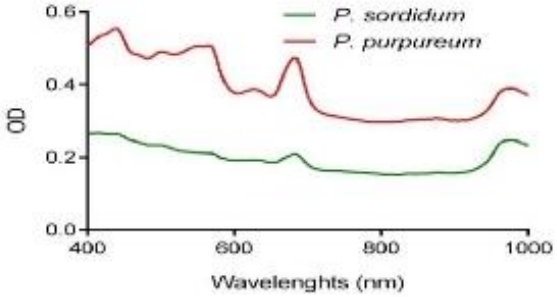
Annex 4. Calculation of surface area and volume for the *P. sordidum* and *P. purpureum* cells

Repetition	Diameter [µm]		Surface [µm^2]		Volume [µm^3]	
	P. sordidum	*P. purpureum*	*P. sordidum*	*P. purpureum*	*P. sordidum*	*P. purpureum*
1	9.6	8.7	287	239	458	348
2	9.8	7.4	304	174	500	216
3	9.7	8.6	295	230	477	329
4	9.3	8.0	274	200	427	266
5	9.9	7.5	306	179	504	225
6	9.6	8.1	287	206	457	278
7	9.0	8.4	255	219	382	306
8	9.8	8.0	303	203	496	273
9	9.2	8.6	265	235	405	339
10	11.5	6.6	419	135	806	148
11	11.5	8.0	416	200	798	266
12	9.2	6.9	265	150	405	173
Average	9.8	7.9	306.4	197.6	509.6	263.8
SD	0.8	0.7	54.5	32.8	142.3	63.6

The diameters of 12 random microalgae of each strain were measured in order to determine the average diameter. The surface of both strains were calculated with the following formula ($S = 4\pi r^2$). The volume was calculated with the sphere volume formula ($V = 4/3\ \pi r^3$). * ($r = D/2$). Finally, a t-student was performed to the diameter of both *Porphyridium* strains, corroborating that there were significant differences between the strains' diameters.

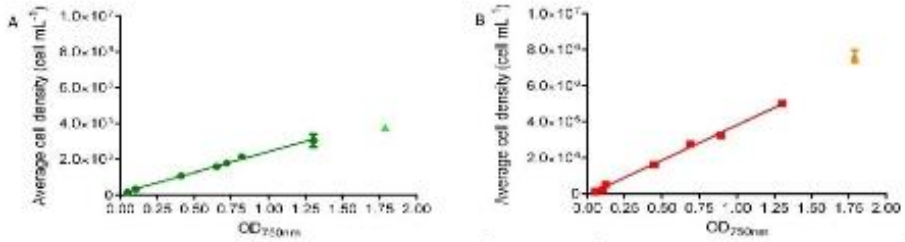
Annex 5. Evaluation of the *P. sordidum* and *P. purpureum* OD response

Annex 5.1. Spectral analysis for the *P. sordidum* and *P. purpureum*



In order to identify the appropriate wavelength, a spectral analysis was performed on the high dense cultures of both *Porphyridium* strains from 400 to 1,000 nm. To observe the differences between the spectra, the *P. purpureum* samples were diluted in half, since the *P. sordidum* (olive-green color) gave a lower response compared to *P. purpureum* (red color). The evaluation was done in quadruplicate in a 96 well plate.

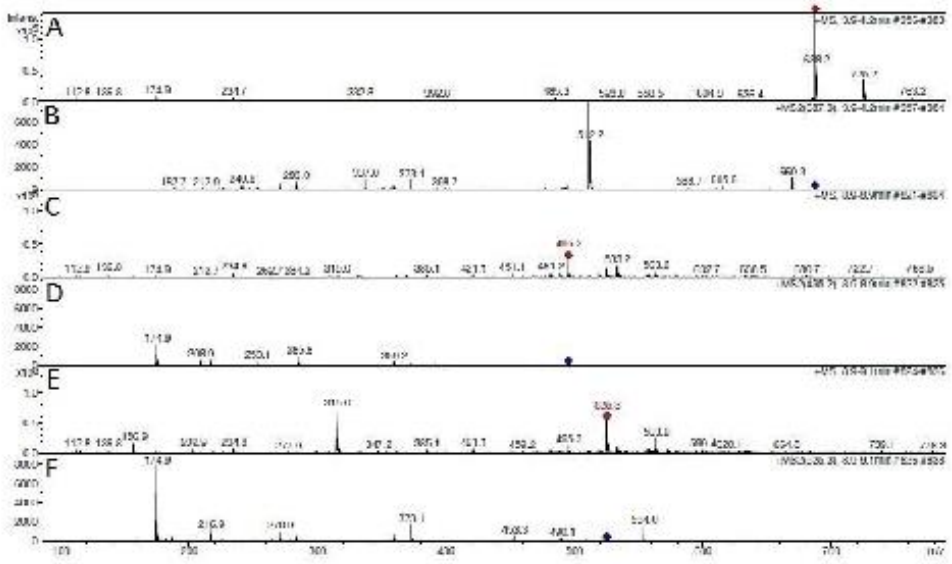
Annex 5.2. Comparison of the OD_{750nm} against the average cell density obtained from the Thomas chamber for both microalgae strains *P. sordidum* and *P. purpureum*.



The response generated for A. *P. sordidum* ($y = 2*10^6 x + 0.9*10^5$) and B. *P. purpureum* ($y = 4*10^6 x - 0.7*10^5$) both with a $R^2 = 0.996$. The OD_{750nm} values above 1.3 were not considered for the linear regression. All the measurements for the growth curves were performed below values of 0.6 for *P. sordidum* and 1.3 for *P. purpureum* at OD_{750nm}. Although both strains had a linear behavior their response was different due to the differently size and shape of the microalgae cells. The graphs are based on the average including standard deviation as variability (*n=3*).

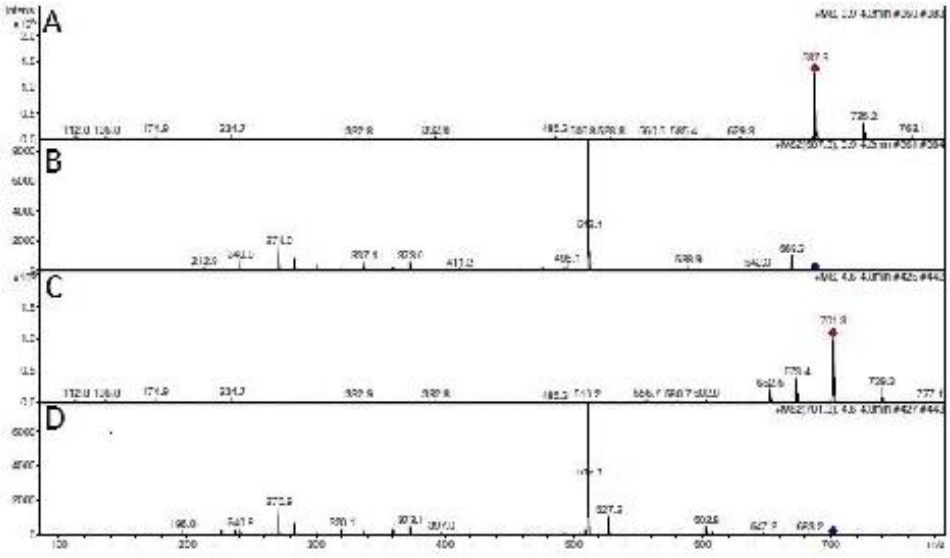
Annex 6. Fragmentation pattern of the methyl carbohydrates detected in *P. sordidum* and *P. purpureum* EPS.

Annex 6.1. Fragmentation pattern of unknown peaks of *P. sordidum* EPS.



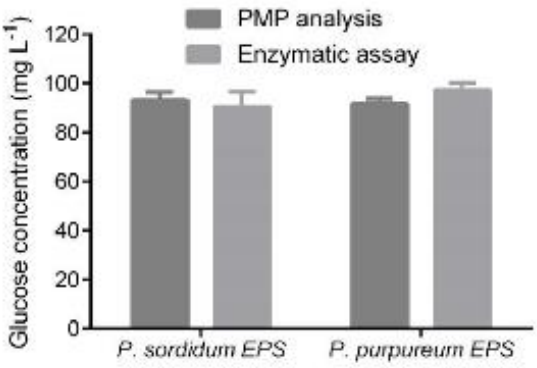
Mass spectra, MS, (*m/z 100-800*) from the *P. sordidum* EPS is presented. (**A**) Uronic acid hexose dimer. (**B**) Fragmentation of Uronic acid hexose dimer. (**C**) methyl pentose with a *m/z* 495 (pentose *m/z* 481 + methyl group *m/z* 14). (**D**) Fragmentation of methyl pentose. (**E**) methyl hexose with a *m/z* 525 (hexose *m/z* 511 + methyl group *m/z* 14). (**F**). Fragmentation of methyl hexose. The characteristic fragmentation of Δ *m/z* 14 was the detection of the methyl group. Although, the methyl carbohydrates are not common in bacterial EPS, it has been reported that microalgae contain methyl carbohydrates [5-7].

Annex 6.2. Fragmentation pattern of unknown peaks of *P. purpureum* EPS.



MS compound spectra (*m/z 100-800*) of different unknown peaks from *P. purpureum* EPS. (**A**) Uronic acid hexose dimer. (**B**) Fragmentation of uronic acid hexose dimer. (**C**) Methyl uronic acid hexose dimer. (**D**) Fragmentation of methyl uronic acid hexose dimer. Since both peaks are dimers, they are very similar (B and D). However, 701 (uronic acid hexose dimer *m/z* 687 + methyl group *m/z* 14) was detected, which indicates methylation of the dimer.

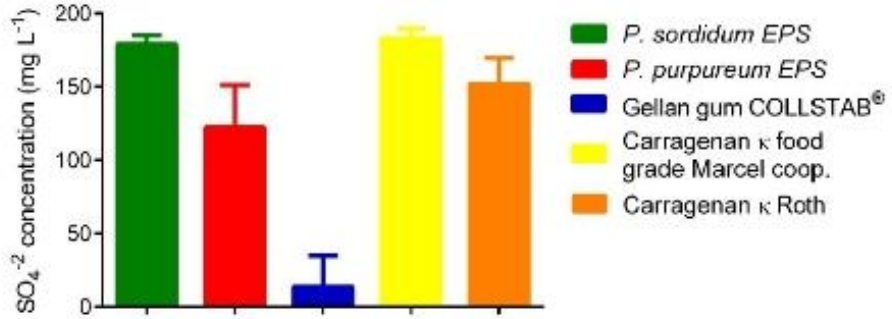
Annex 7. Glucose comparison enzymatic assay and PMP analysis



Comparison of the glucose concentration detected by the PMP analysis and glucose oxidase assay for both the *P. sordidum* and the *P. purpureum* EPSs. The results are similar for both strains using the two methods. The graph is based on the average including standard deviation as variability (*n=3*).

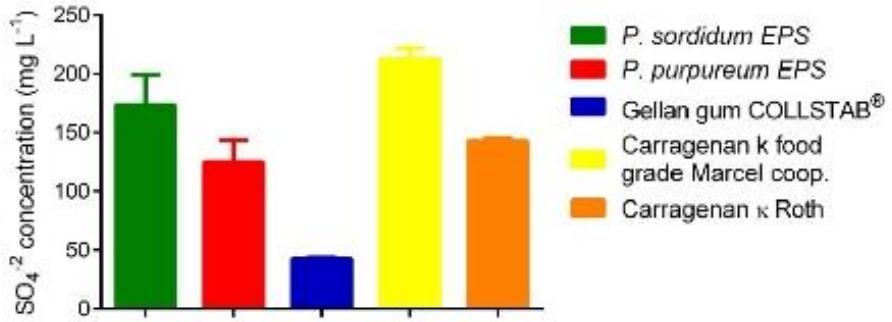
Annex 8. EPS Sulfate validation and determination

Annex 8.1. Sulfate detection with enzymatic assay



Sulfate was reported to be linked to microalgae EPS; EPS samples from both strains were therefore tested [2, 5]. The enzymatic method used by this test indirectly detected the sulfate content by measuring the pyruvate concentration [8]. In order to evaluate the sulfate, two carrageenan κ were used as positive control and for the negative control, gellan was used as negative control [9-11]. It is important to highlight that EPSs containing pyruvate like xanthan are not suitable for this assay. The graph is based on the average including standard deviation as variability (*n=3*).

Annex 8.2. Determination of the sulfate content in EPS samples with the turbidimetric assay base on the use of $BaCl_2$.



The turbidimetric assay allowed the sulfate concentration in samples. The same EPS samples and positive and negative controls were used in the enzymatic assay. The negative control gave a small turbidimetric response since polymers gave signals that could be detected at 600 nm [12]. The graph is based on the average including standard deviation as variability (*n=3*).

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4.2.2. Determination of the methyl position in *P. sordidum* EPS.

Although, *P. sordidum* EPS contained a methyl pentose and methyl hexose in their structure; the position in which methyl groups are attached to the carbohydrates could not be determined because of two reasons. A faint mass-spectra fragmentation pattern was observed due to the small quantities of the methylated carbohydrates detected in the test. And, there were no methyl carbohydrates standards available to compare their fragmentation pattern to the ones generated by the microalgae EPS methyl carbohydrates.

Fortunately, in subsequence runs, stronger fragmentation patterns of the methyl carbohydrates from *P. sordidum* EPS were obtained. Furthermore, four methyl glucose standards were commercially available in positions two, three, four and six, to compare their fragmentation patterns to the ones generated by the methyl carbohydrate patterns from *P. sordidum* EPS.

Table 4.1.
Peaks masses (*m/z*) observed in methyl glucose standards and *P. sordidum* EPS. The mass showed in each glucose standard are presented in order to identify the position in which the methyl group is located in *P. sordidum* EPS samples. The masses are reported by *Sinzinger et al., 2021* [390].

Observed mass (*m/z*)	Methylated carbohydrates					
	2-Methyl glucose	3-Methyl glucose	4-Methyl glucose	6-Methyl glucose	Methyl hexose[1]	Methyl pentose[1]
217	–	+	+	+	+	+
231	+	–	–	–	–	–
241	+	+	–	+	–	+
253	+	+	+	+	+	+
265	–	–	–	+	–	–
271	+	–	+	–	+	–
283	–	–	+	+	+	–
285	–	+	–	–	–	+
351	+	+	–	–	–	n.p.

– absent mass | + present mass | [1] Peak observed in *P. sordidum* EPS | n.p. not possible mass

The fragmentation pattern of the *P. sordidum* methyl hexose (Figure 4.1) were compared to the ones obtained from the four methyl glucose standards (Table 4.1). The combination of the *m/z* (217, 253, 271, and 283) showed a strong resembles to the fragmentation masses obtain from the 4-methyl glucose standard. Additionally, the absence of particular masses displayed by other methyl glucose standards like *m/z* 231 of 2-methyl glucose, *m/z* 285 of 3-methyl glucose and *m/z* 241 of 2-methyl glucose, 3-methyl glucose and 6-methyl glucose standards corroborated that *P. sordidum* hexose is methylated at position 4. The *P. sordidum* EPS methyl hexose retention time was compared to the ones obtained from the methyl glucoses standards to determine which hexose (glucose or galactose) contain the methyl residue. No

retention time matches the four methyl glucose standards, which came earlier than *P. sordidum* EPS methyl hexose. Contrary, galactose which is a C4 glucose epimer, would have a longer retention time if they contain a methyl group; similar to the one shown by the 4-methyl hexose from *P. sordidum* EPS. Gathering the before mentioned results, it was determined that a 4-methyl galactose is part of the *P. sordidum* EPS.

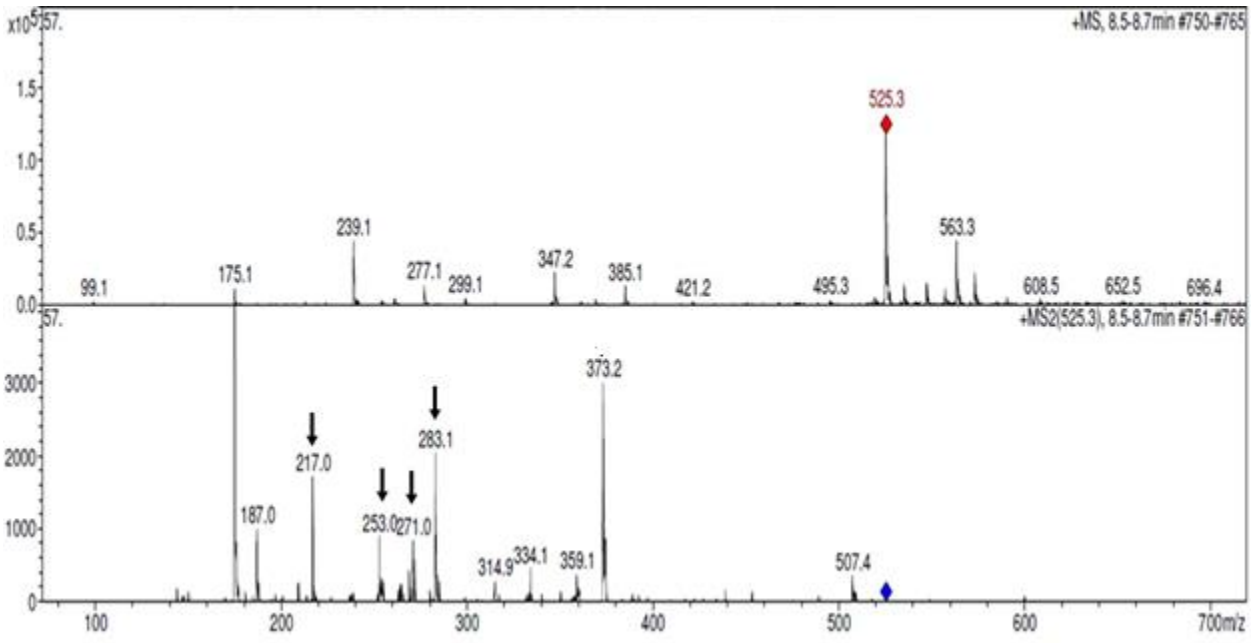

Figure 4.1. *P. sordidum* EPS methyl hexose mass spectra, (m/z 100-700). Common masses to methyl glucose standards (↓) are marked in order to identify the methyl of the methyl hexose.

Methyl position in *P. sordidum* pentose was determined in a similar way. Xylose is the only pentose in *P. sordidum* EPS, so it should be a methyl xylose. A mass delta of *m/z* 14 (267–253) was detected in *P. sordidum* EPS fragmentation pattern, which corresponds to a methyl group. Methyl position was determined to be in position 3, due to several common masses (Figure 4.2) between the methyl pentose and 3-methyl glucose standard: m/z 217, 253, and 285. Also, *m/z* 241 was identified in the methyl pentose as well in the 2-methyl glucose, 3-methyl glucose or 6-methyl glucose standards. In addition, other masses differentiate them from other glucose standard: Like *m/z* 231 which is displayed by 2-methyl glucose standard but not in the *P. sordidum* methyl pentose. And the presence of the m/z 285 which is exclusive of 3-methyl glucose standard. The before presented information show that the *P. sordidum* EPS contain a 3-methyl xylose in its structure.

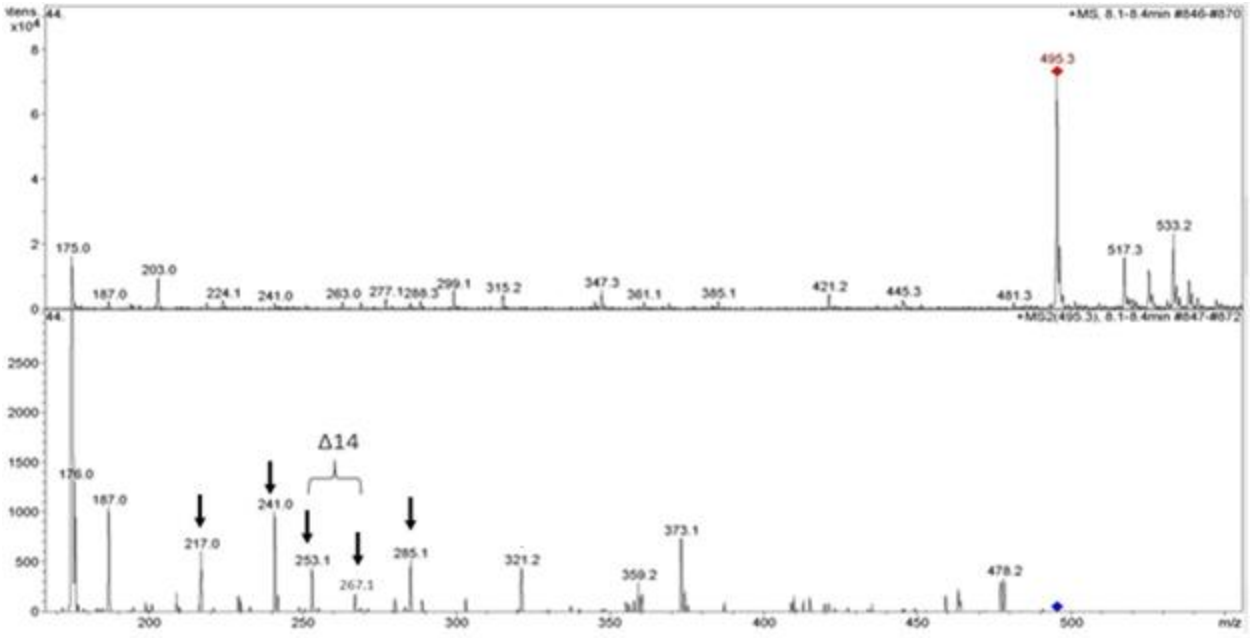

Figure 4.2. *P. sordidum* EPS methyl pentose mass spectra, (*m/z 100-600*). Common masses to methyl glucose standards (↓) are marked.

Unfortunately, because of the lack of methyl hexose uronic acid dimer standards, the methyl position in the *P. purpureum* EPS could not be identified in this study.

4.3. Optimization of growth and EPS production from two *Porphyridium* strains

It was imperative to increase the low EPS yields generated by microalgae to perform further investigation. To achieve this goal, different methods were applied to enhance growth and EPS production on *P. sordidum* and *P. purpureum*. Different wavelengths were tested to identify which one enhance growth and EPS production using the DASGIP® Bioblock system. It was determined that wavelength combination (white light emulation) was the most favorable for growth and EPS production for both *Porphyridium* strains.

The cultivation medium provides the majority of the nutrients required by microalgae development. Hence, its optimization has the potential to enhance growth and EPS yields. This was done by identifying the most important medium components using the Plackett-Burman (PB) design. The selected compound concentrations were optimized using the Taguchi design to develop an improved cultivation media. In this regard, IB medium, which improved growth and EPS production in *P. sordidum* and I3 medium that enhanced EPS production in *P. purpureum* were developed.

Four abiotic factors (pH shift, different NaCl concentrations, temperature variation and the time microalgae were exposed to light) were tested as EPS inducers in both microalgae strains. Abiotic factors were mixed randomly in three levels using the Taguchi design, to identify the most prominent factors on EPS production. In this regard, increasing the time *P. sordidum* was exposed to light and a lower temperature; while pH shift and maintaining light exposure in 12 hours in *P. purpureum* were the most notable factors that increases EPS production.

The optimized parameters (adequate wavelengths, improve cultivation medium and abiotic factors for EPS induction) were combined in a two-stage cultivation system (optimized bioprocess). The newly developed bioprocess was compared to a one-stage cultivation system, which used the initial cultivation conditions. The combination of all optimized parameters increased EPS production 3.4 times for *P. sordidum* and 1.2 times for *P. purpureum*. It was determined that both *Porphyridium* EPSs monomer compositions were not altered by the optimization procedure developed in this investigation. In contrast, *P. purpureum* sulfate fraction doubled in EPS samples obtained from the two-stage cultivation system in comparison to the one generated in the one-stage (initial conditions).

The author designed and performed the experiments, analyzed and interpreted the data. Broder Rühmann analyzed and interpreted the data. Jochen Schmid designed the study, analyzed and interpreted the data. Volker Sieber designed the study. All authors contributed in the manuscript preparation for publication.

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Optimization of growth and EPS production in two *Porphyridium* strains

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ABSTRACT

Due to the relatively low yield of exopolysaccharides from microalgae compared to other microorganisms, their production is more challenging and needs to be optimized. *Porphyridium sordidum* and *Porphyridium purpureum* growth and exopolysaccharide production were optimized by testing influence of light at different wavelengths and varying nutrients as well as abiotic factors. White light was identified as the most suitable for both strains concerning growth and exopolysaccharide production. $CaCl_2$ was identified to have the greatest influence on growth and exopolysaccharide production for *P. sordidum;* NaCl and KH_2PO_4 revealed to be most beneficial for *P. purpureum.* The cultivation medium was optimized to enhance biomass production using the Taguchi design. Additionally, the most prominent abiotic factors for exopolysaccharide induction were determined. Finally, the results were combined in an optimized two-stage cultivation and increased EPS production by 3.4 times for *P. sordidum* and by 1.2 times for *P. purpureum* compared to the initial conditions.

1. Introduction

Microalgae are a source of highly valuable biomolecules such as lipids, proteins, carotenoids and carbohydrate polymers (Gao et al., 2015; Raposo et al., 2013; Slocombe et al., 2013). Carbohydrate polymers represent a unique class of valuable compounds produced by microalgae due to their proposed applications (Raposo et al., 2013). Microbial carbohydrate polymers are classified into intracellular carbohydrates found in the cytoplasm, structural carbohydrates that are attached to cell wall and extracellular polysaccharides (EPS), which are exported to the cell surroundings (Frei and Preston, 1961; Mayers et al., 2018; Paniagua-Michel et al., 2014). EPS are polymers that consist mainly of different carbohydrate monomers linked in different structural arrangements. Also these polymers can contain non-carbohydrate substituents like sulfate or pyruvate groups (Delattre et al., 2016; Raposo et al., 2013).

Currently, bacteria account for the majority of EPS production, of which xanthan is the main representative (Garcıa-Ochoa et al., 2000). Fungi also produce EPS, and are most prominently known for producing scleroglucan and pullulan (Leathers, 2003; Schmid et al., 2011). The applications of microbial EPS are broad, ranging from cosmetics, food, pharmaceuticals and various medical fields (Ates, 2015; Freitas et al., 2011). Therefore, alternative microorganisms such as microalgae are currently of interest for their potential as EPS producers. Until now, very few microalgae EPS are described and studied because of several limitations. Mainly, the very low yields, often in the mg L^{-1} range, are minuscule when compared to scleroglucan (30 g L^{-1}) or xanthan (50 g L^{-1}) (Schmid et al., 2011; Schmid et al., 2015). The microalgae cultivation process is another important limiting factor because it takes significantly longer (15 to 50 days) compared to bacteria or fungi (2 to 5 days) (Farina et al., 1998; Gaignard et al., 2018; Herbst et al., 1992; Lobas et al., 1992; Razaghi et al., 2014; Soanen et al., 2016; Trabelsi et al., 2009a). Nevertheless, interest in the utilization of microalgae EPS is increasing due to the unique and valuable functional groups that they often contain, such as methyl (CH_3) and sulfate (SO_4^{2-}) groups. This increases the potential to use them in other fields such as bio-remediation due to their putative surfactant properties (Paniagua-Michel et al., 2014). In addition, *Porphyridium* EPS as sulfated carbohydrates could be used in a wide range of research fields, however two of the most prominent ones are the pharmaceutical and medical field exploiting their antiviral, anti-inflammatory, antioxidant, anticancer and immunomodulatory properties (Moscovici, 2015; Raposo et al., 2013; Wijesekara et al., 2011). To further evaluate those putative applications larger amounts of EPS is required to be produced by optimizing cell

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growth and EPS yields formation. This could be achieved by a variety of approaches. For example the cultivation parameters (initial pH, light intensity, aeration, temperature and cultivation medium compositions) were tuned using design of experiments (DOE) to enhance *Arthrospira* sp. EPS production (Dejsungkranont et al., 2017). The optimization of the concentration of the two most important compounds of the cultivation medium to increase EPS production for *Chlamydomonas reinhardtii* was achieved using the Placket-Burman design and Response surface methodology (RSM) (Bafana, 2013). Also, the cultivation medium was optimized using a stochiometric approach, in addition the optimal temperature and irradiance were also determined to enhance of *Rhodella violacea* and *Porphyridium marinum* growth and EPS production (Soanen et al., 2016; Villay et al., 2013). Another strategy to optimize/increase microalgae growth rates and EPS yields is the use of different wavelengths of light for their cultivation as reported for Cyanobacteria and Rhodophyta strains (Han et al., 2014; You and Barnett, 2004). Furthermore, an improvement in growth could be achieved by certain wavelengths from white light for instance green light is better absorbed by phycoerythrin, than chlorophyll or other phycobiliproteins (Brody and Brody, 1962). Apparently, different wavelengths can have different (positive or negative) effects on growth and EPS production. Further strategies for increasing EPS production are the utilization of different stress factors: nutrient limitation, high salinity, extreme pH and/or the time cultures are exposed to light (Paliwal et al., 2017). Additionally, two-stage cultivation systems are used for the production of intracellular metabolites such as astaxanthin, lipids and carbohydrates in microalgae, which includes targeting growth in the first-stage and metabolite production in the second-stage. The advantage of this approach is that the growth of microalgae is maximized in the first-stage, so in the next phase a high dense batch is induced (stress or change in cultivation parameters) to produce high concentration of the desired metabolite. Moreover, the two-stage approach was successfully used to increase astaxanthin, carbohydrates and lipids from the biomass, however this technique has never been reported to be used for increasing EPS production in microalgae (Fábregas et al., 2001; Ho et al., 2014; Narala et al., 2016; Xia et al., 2013). Different DOE protocols have been used for EPS optimization in microalgae, such as the Plackett-Burman (PB) method, Surface response methodology (SRM), and Taguchi design (Bafana, 2013; Dejsungkranont et al., 2017). The advantage of DOE resides in the capacity to process multiple different factors in an unbiased fashion with a minimum number of experiments compared to other methods (*AMCTB No* 55, 2013; Rao et al., 2008).

In this study, the optimization procedures used for *Porphyridium sordidum* and *Porphyridium purpureum* are described. Both strains were identified and characterized as EPS producers in a previous report (Medina-Cabrera et al., 2020). Initially, the growth behavior and EPS production was studied under different wavelengths of light to identify the most suitable part of the spectrum for each strain. The identification of the most promising media compounds for growth and EPS production was performed using the PB design. The Taguchi design was used to further optimize the concentration of the selected media compounds and the abiotic factors, which influence EPS production. Finally, all results were combined in a two-stage cultivation process to maximize growth and EPS yields, which were compared to the initial cultivation conditions.

2. Material and methods

2.1. Microalgae strains and cultivation conditions

The *Porphyridium sordidum* (SAG 114.7) and *Porphyridium purpureum* (SAG 1380-1a) inoculums were cultivated in a cylinder air-lift system for 30 days at room temperature (RT) with white (LUXLINE PLUS, F18W, Germany) and blue (AQUA-GLO, 20 W T8, Japan) lights in a 12/12 h' light/dark cycle, and with a 100 mL min^{-1} air flow. The initial cultivation medium used for both strains was the artificial seawater medium (ASW), composed of the following: NaCl (27 g), $MgSO_4{\cdot}7H_2O$ (6.3 g), $MgCl_2{\cdot}6H_2O$ (5.6 g), $CaCl_2{\cdot}2H_2O$ (1.13 g), $NaNO_3$ (0.84 g), KH_2PO_4 (0.07 g), $NaHCO_3$ (0.04 g), $EDTANa_2$ (0.08 g), $FeSO_4{\cdot}7\ H_2O$ (0.01 g) and 1 mL of microelement solution in a final volume of 1 L. The composition of the microelement solution was the following: $MnCl_2{\cdot}4H_2O$ (1.81 g), $Na_2MoO_4{\cdot}2H_2O$ (0.39 g), $ZnSO_4{\cdot}7H_2O$ (0.22 g), $CuSO_4{\cdot}5H_2O$ (0.08 g), $CoCl_2$ (0.05 g) and H_3BO_3 (0.05 g) in 1 L. The cultivation medium was autoclaved at 121 °C for 20 min.

2.2. Evaluation of different wavelengths on cell growth and EPS production

The DASGIP® Bioblock system (Eppendorf, Germany) was equipped with three internal LED-probes per reactor which were used to evaluate the effect of different wavelengths on the growth and EPS production. The reactor LED setup was the following: in reactor 1 blue light (430 nm) was tested; in reactor 2 green/yellow/orange light (572/625/640 nm) was used; in reactor 3 orange/red light (660/780 nm) was applied and in reactor 4 a combination of wavelengths (white light emulation) was tested. The reactors were covered with opaque sheathings to avoid light interference. The irradiance increased following a cosine function, reaching a maximum of 1.87 μE s^{-1} at noon followed by a decrease to zero, emulating 12/12 h' day/night cycle. The volume of the reactor was 1.5 L, the temperature was maintained at 25 °C, and the system was agitated at 150 rpm and aerated at 50 L h^{-1}.

For 17 days, the strains were adapted to each light evaluation. 0.12 L of inoculum from the cylinder system was transferred into 1.2 L working volume of fresh ASW medium. At the end of the adaptation process, the biomass concentration was normalized in all reactors by adding an adequate volume of fresh medium until reaching similar optical density (OD) values.

The growth behavior was evaluated for 7 days by taking daily samples to be measured at OD_{750nm}, which is a reported benchmark to evaluate *P. sordidum* and *P. purpureum* growth (Medina-Cabrera et al., 2020). At the end of the growth phase, the supernatant was separated from the biomass by centrifugation at 4,000 × *g* for 10 min at 20 °C. The EPS was precipitated by mixing the supernatant with two volumes of 2-propanol. The precipitated EPSs were further purified by re-dissolving them in 140 mL of ultrapure water (RT, overnight) followed by centrifugation at 21,000 × *g* for 15 min and 20 °C. A second alcohol precipitation was performed as described above, and then the EPS fibers were collected and dried in a vacuum oven at 45 °C for 48 h. All experiments were performed in biological triplicates.

2.3. Selection of the most important medium components by Placket-Burman (PB) design

The effect and significance of the medium components on the growth and EPS production were determined utilizing the PB design for both strains. The nine components of ASW medium were tested in two levels: presence (+) and absence (−). 1 L homogenous pre-culture from the cylinder system was split into 25 mL aliquots and centrifuged at 4,000 × *g* for 10 min at RT. The cell pellets were re-suspended in a final volume of 75 mL in sterile Erlenmeyer flasks. Experiments were done in technical triplicates. Cultivation was performed for 7 days at RT and 120 rpm with a 12/12-h light/dark cycle under a white warm LED (MÜLLER licht, 15 W 1000 lm, Germany). The biomasses from each evaluation were harvested and dried at 105 °C for 3 days for gravimetrical analysis, while the EPS were harvested as described above. Confirmation runs were performed in biological duplicates, using the cultivation parameters previously described.

2.4. Media optimization by Taguchi design

The cultivation medium for each strain was optimized using the Taguchi design. The concentrations of the six most important medium compounds selected by the PB design for each strain were varied

simultaneously. The selected compounds for *P. sordidum* were NaCl, $MgSO_4$, $CaCl_2$, $NaNO_3$, microelements and KH_2PO_4, while $EDTANa_2$, $FeSO_4$ and $NaHCO_3$ concentrations were maintained at the concentrations present in the original ASW medium. The compounds selected for *P. purpureum* were: NaCl, $MgSO_4$, $NaNO_3$, KH_2PO_4, $NaHCO_3$ and $CaCl_2$, while the microelements, $EDTANa_2$ and $FeSO_4$ concentrations were held constant. Five compounds were evaluated in three levels (g L^{-1}) and the last compound was evaluated in two levels in an orthogonal array (L_{18}). The larger the better definition was used because the objective of the Taguchi design was to enhance the growth. The different runs were generated by the Minitab v17 program. The evaluations (biological triplicates) were cultivated for 7 days and the biomass was harvested as previously described. A confirmation run for both Rhodophyta strains was performed to compare the optimal medium against ASW medium for 40 days using the same cultivation parameters and EPS harvesting procedures as described in Section 2.2.

2.5. Comparison of abiotic stress factors on EPS production by Taguchi design

Four abiotic factors were tested on EPS induction using the Taguchi design in three levels (L_9). The factors evaluated were the NaCl concentration (0, 13.5, 27 g L^{-1} for *P. sordidum* and 0, 27 and 50 g L^{-1} for *P. purpureum*), pH (Bafana, 2013; Fábregas et al., 2001; Freitas et al., 2011), hours of light exposure (12, 18 and 24 h) and temperature (20, 25 and 30 °C). The combination of factors was again generated by the Minitab v17 program. The larger the better definition was used as the objective was to increase in the EPS production. The factor combination was achieved by adding 25 mL of different buffers with fixed pH and NaCl concentrations to 50 mL of microalgae culture obtained from the cylinder system. Aluminum shades were used to vary the time which cultures were exposed to light. The induction was performed for 5 days in biological triplicate. The EPS was separated from the biomass as described in Section 2.2.

2.6. Two-stage cultivation system

The DASGIP® Bioblock system was used to compare two-stage and one-stage systems with the initial cultivation parameters used in this investigation (biological duplicate). For the one-stage cultivation, the parameters described in Section 2.2 were used; the wavelengths combination (white light emulation) was used for both *Porphyridium* strains. The two-stage cultivation was performed as follows: the first stage focused on the microalgae growth using the same cultivation parameters that were used in the one-stage process for 15 days, followed by a second stage that focused on induction of EPS production for 5 days. The induction parameters for *P. sordidum* were the following: temperature decreased to 21 °C and light exposure increased to 24 h. The EPS induction phase for *P. purpureum* was achieved by adding 180 mL of 20 X improved medium 3 (I3) to decrease the pH to 6. All other parameters were the same as for the growth phase.

The EPS was obtained by centrifuging 1 L of cultivation broth at 4,000 × *g*, 20 °C for 30 min. The cell-free supernatant was diluted 1:5 with ultrapure water, and the EPS was concentrated using the Sartorius crossflow system with a 100 kDa ultrafiltration cassette (Hydrosart®, AG, Germany) until reaching a volume of 0.75 L. The salt-free concentrated supernatant was precipitated with two volumes of 2-propanol and dried at 45 °C for 3 days in a vacuum oven.

2.7. Analysis of the EPS composition

The chemical composition of the EPS was determined from 0.1% solutions that were prepared in ultra-pure water. The carbohydrate monomer composition was determined by the HT-PMP method as described previously (Medina-Cabrera et al., 2020; Rühmann et al., 2014). Briefly, the EPS samples were mixed, hydrolyzed by Trifluoroacetic acid (TFA) and neutralized. The neutralized solution was derivatized using the HT-PMP method. The reaction was stopped and filtered for the following steps of the analysis. All analytical samples were evaluated in technical triplicates. The sulfate content was determined by modified turbidimetry using a barium chloride/PEG 6000 master mix in three technical replicates (Medina-Cabrera et al., 2020). The protein content was quantified by a modified Bradford method in three technical replicates (Medina-Cabrera et al., 2020). The EPS recovery rate was calculated as was previously reported (Medina-Cabrera et al., 2020).

3. Results and discussion

3.1. Evaluation of the influence of different wavelengths on the growth and EPS production

Different wavelengths of light can exert strong effects on microalgae due to their photosynthetic nature (Li et al., 2019). Therefore, the DASGIP® Bioblock system was used to test different wavelengths on the growth and EPS production for *P. sordidum* and *P. purpureum*. To assure that the strains were adapted to each wavelength, they were cultivated for 17 days, followed by the evaluation of growth and EPS production.

The effects of the different wavelengths on the growth are shown in Fig. 1. It was previously reported that blue and red light were favorable for growth and EPS production for microalgae and cyanobacterial strains (Han et al., 2014; You and Barnett, 2004). Conversely, our results indicate that blue light (430 nm) was the least favorable for growth and EPS production for *P. sordidum* (yield: 0.01 ± 0.00 g L^{-1} and EPS recovery rate of the quantifiable fraction: 45 ± 1.4%) and for *P. purpureum* (yield: 0.03 ± 0.01 g L^{-1} and recovery rate: 27 ± 1.0%). Slightly better growth and EPS production were observed in the cultures exposed to green/yellow/orange (572/625/640 nm) light for *P. sordidum* (yield: 0.08 ± 0.01 g L^{-1} and recovery rate: 44 ± 2.8%) and *P. purpureum* (yield: 0.08 ± 0.01 g L^{-1} and recovery rate: 54 ± 3.4%). White light was generally most suitable for growth rate and EPS production for *P. sordidum* (yield: 0.10 ± 0.00 g L^{-1} and recovery rate: 50 ± 7.0%) and *P. purpureum* (yield: 0.14 ± 0.01 g L^{-1} and recovery rate: 55 ± 6.6%). A remarkable difference between the strains was seen when growing in orange/red (660/780 nm) light. The *P. sordidum* growth and EPS production (yield: 0.12 ± 0.01 g L^{-1} and recovery rate: 37 ± 0.4%) were as good as for white light, but for *P. purpureum* it was less optimal than white light (yield: 0.09 ± 0.01 g L^{-1} and recovery rate: 43 ± 0.6%) and only as good as green/yellow/orange light (572/625/640 nm). These results might be explained because the illumination sources used to cultivate microalgae being located outside of the cultivation vessel (Patel et al., 2013; Roussel et al., 2015; Soanen et al., 2016; Velea et al., 2011; You and Barnett, 2004). Such setups could lead to light deflection and thus a decrease in light intensity and wavelength range received. In contrast, the LEDs used in this study were placed inside the cultivation medium which could lead to the observed negative influence of the blue and red light on microalgae cultivation and EPS production.

In general the chemical compositions of the EPS obtained in the different light treatments were identical to the ones previously reported for both strains and included glucose, galactose, xylose, glucuronic acid and sulfate (Medina-Cabrera et al., 2020). The differences in growth rates, EPS yields and recovery rates generated by the different wavelengths were possibly mediated by the pigments synthetized in each *Porphyridium* strain (Phycobiliproteins and Chlorophyll), which are manifested in the red color of the *P. purpureum* and olive-green color of the *P. sordidum* and affect the wavelengths that are harvested (Medina-Cabrera et al., 2020). The results demonstrated that the white light emulation (wavelength combination) was most suitable for growth and EPS production for both strains, and therefore it was used in all subsequent experiments.

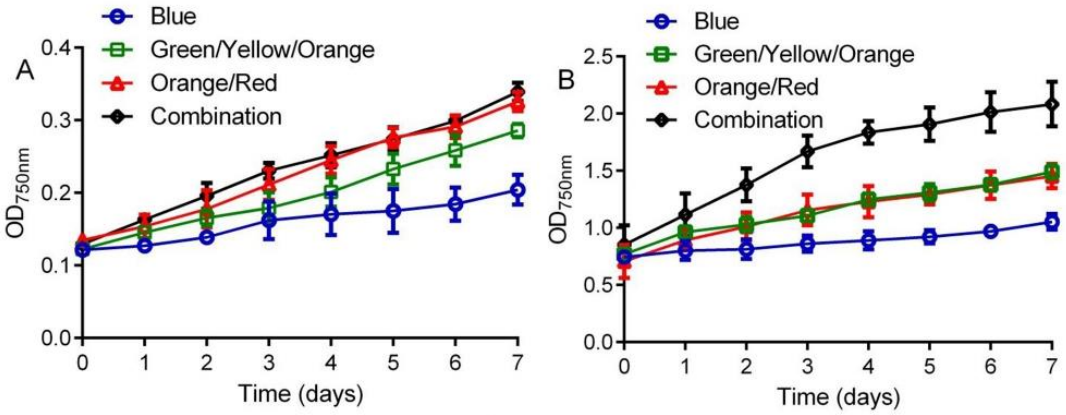

Fig. 1. Influence of different wavelengths on the growth rate for *P. sordidum* (A) and *P. purpureum* (B) The growth was measured at OD_{750nm} in the DASGIP Bioblock system. Four conditions were tested: blue, green/yellow/orange, orange/red and the three mode combination (white light). The difference in the OD_{750nm} registered for both strains was related to the microalgae color (olive-green for *P. sordidum* and red for *P. purpureum*) as it was previously reported (Medina-Cabrera et al., 2020). The graph is based on the average including the standard deviation as variability ($n = 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Selection of the most prominent medium components by Placket-Burman (PB) design

Photosynthetic microalgae gain energy from light; however, the cultivation medium provides the other compounds that enable their growth and EPS production. After identifying the optimal light, the most important cultivation medium compounds were selected for further optimization. The chelating agent EDTA-Na_2 was used to stabilize the $FeSO_4$ in water, so the two compounds were considered to be one variable. Additionally, the microelements solution was considered to be one factor to decrease the PB design complexity.

The microalgae growth was evaluated by weighting the dry biomass from each run after the cultivation process. For *P. sordidum*, the presence of $CaCl_2$, microelements, $NaNO_3$ and NaCl and the absence of $MgSO_4$ favored biomass generation. *P. purpureum* growth is positively affected by all medium compounds. The rest of the ASW medium compounds were not selected for optimization due to the small effect that they had on the biomass generation.

The effect of the medium compounds on EPS production was evaluated by determining the EPS yields. In this regard, the presence of $CaCl_2$, $NaNO_3$ and microelements and the absence of NaCl, $MgSO_4$ and KH_2PO_4 were favorable for EPS generation in *P. sordidum*. In contrast, the presence of KH_2PO_4, $MgSO_4$ and $NaHCO_3$ and the absence of the $NaNO_3$, NaCl and $CaCl_2$ favored EPS generation for *P. purpureum*. The positive effect of the absence of several compounds in this approach could be interpreted as an indicator to decrease the concentration of these compounds in the cultivation medium.

The combination of the results for biomass and EPS generation allowed the selection of the most important compounds for optimization. For *P. sordidum*, the five most important compounds were the same in both tests: $CaCl_2$, $NaNO_3$, NaCl, microelements and $MgSO_4$. KH_2PO_4 was selected as the last compound for optimization, as it was the sixth most important compound for the EPS production. However, because the compounds have a different effect for *P. purpureum*; the two most important compounds from each test were selected: NaCl and $NaHCO_3$ for growth and KH_2PO_4 and $MgSO_4$ for EPS production. The other two compounds selected were $NaNO_3$ and $CaCl_2$ because they were important in both PB designs. Furthermore, this design allowed the identification of the least important compounds that affected the growth and EPS production.

A confirmation test was completed to consider the elimination of these compounds in order to simplify the cultivation medium. It was determined that $MgCl_2$ did not have a significant effect on growth and EPS production in both microalgae strains, so it was eliminated from the medium. The $NaHCO_3$ for *P. sordidum*, the microelements for *P. purpureum*, and EDTA-$FeSO_4$ for both strains were maintained because their elimination from the cultivation medium had a negative effect on the growth and/or the EPS production. The confirmation test results are presented in Fig. 2. The PB design allowed to screen and to adequately identify the most important medium compounds to be optimized in the following steps and, accordingly, to eliminate the less important compounds of the ASW medium.

3.3. Cultivation medium optimization using the Taguchi method

The Taguchi design is a valuable tool to optimize biotechnological systems, including for increasing the production of EPS in *A. platensis* using the larger the better definition to determine the optimal cultivation conditions for EPS production (Dejsungkranont et al., 2017; Ranganathan et al., 2016; Rao et al., 2008). The levels investigated in the Taguchi design were fixed and the following points were taken into account: i) The results from the PB design for growth and EPS production were considered as follows: if the presence of the compound was favorable, the concentration was increased and when the absence was favorable, the concentration was decreased or eliminated. ii) The concentrations used in this research were fixed using the cultivation medium concentration reported in other media used for other *Porphyridium* strains (Kathiresan et al., 2007; Nikolova et al., 2019; Ott, 1987; Razaghi et al., 2014; Soanen et al., 2016; Velea et al., 2011). The levels used in the Taguchi design are shown in Table 1. The variation of concentrations of many compounds at the same time increases the complexity of the procedure. For that reason, the Taguchi design was used to determine the optimal concentrations of six medium compounds and so develop an improved cultivation medium that enhances microalgae growth using only 18 runs, instead of 54 runs using RSM and 64 runs by a full factorial design.

The Taguchi design ranked the most important compounds for biomass production. The ranking was as follows for *P. sordidum*: KH_2PO_4 > NaCl > $MgSO_4$ > $CaCl_2$ > microelements > $NaNO_3$; for *P. purpureum*, KH_2PO_4 > $NaHCO_3$ > $MgSO_4$ > $NaNO_3$ > NaCl. Additionally, the Taguchi design allows the identification of the optimal levels for each compound, which are the ones above the reference

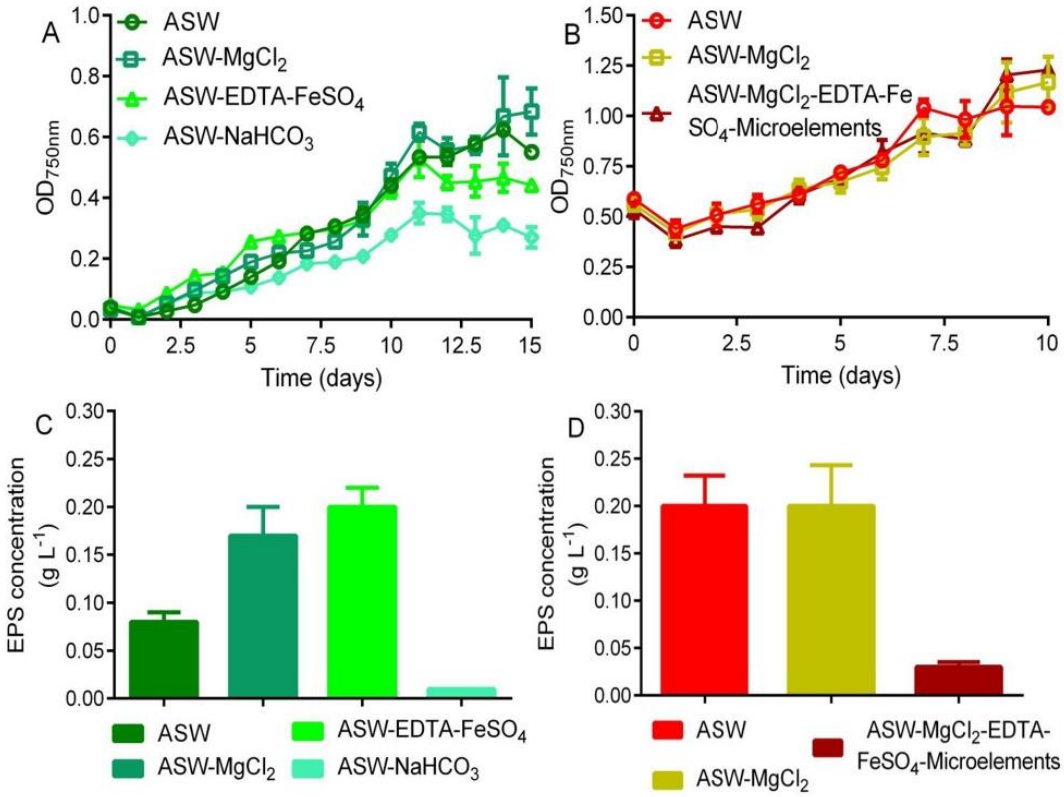

Fig. 2. PB design confirmation on compound elimination from the ASW medium in both *Porphyridium* strains. The growth behaviors were evaluated for *P. sordidum* (A) and *P. purpureum* (B). The EPS production can be observed in (C) for *P. sordidum* and in (D) for *P. purpureum*. The graph is based on the average including the standard deviation ($n = 2$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

line in the signal to noise ratio (S/N) graph. All compounds showed a clear optimum, except for NaCl (3 and 15 g L^{-1}) and the microelements (1.5 and 2 mL), which each had two optimal levels for *P. sordidum*. Similarly, for *P. purpureum*, the optimum values were easily detected except for $NaNO_3$ (1.5 and 1.8 g L^{-1}) and NaCl (15 and 27 g L^{-1}), which also had two optimal levels. As two optimal were identified in some compounds, a confirmation test was performed by combining the two optimal concentrations in three different cultivation media for each strain. The composition of the three improved media (IA, IB and IC) for *P. sordidum* and the three improved media (I1, I2 and I3) for *P. purpureum* are shown in Fig. 3. For improving IC, a higher KH_2PO_4 concentration (0.9 g L^{-1}) was tested to reach the initial N/P suggested for other *Porphyridium* strains (Soanen et al., 2016). The cultivation process proceeded for 40 days to allow a complete adaptation to the new media. These three improved media significantly enhanced the growth of *P. sordidum* in comparison to the ASW medium. In contrast, the three improved media decreased *P. purpureum* growth in comparison to the initial conditions. Moreover, the EPS were harvested, dried and quantified. For *P. sordidum*, all improved media resulted in higher EPS yields, whereas for *P. purpureum* the three improved media did not generate more EPS.

Combining the growth and EPS production results clearly illustrated that lowering the NaCl and $MgSO_4$ concentrations and increasing the $NaNO_3$, KH_2PO_4 and $CaCl_2$ concentrations positively influence the *P. sordidum* growth rate and EPS production. Because the three improved media produced quite similar amounts of EPS, IB medium, which has the lowest concentrations, was selected. Furthermore, the IB medium produced slightly more EPS than the other media (Fig. 3). In contrast, the three newly developed media for *P. purpureum* did not enhance the growth and the EPS production. However, the I3 medium produced a similar EPS yield with lower cell numbers, based on the OD_{750nm}, compared to the cultivation in ASW medium. This can be explained via the initial N/P ratio (1.5) of the I3 medium, which allowed it to reach the 4.9 N/P ratio via nutrient uptake, enhancing the EPS production although less biomass was produced (Razaghi et al., 2014; Soanen et al., 2016). A clear outcome is that the NaCl concentration should be kept constant at 27 g L^{-1}, since lower concentrations (I1 and I2 media) resulted in reduced EPS production. The results of the confirmation tests are summarized in Fig. 3, which indicates that the ASW medium is the most suitable for *P. purpureum* cultivation (Kathiresan et al., 2007; Velea et al., 2011).

3.4. Evaluation of abiotic factor on the EPS production

The Taguchi design was also used to determine if abiotic factors can trigger the production of secondary metabolites, which in this study are EPS (Paliwal et al., 2017; Trabelsi et al., 2009b). In previous studies only two factors (temperature and light intensity) were tested at the same time as EPS inducers (Mezhoud et al., 2014; Trabelsi et al., 2009b). Contrary, in this investigation, double the number of abiotic factors were tested at the same time using the Taguchi design (pH, time exposure to light, NaCl concentration and temperature) using a minimum number of runs to determine the most prominent factors that induce EPS production. The Taguchi design was again used because it

Table 1
Summary of the Taguchi design levels used for the optimization of the cultivation medium and EPS production.[a] The changes made to the reference medium (ASW) and to the abiotic factors use for cultivation are indicated by: decrease (↓) and increase (↑).

Media optimization					
P. sordidum					
Compounds (g L^{-1})	Evaluations			Variation from original media	References
	1	2	3		
NaCl	3	15	27[b]	↓	PB_{EPS} and (Nikolova et al., 2019)
$MgSO_4$	4.3	5.3	6.3[b]	↓	PB_{EPS}, PB_B and (Ott, 1987)
$CaCl_2$	1.13[b]	1.5	1.8	↑	PB_{EPS} and PB_B
$NaNO_3$	1.0[b]	1.5	1.8	↑	PB_B and (Razaghi et al., 2014; Soanen et al., 2016)
Microelements (mL)	1.0[b]	1.5	2	↑	PB_{EPS} and PB_B
KH_2PO_4	0.07[b]	0.7		↑	(Nikolova et al., 2019)
P. purpureum					
Compounds (g L^{-1})	Evaluations			Variation from original media	References
	1	2	3		
NaCl	15	27[b]	39	↓	PB_{EPS} and (Velea et al., 2011)
				↑	PB_B
$MgSO_4$	5.3	6.3[b]	7.3	↓	(Nikolova et al., 2019)
				↑	PB_{EPS} and PB_B
$NaNO_3$	1.0[b]	1.5	1.8	↑	PB_B and (Razaghi et al., 2014; Soanen et al., 2016)
KH_2PO_4	0.07[b]	0.5	0.9	↑	PB_B and (Razaghi et al., 2014; Soanen et al., 2016)
$NaHCO_3$	0.04[b]	0.08	0.12	↑	PB_{EPS} and PB_B and (Velea et al., 2011)
$CaCl_2$	1.13[b]	1.5		↑	PB_B and (Kathiresan et al., 2007)
EPS production optimization					
Factors	Evaluation			Abiotic factor changes	References
	1	2	3		
NaCl (g L^{-1})	0	13.5	27[c]	↓ for *P. sordidum*	PB_{EPS} and Taguchi design for optimization
		27[c]	50	↓ and ↑ for *P. purpureum*	PB_{EPS}, PB_B and (Paliwal et al., 2017)
pH	3	7[c]	11	↓ and ↑ for both strains	(Paliwal et al., 2017)
Light exposure (h)	12[c]	18	24	↑ for both strains	(Paliwal et al., 2017)
Temperature (°C)	20	25[c]	30	↓ and ↑ for both strains	Determine by pre-test, data not shown and (Trabelsi et al., 2009b)

[a] Table 1. Results and discussion (Sections 3.3 and 3.4). Levels used in the optimization.
[b] Concentration in the ASW medium.
[c] Parameters used for microalgae cultivation

allowed achieving the optimization with at least 3 times less runs as other DOE, like the RSM (Narala et al., 2016) and full factorial design (81) required. The levels used were determined based on the results from the PB design. The Taguchi design for medium optimization, together with results from previous reports is shown in Table 1.

The optimal levels were selected using the values above the S/N line. The Taguchi design ranks the stress factors on the EPS production. For *P. sordidum,* the ranking was as follows: temperature (°C) > light exposure (h) > pH > NaCl (g L^{-1}). In contrast, the ranking for *P. purpureum* was pH > light exposure (h) > NaCl (g L^{-1}) > temperature (°C). The optimal parameters for EPS production in *P. sordidum* were lower temperatures (20 or 25 °C) and an increase in light exposure to 18 or 24 h. The change in pH towards an acidic and alkaline environment and a decrease in NaCl concentration were revealed to be the two least important factors for EPS production. In contrast, the most prominent abiotic factors for EPS production in *P. purpureum* were the variations of the pH towards the alkaline (pH 8) or acidic (pH 6) environments and maintaining the light exposure at 12 h. The two less significant factors for EPS production for *P. purpureum* were the decrease of the NaCl content and the temperature.

The two most important abiotic factors for *P. sordidum* and *P. purpureum* EPS induction were tested in un-induced cultures as follows: for *P. sordidum,* the temperature was set to 20 °C and the light exposure was increased to 24 h. The other two stress factors were indirectly achieved by using the IB medium (low NaCl concentration and pH 6). For *P. purpureum*, the first factor tested as EPS inducer was the pH shift to an alkaline or acidic environment. For this reason, a controlled decrease of the pH was achieved using a 20 x I3 medium by exploiting its buffering effect (0.9 g L^{-1} of KH_2PO_4), fixing the pH at 6 and reaching the I3 medium concentration. A shift to alkali pH was not used because compounds in enriched medium precipitated in environments over pH 7.5. Additionally, the light exposure was maintained at 12 h because it was the favorable abiotic factor for EPS induction. In order to validate the results obtained from the Taguchi design, a factor that did not increase the EPS yield (24 h of light) was also tested in I3 medium. In addition to the two inducted EPS productions, these systems were compared to the ASW medium and the reconstituted ASW to its initial conditions with 12 h of light exposure. It was confirmed that the EPS production was increased using the optimal abiotic factors for both *Porphyridium* strains. Moreover, non-optimal levels such as an increase to 24 h of light for *P. purpureum* decreased the EPS production, validating the results obtained by the Taguchi design.

3.5. Two-stage cultivation system

The final step of the optimization was to combine medium optimization and EPS-inducing abiotic factors in a two-stage cultivation process consisting of the growth and induction phases. This strategy has been used for microalgae to enhance the production of different compounds, such as lipids and biodiesel (Farooq et al., 2013; Narala et al., 2016). The two-stage system was compared to the initial one-stage cultivation conditions in the DASGIP® Bioblock system using the white light emulation. A 10% inoculum was used to decrease the cultivation time for reaching an initial OD_{750nm} of 0.06 for *P. sordidum* and 0.3 for *P. purpureum*. The differences in the initial OD_{750nm} values are related to the shape and size of both *Porphyridium* strains (Medina-Cabrera et al., 2020).

A slight difference in growth was detected for *P. sordidum* on day 10 which increased in the subsequent days. On day 15, the induction phase was initiated by decreasing the temperature to 21 °C and increasing the

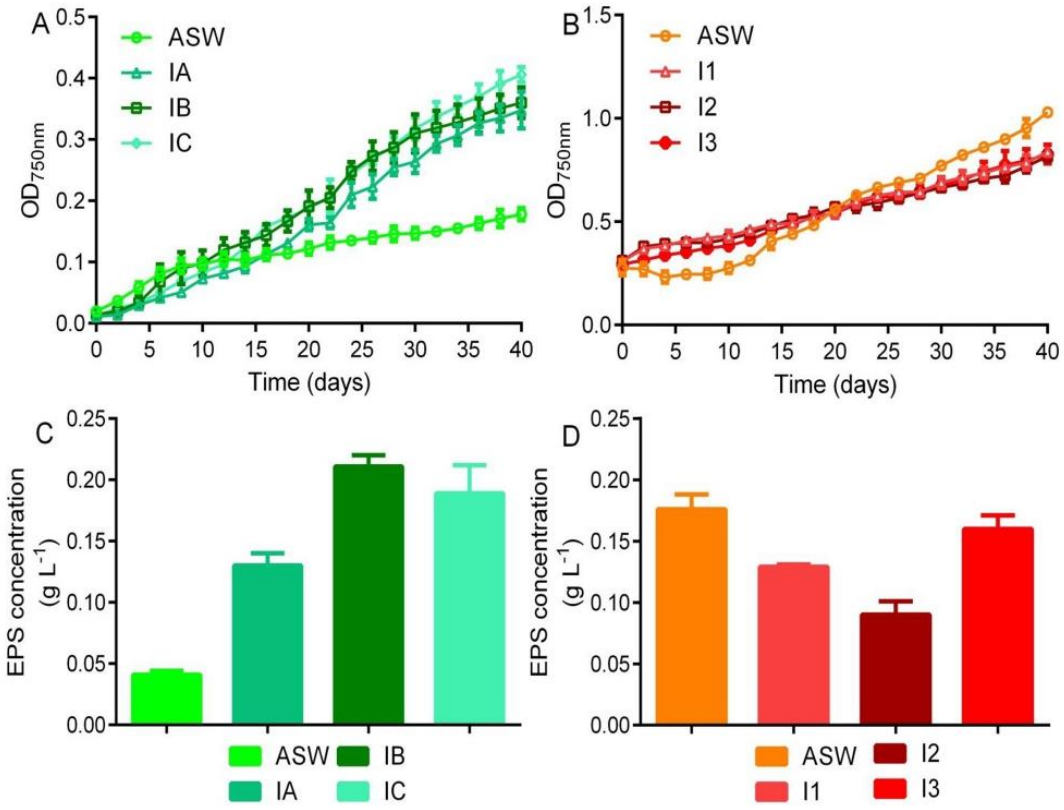

Fig. 3. Comparison of the three improved media generated by the Taguchi design against the ASW medium. The growth is presented for *P. sordidum* (A) and *P. purpureum* (B) and EPS production is shown for *P. sordidum* (C) and *P. purpureum* (D). The evaluation was conducted over the course of 40 days of cultivation. The graph is based on the average including the standard deviation as variability ($n = 3$). For *P. sordidum*: Improved medium A (IA): NaCl (15 g L^{-1}), $CaCl_2$ (1.8 g L^{-1}), $NaNO_3$ (1.5 g L^{-1}), $MgSO_4$ (4.3 g L^{-1}), KH_2PO_4 (0.7 g L^{-1}) and microelements solution 2 mL; improved medium B (IB): NaCl (3 g L^{-1}), $CaCl_2$ (1.8 g L^{-1}), $NaNO_3$ (1.5 g L^{-1}), $MgSO_4$ (4.3 g L^{-1}), KH_2PO_4 (0.7 g L^{-1}) and microelements solution 1.5 mL and improved medium C (IC): NaCl (3 g L^{-1}), $CaCl_2$ (1.8 g L^{-1}), $NaNO_3$ (1.5 g L^{-1}), $MgSO_4$ (4.3 g L^{-1}), KH_2PO_4 (0.9 g L^{-1}) and microelements solution 2 mL. The $FeSO_4$, EDTA and $NaHCO_3$ concentrations were maintained as in the ASW media for IA, IB and IC. For *P. purpureum*: The improved medium 1 (I1): NaCl (15 g L^{-1}), $CaCl_2$ (1.5 g L^{-1}), $NaNO_3$ (1.8 g L^{-1}), $NaHCO_3$ (0.12 g L^{-1}), $MgSO_4$ (5.3 g L^{-1}) and KH_2PO_4 (0.9 g L^{-1}); improved medium 2 (I2): NaCl (15 g L^{-1}), $CaCl_2$ (1.5 g L^{-1}), $NaNO_3$ (1.5 g L^{-1}), $NaHCO_3$ (0.12 g L^{-1}), $MgSO_4$ (5.3 g L^{-1}) and KH_2PO_4 (0.9 g L^{-1}) and improved medium 3 (I3): NaCl (27 g L^{-1}), $CaCl_2$ (1.5 g L^{-1}), $NaNO_3$ (1.8 g L^{-1}), $NaHCO_3$ (0.12 g L^{-1}), $MgSO_4$ (5.3 g L^{-1}) and KH_2PO_4 (0.9 g L^{-1}). The $FeSO_4$, EDTA and microelements concentrations were maintained as in the ASW medium for I1, 2 and 3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

time of light exposure to 24 h. The induction phase was maintained for 5 days and could not be extended further, because on day 18 the sparger was blocked with microalgae biomass and EPS. At the end of the cultivation process, the EPS produced by the two-stage system (0.17 ± 0.01 g L^{-1}) was 3.4 times higher compared to the initial conditions (0.05 ± 0.01 g L^{-1}). The growth difference between the one- and two-stage systems was more evident in the induction phase (Fig. 4A). A possible explanation is that the EPS induction factors also enhanced *P. sordidum* growth. To confirm the previous explanation, the *P. sordidum* growth was tested under the influence of EPS inducing abiotic factors (20 °C and 24 h of light) and using the normal cultivation parameters (12/12 h day/night cycle and RT, in biological triplicate). No significant differences in the growth behavior were observed. Therefore, the increase in the growth rate observed in the two-stage system was only mediated by the improved medium B.

The *P. purpureum* two-stage system was compared to the initial cultivation. No differences were detected in the growth phase, as the same parameters are used in both approaches (Fig. 4B). When the EPS yields were compared, the two-stage system (0.12 ± 0.01 g L^{-1}) produced 1.2 times more EPS than the one-stage system (0.10 ± 0.00 g L^{-1}), possibly mediated by the previously described I3 medium effect. Moreover, this effect could be enhanced by the ability of the large microalgae concentration at the end of the first stage to rapidly uptake the nutrients, reaching an adequate ratio for EPS production. In previous investigations the EPS production in microalgae has been improved using different methodologies. Specifically the EPS production has been increased 1.6 times for *Chlamydomonas reinhardtii*, 2.9 times for *Rhodella violacea* and 4 times for *P. marinum* (Bafana, 2013; Soanen et al., 2016; Villay et al., 2013). Typically two common approaches to enhance the EPS production are used, optimization of the medium and/or the cultivation parameters (pH, temperature, irradiance etc.) (Dejsungkranont et al., 2017; Villay et al., 2013). These approaches are successful since optimal cultivation conditions allowed to reach a dense cell growth that produce high amounts of EPS when nutrients are consumed and thus appropriate ratios are reaching between them (Razaghi et al., 2014; Soanen et al., 2016). In this investigation the determination of the most appropriate cultivation conditions (medium and wavelengths) for optimal microalgae growth and

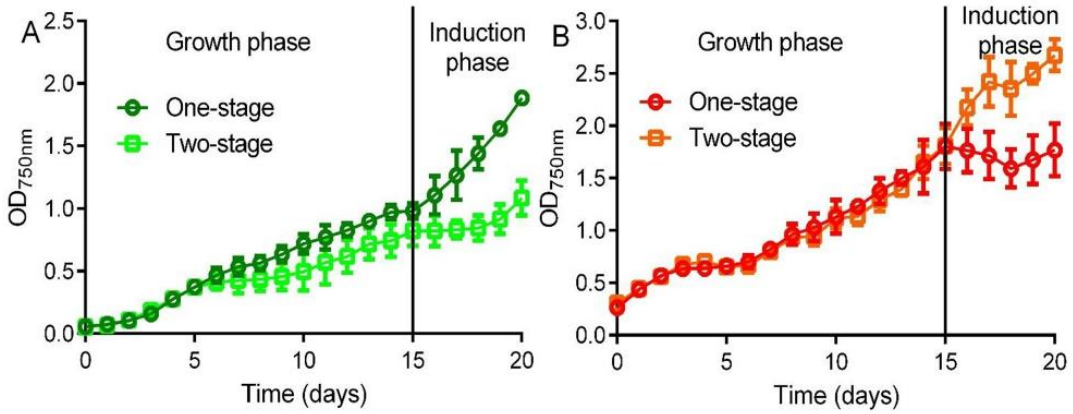

Fig. 4. Comparison of growth behavior (OD_{750nm}) comparing the one-stage and the two-stage cultivation (growth phase and induction phase). For *P. sordidum* (A) and *P. purpureum* (B), the evaluation was done in biological duplicates ($n = 2$) and the graphs show the average with the standard deviation as variability. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

EPS production (Section 3.3) as well as abiotic induction and two-stage cultivation system were combined. The optimal cultivation conditions (first stage) allowed to obtain a high-density EPS producing microalgae culture. This was followed by a second stage with the objective to further enhance the EPS production by changing the selected abiotic factors (Section 3.4).

Both *Porphyridium* strains showed improved EPS production, with a 1.2 folds increase for *P. purpureum*, although this strain was already cultivated in an optimal medium (ASW) and white light as the most appropriate source for optimal growth and EPS production. The improvement was mediated by the abiotic factor induction in the second cultivation stage. For *P. sordidum* the combination of the two-stages increased the EPS production 3.4 folds by the optimal cultivation conditions (improve medium and appropriate wavelengths) in the first cultivation-stage (2.3 folds) of the cultivation. Additionally, the EPS production was further increased by the abiotic factors in the second cultivation-stage (1.1 folds). To best of our knowledge, the two-stage cultivation system has never been tested for EPS production in microalgae.

The EPS compositions from the one- and two-stage system were compared. No changes were observed in the *P. sordidum* EPS composition (Table 2). In the case of the *P. purpureum* EPS, the carbohydrate fraction did not change, but the sulfate content increased two-fold in the two-stage system sample compared to the EPS obtained from the initial conditions (Table 2). An increase in the sulfate content was also reported for the *P. marinum* EPS when it was produced in an improved medium (Soanen et al., 2016). The average EPS recovery of the quantifiable fractions in *P. sordidum* EPS were 46 ± 5.4% (two-stage) and 47 ± 3.7% (one-stage); for *P. purpureum*, the EPS recovery of the quantifiable fractions were 45 ± 1.6% (two-stage) and 50 ± 0.5% (one-stage). These recoveries are in the same range as reported for other EPS analyzed by the HT-PMP method (Gansbiller et al., 2019; Rütering et al., 2016). Furthermore, the compositions and recoveries were similar to the ones previously reported for those strains (Medina-Cabrera et al., 2020).

Table 2
The percentage of the mass ratios of quantifiable EPS constituents were compared between the two-stage and one-stage setups for both strains.[a] Additionally, the EPS production from each evaluation is shown. The values present the average of the EPS constitutes with their standard deviation. The analysis was performed in technical triplicate.

	Compounds	*P. sordidum*		*P. purpureum*	
		One-stage	Two-Stage	One-stage	Two-Stage
% EPS Quantifiable molecules	Glucose	19 ± 1.7	19 ± 0.5	17 ± 0.6	13 ± 1.5
	Galactose	21 ± 2.5	23 ± 1.4	23 ± 0.0	19 ± 4.8
	Xylose	38 ± 1.2	36 ± 1.7	41 ± 0.4	28 ± 7.7
	Glucuronic acid	3.4 ± 0.9	2.2 ± 0.1	1.8 ± 0.1	2.9 ± 0.1
	Sulfate	16 ± 1.5	19 ± 3 .3	14 ± 0.3	36 ± 14
	Protein [b]	2.7 ± 0.3	1.8 ± 0.3	2.9 ± 0.1	0.7 ± 0.2
	Recovery	47 ± 3.7	46 ± 5.4	50 ± 0.5	45 ± 1.6
EPS yields, g L^{-1}		0.05 ± 0.01	0.17 ± 0 .01	0.10 ± 0.00	0.12 ± 0.01
OD_{750nm} [c]		1.1 ± 0.5	1.9 ± 0.1	2.7 ± 0.6	2.2 ± 0 .3

[a] Table 2. Results and discussion (Section 3.5). Comparison of initial-stage to the two-stage cultivation system.
[b] Protein detected was considered as contamination.
[c] Value of OD_{750nm} obtained at the end of the cultivation process.

4. Conclusions

Four optimization strategies were tested to enhance the growth rate and EPS production for both *Porphyridium* strains. White light was identified as most suitable for both strains. The PB and Taguchi design were used to optimize the cultivation medium and EPS production. The implementation of a two-stage cultivation process increased the EPS production by 3.4 times in *P. sordidum* and by 1.2 times in *P. purpureum* compared to the initial conditions; no significant change in composition of the P. *sordidum* EPS was detected, but the sulfate content increased in the *P. purpureum* EPS obtained from the two-stage cultivation.

Credit author statement

Edilberto Vicente Medina-Cabrera performed the experiments. Jochen Schmid, Volker Sieber and Edilberto Vicente Medina-Cabrera designed the study. Edilberto Vicente Medina-Cabrera, Jochen Schmid and Broder Rühmann analyzed and interpreted the data. Edilberto Vicente Medina-Cabrera, Jochen Schmid and Broder Rühmann wrote the manuscript. All authors revised it critically for scientifically as well as technically soundness. All authors approved of the final version to be submitted.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

EVMC performed the experiments. JS, VS and EVMC designed the study. EVMC, JS and BR analyzed and interpreted the data. EVMC, JS and BR wrote the manuscript. All authors revised it critically for scientifically as well as technically soundness. All authors approved of the final version to be submitted.

Appendix A. Supplementary data

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Bioresource technology reports

Optimization of growth and EPS production in two *Porphyridum* strains

Appendix A: Supplementary material

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Table S1. The effect of different wavelengths on EPS production. The percentage mass ratios of the quantifiable molecules that compose the EPSs are presented. Additionally, the recovery rate determined by the addition of the carbohydrate and sulfate fraction is shown. The evaluation was performed in technical triplicates. The average is presented including the standard deviation as variability ($n=3$).

Strains	Compounds		Blue, 430 nm	Green, 572 nm Yellow, 625 nm Orange, 640 nm	Orange, 660 nm Red, 780 nm	Combination of lights
P. sordidum	% EPS Quantifiable molecules	Glucose	22 ± 1.8	15 ± 1.5	14 ± 0.2	17 ± 0.8
		Galactose	26 ± 1.0	21 ± 0.8	20 ± 0.3	22 ± 1.4
		Xylose	36 ± 1.4	30 ± 0.8	31 ± 0.4	30 ± 2.1
		Glucuronic acid	4.1 ± 0.3	4.7 ± 0.5	5.6 ± 0.2	4.6 ± 0.5
		Sulfate	10 ± 0.4	27 ± 1.8	28 ± 2.6	25 ± 3.5
		Protein*	1.4 ± 0.1	2.1 ± 0.1	2.6 ± 0 .3	1.6 ± 0.3
		Recovery	45 ± 1.4	44 ± 2.8	37 ± 0.4	50 ± 7.0
	EPS yield, g L^{-1}		0.01 ± 0.00	0.08 ± 0.01	0.12 ± 0.01	0.10 ± 0.00
	OD_{750nm}[§]		0.20 ± 0.02	0.28 ± 0.01	0.33 ± 0.01	0.34 ± 0.01
P. purpureum	% EPS Quantifiable molecules	Glucose	10 ± 0.2	14 ± 0.5	10 ± 1.8	16 ± 1.0
		Galactose	29 ± 0.7	16 ± 0.7	14 ± 0.4	21 ± 1.7
		Xylose	32 ± 0.3	30 ± 2.5	21 ± 0.1	28 ± 2.4
		Glucuronic acid	7.8 ± 0.8	3.5 ± 0.2	5.0 ± 0.1	4.8 ± 0.7
		Sulfate	18 ± 0.3	36 ± 2.5	49 ± 2.0	31 ± 4.2
		Protein*	2.9 ± 0.1	0.9 ± 0.1	1.2 ± 0.3	0.31 ± 0.1
		Recovery	27 ± 1.0	54 ± 3.4	43 ± 0.6	55 ± 6.6
	EPS yield, g L^{-1}		0.03 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.14 ± 0.01
	OD_{750nm}[§]		1.05 ± 0.07	1.45 ± 0.11	1.49 ± 0.06	2.08 ± 0.19

* Protein detected was considered as contamination

§ Value of OD_{750nm} obtained at the end of the culturing process

Table S2. The effect and rank of the compounds of the ASW media on the biomass generation by PB design for *P. sordidum* and *P. purpureum*. (+ indicates the presence and – indicates the absence of the compound favoring the biomass and EPS generation.

Biomass							
P. sordidum				*P. purpureum*			
Rank	Compound	Effect	%	Rank	Compound	Effect	%
1	$CaCl_2$	+	75	1	NaCl	+	51
2	Microelements	+	12	2	$NaHCO_3$	+	22
3	$MgSO_4$	–	6.4	3	$CaCl_2$	+	18
4	$NaNO_3$	+	3.2	4	$NaNO_3$	+	4.0
5	NaCl	+	1.8	5	Microelements	+	1.7
6	EDTA-$FeSO_4$	+	0.5	6	$MgCl_2$	+	1.3
7	$NaHCO_3$	–	0.1	7	KH_2PO_4	+	1.1
8	KH_2PO_4	–	0.1	8	$MgSO_4$	+	0.3
9	$MgCl_2$	+	0.02	9	EDTA-$FeSO_4$	+	0.02
EPS							
P. sordidum				*P. purpureum*			
Rank	Compound	Effect	%	Rank	Compound	Effect	%
1	$CaCl_2$	+	21	1	KH_2PO_4	+	42
2	$NaNO_3$	+	21	2	$MgSO_4$	+	19
3	NaCl	–	11	3	$NaHCO_3$	+	10
4	$MgSO_4$	–	11	4	$NaNO_3$	–	10
5	Microelements	+	11	5	NaCl	–	8.9
6	KH_2PO_4	–	7.7	6	$CaCl_2$	–	4.2
7	$NaHCO_3$	+	7.7	7	$MgCl_2$	+	3.8
8	EDTA-$FeSO_4$	–	7.7	8	EDTA-$FeSO_4$	–	1.6
9	$MgCl_2$	+	2.0	9	Microelements	+	0.8

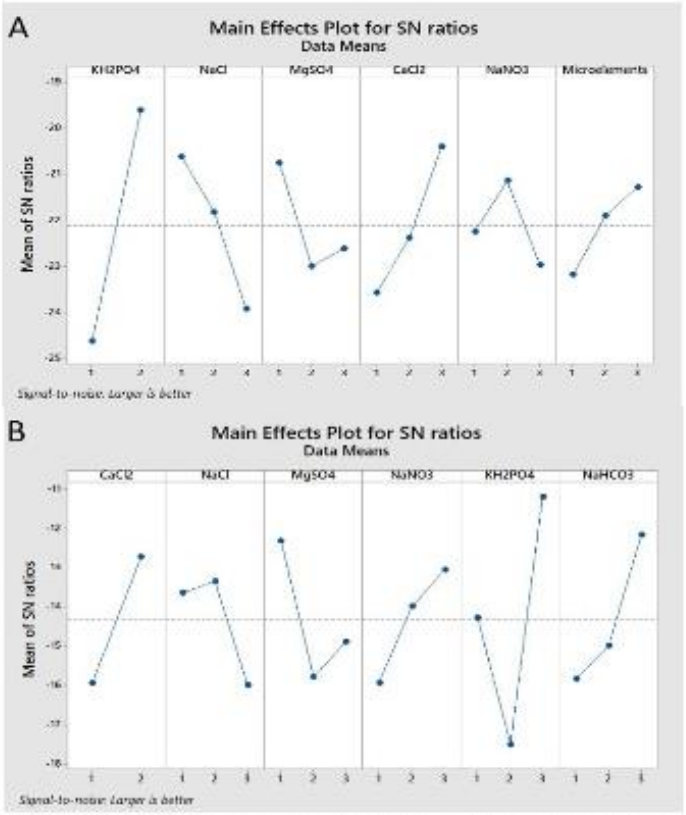

Fig S1. Signal to noise (S/N) ratio obtained from the Taguchi design for media optimization for each strain. (A) *P. sordidum* (KH_2PO_4: 0.07 and 0.7 g L^{-1}; NaCl: 3.0, 15 and 27 g L^{-1}; $MgSO_4$ 4.3, 5.3 and 6.3 g L^{-1}; $CaCl_2$:1.13, 1.5 and 1.8 g L^{-1}; $NaNO_3$:1.0, 1.5 and 1.8 g L^{-1}; microelements: 1, 1.5 and 2 mL) and (B) *P. purpureum* ($CaCl_2$: 1.13 and 1.5 g L^{-1}; NaCl: 15, 27 and 39 g L^{-1}; $MgSO_4$ 5.3, 6.3 and 7.3 g L^{-1}; $NaNO_3$:1.0, 1.5 and 1.8 g L^{-1}; KH_2PO_4: 0.07, 0.5 and 0.9 g L^{-1}; $NaHCO_3$: 0.04, 0.08 and 0.12 g L^{-1}) for the media compounds evaluation, using the L_{18} orthogonal array and the "larger the better" definition, evaluating three and two levels.

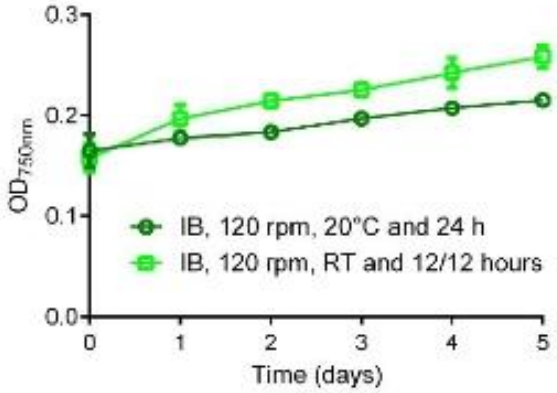

Fig S3. *P. sordidum* growth behavior comparison. The induction mode parameters (24 hours of light and 20°C) were tested against the normal culturing parameters (12 hours of light and RT) using the same agitation (120 rpm) and culture media (IB).

4.4. Rheological characterization of *Porphyridium sordidum* and *Porphyridium purpureum* exopolysaccharides

P. sordidum and *P. purpureum* EPS rheological properties were evaluated in this study. The importance of this research lies in fact that putative application can be postulated after identifying EPSs rheological properties.

The most adequate media, IB for *P. sordidum* and ASW for *P. purpureum*, determined in Result, 4.3, Fig. 3 and 4.5 L cultivation system (Result 4.4, Fig. S 1) were used to produce sufficient EPS for this study.

After the cultivation-stage microalgae EPS were harvested using the cross-flow filtration strategy (Result 4.3, 2.2). This harvesting strategy was selected because it assured the elimination of salt traces from EPS, obtaining a highly pure sample.

1 % *P. sordidum* and *P. purpureum* EPSs solutions were prepared for rheological evaluation. Five moduli: flow curve, amplitude-, frequency-, temperature- sweeps and thixotropy were tested. In addition, the effect of monovalent (NaCl) and divalent ($CaCl_2$) salts were also evaluated on the rheological behavior. Furthermore, the effects of high salinity media (HSM) were also investigated on microalgae EPS rheological behavior.

In this study a remarkable stability under the effect of salts and temperature were observed on the rheological properties displayed by *P. sordidum* and *P. purpureum* EPSs.

The author conducted the investigation, developed the methodology and analyzed the data. Moritz Gansbiller developed the methodology and analyzed the data. Broder Rühmann analyzed the data and supervised the project. Jochen Schmid analyzed the data and supervised the project. Volker Sieber supervised the project. All authors contributed in the manuscript preparation for publication.

Rheological characterization of *Porphyridium sordidum* and *Porphyridium purpureum* exopolysaccharides

Edilberto Vicente Medina-Cabrera, Moritz Gansbiller, Broder Rühmann, Jochen Schmid and Volker Sieber

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Rheological characterization of *Porphyridium sordidum* and *Porphyridium purpureum* exopolysaccharides

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ABSTRACT

Porphyridium exopolysaccharides (EPSs), which contain sulfate and methyl groups, have a similar potential for use in multiple industrial applications as macroalgae counterparts but lack detailed characterization. For this reason, we produced 0.21 g L^{-1} of *P. sordidum* EPS and 0.17 g L^{-1} *P. purpureum* EPS, followed by a thorough rheological characterization in respect to their differences in monomer composition, sulfate concentrations and methyl patterns. Furthermore, the effect of NaCl and $CaCl_2$ was evaluated, and the effect of high salinity media on the rheological properties of the biopolymers was analyzed. Both *Porphyridium* EPSs show a remarkable stability at high temperature and under the effect of mono- and divalent cations, and high salinity cultivation medium, which was evidenced by the rheological properties of the EPS. This feature is not displayed by many carbohydrate polymers, making it possible to enrich current applications in which EPS are used.

1. Introduction

In recent decades, the importance of bio-based polymers, such as exopolysaccharides (EPSs) and sulfated carbohydrate polymers, has been increasing because of their use in industries, including textile, cosmetics, food, agriculture, and medical. Xanthan, levan, scleroglucan, pullulan, carrageenan, and alginates are several of the commercial carbohydrate polymers that are mainly synthesized by bacteria, fungi, and macroalgae (Ates, 2015; Freitas, Alves, & Reis, 2011; Mahapatra & Banerjee, 2013; Necas & Bartosikova, 2013; Nwodo, Green, & Okoh, 2012; Schmid, Meyer, & Sieber, 2011; Sutherland, 1991). By contrast, carbohydrate polymers obtained from microalgae are least studied. In this regard, there are three main types of carbohydrate polymers produced by microalgae: i) intracellular polysaccharides, which are stored in the cytoplasm; ii) structural polysaccharides, which are attached to the cell wall; and iii) EPSs, which are excreted to the cell surroundings (Bernaerts et al., 2018; Frei & Preston, 1961; Raposo, de Morais, & Bernardo de Morais, 2013). Besides sulfate residues, microalgae carbohydrate polymers contain other unusual decorations such as methyl groups, which are uncommon in polymers obtained from other microorganisms (Delattre, Pierre, Laroche, & Michaud, 2016; Geresh, Lupescu, & Arad, 1992; Staats, De Winder, Stal, & Mur, 1999).

Presently, carrageenan polymers, are the most prominent sulfated carbohydrate polymers used in industry because of their thermoreversible, thixotropic, and gelling properties (Hotchkiss, Brooks, Campbell, Philp, & Trius, 2016; Necas & Bartosikova, 2013; Saha & Bhattacharya, 2010; Therkelsen, 1993). Carrageenan polymers are industrially produced from red seaweed biomass that is seasonably farmed and/or collected for carrageenan production. Because of uncontrollable factors in their natural environment, such as fluctuation of water temperature, changes in exposure of microalgae to light, and changes in nutrient availability for growth, each macroalgae batch is slightly different from the previous one, resulting in different rheological properties of the various carrageenan batches (Necas & Bartosikova, 2013; Simpson & Shacklock, 1979; Therkelsen, 1993). To address this problem, seaweed can be cultivated in controlled environments to produce more homogenous carrageenan batches. However, seaweed cultivation is expensive, and thus, the production of carrageenan by a highly

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controlled procedure is economically challenging (Therkelsen, 1993). By contrast, bacterial and fungal polysaccharides can be produced by highly reproducible processes in a bioreactor (Farina, Sineriz, Molina, & Perotti, 1998; Rütering, Schmid, Rühmann, Schilling, & Sieber, 2016). Although sulfated polysaccharides are mainly produced by marine organisms, with no equivalents in bacteria and fungi, microalgae can be used for the production of sulfated EPS in a controlled cultivation process (Raposo et al., 2013).

The production of carbohydrate polymers from microalgae has been explored for decades, besides the established extraction of carrageenan from macroalgae; however, further investigation is needed to clearly identify the potential and characteristics of microalgae polymers (e.g., their rheological properties). The rheological properties of two cyanobacteria EPS have been partially described, *Anabaena* sp. ATCC 33047 and *Nostoc calcicola* (Bhatnagar, Pareek, Bhatnagar, & Ganguly, 2014; Moreno et al., 2000). *Porphyridium* sp. (Rhodophyta) have been described as source for sulfated carbohydrate polymers (Arad, Adda, & Cohen, 1985; Arad, Friedman, & Rotem, 1988; Geresh & Dawadi, 2000; Li, Shabtai, & Arad, 2000; Singh, Arad, & Richmond, 2000). Its monomer composition was elucidated to consist of xylose, galactose, glucose, glucuronic acid and methylated carbohydrates as well as sulphate decorations (Geresh & Arad, 1991; Lupescu, Arad, Geresh, Bernstein, & Glaser, 1991). Those monomers are possibly grouped in a specific sequence in repeating units, which is also underpinned by the molar ratios of the monomers (Gloaguen et al., 2004). (Gloaguen et al., 2004). Moreover, *Porphyridium* EPS structures likely consist of a linear carbohydrate backbone to which branching carbohydrate chains are connected (Geresh et al., 2009). Moreover, via X-ray analysis it has been postulated that *Porphyridium* EPS has a helical structure (Eteshola, Karpasas, Arad, & Gottlieb, 1998). This data allows to postulate that *Porphyridium* sp. EPS have a branched structure, which forms helices with repeating units. Also, initial rheological evaluation has been done for *Porphyridium* sp. and *Porphyridium cruentum* EPS (Eteshola et al., 1998; Geresh, Adin, Yarmolinsky, & Karpasas, 2002; Patel et al., 2013). In addition, flow curves, frequency-, and temperature sweeps of different fractions from the cell wall of *Porphyridium cruentum* were investigated (Bernaerts et al., 2018). However, further investigation on microalgae EPS is of high priority as they are similar to sulfated carbohydrate polymers such as carrageenan-derived molecules, glycosaminoglycan (GAG) and chondroitin, which are used in different areas, and especially in the medical field for antiviral, anti-inflammatory, antioxidant, anticancer, bio-lubricant. immunomodulatory hypocholesterolemic and nutraceutical purposes (Arad et al., 2006; Dvir et al., 2000; Dvir, Stark, Chayoth, Madar, & Arad, 2009; Harden, Falshaw, Carnachan, Kern, & Prichard, 2009; Huheihel, Ishanu, Tal, & Arad, 2002; Nakano, Nakano, & Sim, 1998; Schiraldi, Cimini, & De Rosa, 2010; Tannin-Spitz, Bergman, van-Moppes, Grossman, & Arad, 2005; Wijesekara, Pangestuti, & Kim, 2011).

In this study, EPS from Rhodophyta strains *Porphyridium sordidum* and *Porphyridium purpureum* were produced under optimized conditions, and their EPS were harvested, and dried. A thorough rheological characterization was conducted, including flow curve, amplitude-, frequency-, temperature sweeps and thixotropy. Salts are reported to alter carbohydrate polymers rheological properties and possibly influencing putative application (Rochefort & Middleman, 1987; Xu, Dong, Gong, Sun, & Li, 2015, 2019). Because, the effect of salts on the rheological properties of microalgae EPS remains unclear; in this investigation the effect of salts was tested on *P. sordidum* and *P. purpureum* EPSs. On this subject, three samples were prepared: one was dissolved in ultrapure water, one with additional monovalent salt (NaCl), and one with additional divalent salt ($CaCl_2$). Furthermore, the effect of high salinity media (HSM) on the rheological properties of both *Porphyridium* EPS was evaluated. It was hypothesized that because both *Porphyridium* strains show optimal growth in HSM which contains several salts, the rheological properties of the EPS produced by those microalgae will be minimally affected by mono- and divalent ions.

2. Material and methods

2.1. Materials

Ultrapure water was obtained with the Milli Q-POP system (Merck). The purchased media compounds used in this investigation are as follows: sodium chloride (Roth), magnesium chloride hexahydrate (Roth), calcium chloride dihydrate (Roth), sodium nitrate (Merck), sodium hydrogen carbonate (Sigma-Aldrich), magnesium sulfate heptahydrate (Roth), monopotassium phosphate (Roth), ethylene diamine tetraacetic acid disodium salt (Roth), iron (II) sulfate (Merck), zinc sulfate heptahydrate (Merck), manganese chloride tetrahydrate (Merck), copper sulfate pentahydrate (Fluka), cobalt chloride (Alfa Aesar), sodium molybdate dihydrate (Sigma), and boric acid (Sigma).

2.2. Strains and cultivation conditions

Porphyridium sordidum (SAG 114.7) and *Porphyridium purpureum* (SAG 1380-1a) were used in this investigation. Microalgae were cultivated in 5 L systems; the complete set-up and specifications are shown in the supplemental material Fig. S 1. *P. sordidum* was cultivated in improved medium B (IB) and *P. purpureum* in artificial sea water (ASW) medium, as these are reported to be the most suitable for each strain (Medina-Cabrera, Rühmann, Schmid, & Sieber, 2020). Daily samples were taken to evaluate the growth using 200 µL of cultivation volume (technical quadruplicate) in a 96-well flat-bottom plate (Greiner Bio-one) in a Varioskan™ microplate reader at OD_{750nm} for 30 days.

2.3. EPS harvesting

At the end of the cultivation process, the broth was centrifuged at 4000 ×*g* for 30 min at 20 °C to separate the biomass from the cultivation media. The cell-free supernatant was 1:1 diluted with ultrapure water and concentrated with an ultrafiltration cassette (Hydrosart®, Sartorius AG) with a molecular weight cutoff of 100 kDa. The concentrated supernatant was precipitated with two volumes of 2-propanol and dried at 45 °C for 3 days in a vacuum oven.

2.4. Sample preparation

Solutions (1 % w/v) of *P. sordidum* and *P. purpureum* EPSs (60 mL) were prepared by stirring at 500 rpm overnight in ultrapure water in 100 mL conical flask. 20 mL of salt free polymers were separated to be used for rheological evaluation. 0.4 mL of 4.3 M solution NaCl and 0.8 mL of 2.1 M $CaCl_2$ were mixed with the 1 % EPS solution reaching a final volume of 20 mL and a final salt concentration of 85 mM. Additionally, microalgae EPS samples were dissolved in their respective cultivation media at a 1 % (w/v) concentration. The flasks were hermetically sealed to avoid water loss during the dissolution process.

2.5. EPS chemical composition

The chemical composition of the EPS produced by the two strains was evaluated using the following techniques. The HT-PMP analysis was used to determine the carbohydrate monomer composition (Rühmann, Schmid, & Sieber, 2014). A turbidimetry method was used to determine the EPS sulfate concentration (Medina-Cabrera, Rühmann, Schmid, & Sieber, 2020). Protein contamination of the EPS was quantified using the Roti®-Quant (Carl Roth) (Medina-Cabrera et al., 2020a).

2.6. Rheological evaluation

Rheological measurements were conducted with an air-bearing MCR300 stress-controlled rheometer (Anton Paar, Germany), using a cone-and-plate geometry (50 mm diameter, 1° cone angle, 0.05 mm gap). The data were collected and analyzed with Rheoplus V.3.61

software (Anton Paar, Germany). The samples (830 μL) were equilibrated at a fixed temperature of 20 ± 0.1 °C for 5 min before measuring.

2.6.1. Flow curves

Viscosity curves were analyzed during a logarithmic shear rate ramp ($\dot{\gamma}$, 0.001–1000 s^{-1}) with 25 points with a gradually decreasing measurement time from 100 to 5 s per data point.

2.6.2. Amplitude sweeps

Amplitude sweeps were measured logarithmically by an increased shear stress amplitude from 10^{-1} to 10^{2}Pa at a fixed frequency of 1 Hz. Amplitude sweeps were used to determine the linear viscoelastic (LVE) region.

2.6.3. Frequency sweeps

Frequency sweeps were measured at constant shear stress amplitude within the LVE from 10^{-2} to 10^{1}Hz.

2.6.4. Temperature sweeps

Temperature sweeps were conducted within the LVE region at a frequency of 1 Hz. A discrete temperature ramp from 20 °C to 75 °C at a heating ramp of 4 °C min^{-1}. To prevent evaporation, the edge of the sample was covered with low viscosity paraffin oil (Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

2.6.5. Thixotropy

Thixotropic behavior of the samples was evaluated in a three-stage oscillatory shear test. During the first stage, the sample was measured within the previously determined LVE region followed by a high oscillatory shear of 10^{2}Pa. The recovery of the structure was then measured over 10 min within the LVE region.

3. Results

3.1. Evaluation of the production and chemical characterization of *P. sordidum* and *P. purpureum* EPS

P. sordidum and *P. purpureum* were described as EPS producers by previous investigations (Medina-Cabrera et al., 2020a; Nikolova et al., 2019; Roussel et al., 2015). To obtain sufficient amounts of EPSs, the strains were successfully cultivated in 4.5 L of working volume using IB medium for *P. sordidum* and ASW medium for *P. purpureum*, as these have been reported to be the best for growth and EPS production (Medina-Cabrera et al., 2020b). Both *Porphyridium* strains successfully grew in the system developed for this evaluation (supplemental material Figs. S 1 and S 2). In this study, soluble EPS was harvested from the cell-free supernatants after the cultivation process to obtain 0.21 and 0.17 g L^{-1} EPS from *P. sordidum* and *P. purpureum* cultures, respectively. The chemical composition for both EPSs were elucidated in a previous investigations (Medina-Cabrera et al., 2020a). In summary, the carbohydrate fraction of both *Porphyridium* EPSs was quantified, with xylose being the most abundant monomer, followed by galactose, glucose and glucuronic acid (in that order). Moreover, uncommon monomeric carbohydrates were identified in both *Porphyridium* EPSs. A methyl pentose and methyl hexose were detected in *P. sordidum* EPS while *P. purpureum* EPS contained a methyl uronic acid connected to a hexose. In addition, the sulfate content was higher for the EPS of *P. sordidum* than the EPS of *P. purpureum*. The monomer compositions for both *Porphyridium* EPSs were consistent with the ones previously reported (Medina-Cabrera et al., 2020a, 2020b). The monomer compositions of both EPS are summarized in Table 1.

3.2. General rheological behavior

Flow curves were analyzed by measurement of the viscosity (η) over an increasing shear rate ($\dot{\gamma}$). The shear thinning or pseudoplastic behavior of *P. sordidum* and *P. purpureum* EPSs is similar to the ones obtained from *Porphyridium* sp. and *P. cruentum* (Bernaerts et al., 2018; Geresh et al., 2002; Geresh & Arad, 1991; Patel et al., 2013). Within the strictly exponential relation between shear rate and viscosity, the power law ($\eta = K\dot{\gamma}^{n-1}$) was applied to each viscosity curve. The obtained values of the consistency index (K) and flow index (n) are shown in Table 2.

Table 1
Chemical composition of the quantifiable section of *P. sordidum* EPS and *P. purpureum* EPS. The carbohydrate section was measured by HT-PMP, sulfate by turbidimetry, and protein by Bradford assay. All measurements were in triplicates ($n = 3$).

Compounds		*P. sordidum* EPS	*P. purpureum* EPS
% EPS Quantifiable molecules	Xylose	29 ± 0.6	35 ± 0.2
	Galactose	16 ± 0.1	20 ± 0.2
	Glucose	9.5 ± 0.2	12 ± 0.1
	Glucuronic acid	3.3 ± 0.04	2.7 ± 0.2
	Sulfate	39 ± 0.4	27 ± 2.8
	Protein*	2.5 ± 0.5	1.6 ± 0.4
	Recovery	41 ± 0.7	43 ± 2.6
EPS Un-quantifiable substituents	Uronic acid hexose dimer	+	+
	Methyl pentose	+	–
	Methyl hexose	+	–
	Methyl uronic acid hexose dimer	–	+

* Protein detected was considered as contamination.

P. sordidum EPS (Fig. 1 A) and *P. purpureum* EPS (Fig. 1 B) showed shear thinning behavior and lower exponents n, indicating a higher shear rate dependency of the viscosities, whereas the EPS from *P. purpureum* had a higher dependency than that from *P. sordidum*. Consistency indices also indicate an overall higher viscosity of EPS from *P. purpureum* than that from *P. sordidum*. A similar thinning behavior was reported in an preliminary rheological evaluation of *P. sordidum* or *P. purpureum* EPSs (Medina-Cabrera et al., 2020a). The changes in consistency index (K) and flow index (n) between the studies could derive from the different cultivation and harvesting strategies used to obtain the EPS impacting their purity (recovery) of the quantifiable fraction. However, from both studies it can be extracted that *P. purpureum* EPS free of salts showed a higher viscosity, consistency index (K) and flow index (n) throughout the entire shear rate range in comparison to *P. sordidum* EPS.

The addition of NaCl resulted in no significant change of viscosity for either *P. sordidum* or *P. purpureum* EPS, as seen by the consistency index (K) in the power law model (Table 2). The addition of $CaCl_2$ and the IB medium (HSM) led to a decrease of overall viscosity, as indicated by a decreased consistency index K of the *P. sordidum* EPS samples. A similar behavior was observed when $CaCl_2$ and ASW medium *P. purpureum* EPS was added, decreasing the shear rate dependency of the viscosity.

3.3. Viscoelasticity

The LVE for all polymers with and without the influence of NaCl,

Table 2
Consistency parameter K and exponent n of the power law fit calculated using technical triplicates (n = 3). The data were fitted in the strictly exponential region between 0.016 and 383 s^{-1}.

1 % carbohydrate polymer solutions	K [Pa s]	n
P. sordidum EPS	4.98 ± 0.49	0.118 ± 0.001
P. sordidum EPS +85 mM NaCl	4.30 ± 0.11	0.115 ± 0.001
P. sordidum EPS +85 mM $CaCl_2$	3.82 ± 0.17	0.118 ± 0.002
P. sordidum EPS + IB medium	3.18 ± 0.02	0.153 ± 0.001
P. purpureum EPS	7.42 ± 0.40	0.097 ± 0.013
P. purpureum EPS +85 mM NaCl	7.86 ± 0.27	0.099 ± 0.011
P. purpureum EPS +85 mM $CaCl_2$	6.96 ± 0.12	0.097 ± 0.006
P. purpureum EPS + ASW medium	4.72 ± 0.10	0.112 ± 0.004

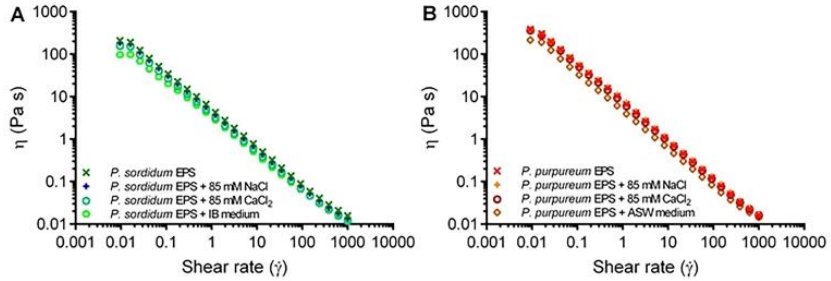

Fig. 1. Flow curves of 1 % EPS solutions of A) *P. sordidum* EPS, B) *P. purpureum* EPS. Samples were tested without salts (x), with additional NaCl (+), with additional $CaCl_2$ (○), and high salinity media (◇), improved medium B (IB) for *P. sordidum* and artificial sea water media (ASW) for *P. purpureum*. Plots are based on the average results ($n = 3$) with standard deviation.

$CaCl_2$, or cultivation media was determined by the amplitude sweeps. These amplitude sweeps also describe the basic viscoelastic properties, such as gel strength (G′ value within the LVE), and gel character (loss factor tanδ) as well as yield point, the shear stress where the samples start to show predominant liquid character (G′ < G″). The respective values are given in Table 3. The amplitude sweeps are presented for *P. sordidum* EPS in Fig. 2A and B and for *P. purpureum* EPS in Fig. 2C and D.

The EPSs from *P. purpureum* and *P. sordidum* displayed similar characteristics, although the EPS from *P. purpureum* exhibited a higher gel strength with a lower loss factor and higher yield stress than that from *P. sordidum*. Furthermore, the addition of individual mono-, divalent salts and HSM only marginally decreased the LVE, reaffirming the salt stability shown in the flow curves. The amplitude sweep allowed to set the shear stress (1 Pa) for the following oscillatory tests, as they should be conducted in the LVE region.

3.4. Gel character

Frequency sweeps were conducted by changing the frequency at a constant shear stress (Pa) within the LVE region. The two biopolymers showed predominant elastic behavior (G′ > G″) between 0.01 and 10 Hz, with no crossover point (G′ = G″) throughout the investigated frequency range. A similar gel behavior has been reported in *Porphyridium* sp. and *P. cruentum* EPSs (Bernaerts et al., 2018; Geresh et al., 2002). The frequency sweeps of *P. sordidum* are presented in Fig. 3A and B and for *P. purpureum* in Fig. 3C and D.

Furthermore, the addition of individual salts (mono- and divalent) to either microalgae EPSs did not show an apparent effect on the frequency dependency of G′ and G″. However, when the EPSs were dissolved in their respective cultivation media, both G′ and G″ decreased in comparison with those of other samples, and although the overall frequency dependency of G′ was unaltered, the G″ dependency decreased more at lower frequencies. The difference of G″ values between the EPS dissolved in HSM and salt-free media was more noticeable at low frequencies but reduced with increasing frequency to an almost negligible difference with the *P. sordidum* EPS. The *P. purpureum* EPS behavior changed throughout the entire frequency range under the influence of the ASW medium compared with that of the other samples, possibly because of the high salt concentration compared with the other treatments.

Table 3
Plateau values of storage (G′) and loss (G″) moduli within the LVE as well as yield stress, where G′ ≤ G″. G′ and G″ values are averages of 15 data points of the plateau values within the LVE.

1 % of carbohydrate polymer samples	G′ [Pa]	G″ [Pa]	tanδ	Yield stress [Pa]
P. sordidum EPS	11	2.8	0.26	8.1
P. sordidum EPS +85 mM NaCl	10	2.6	0.26	8.1
P. sordidum EPS +85 mM $CaCl_2$	9.8	2.5	0.25	7.2
P. sordidum EPS + IB medium	7.0	2.2	0.32	6.4
P. purpureum EPS	22	4.4	0.24	13
P. purpureum EPS +85 mM NaCl	21	4.3	0.21	13
P. purpureum EPS +85 mM $CaCl_2$	18	4.3	0.24	12
P. purpureum EPS + ASW medium	12	2.8	0.23	8.2

3.5. Temperature dependence

Temperature sweeps were conducted by increasing the temperature from 20 °C to 75 °C and subsequent cooling down to 20 °C at a fixed shear stress and frequency (1 Hz) within the LVE region. The rheological properties of *P. sordidum* as presented in Fig. 4A and B and in Fig. 4C and D for *P. purpureum* were highly stable across the investigated temperature range as it was also observed in the *P. cruentum* cell wall carbohydrate polysaccharides even reaching stability at 80 °C (Bernaerts et al., 2018).

Furthermore, the microalgae EPS were unaffected by the addition of individual salts and were only slightly affected by the addition of HSM, with a temperature stability superior to the other investigated polymers. Similar results to the temperature sweeps have also been observed in different fractions obtained from the *P. cruentum* cell wall without the effect of salts.

3.6. Thixotropy

In the thixotropy studies, G′ and G″ recovery after high shear stress (10^2 Pa) were evaluated. The *P. sordidum* EPS in Fig. 5A and B had a generally faster recovery rate for G′ and G″ compared with that of *P. purpureum* EPS G′ and G″ observed in Fig. 5C and D; additionally, an overshoot of G″ was observed with the *P. sordidum* EPS samples.

The structural recovery is given as percent relative to the plateau values within the first low shear region. Structural recovery of *P. sordidum* EPS and *P. purpureum* EPS was similar in all samples with or without, salts, with a total recovery close to 96 % after 8 min. Both microalgae EPSs had a slower G′ recovery compared with that of the G″, and a short but distinct overshoot of G″ occurred in the initial stage of the recovery. The EPSs recovery rates were not affected by the addition of salts, as they behave alike the free salt samples.

4. Discussion

To improve the understanding of the properties of the EPS from *P. sordidum and P. purpureum*, information related to their composition and structural conformation are required. The chemical composition of both EPSs has been previously described and corroborated (Medina-Cabrera et al., 2020a, 2020b). The limited amount of EPS produced by microalgae normally limits the range of measurements especially of various concentrations, so the first goal was the selection of an adequate

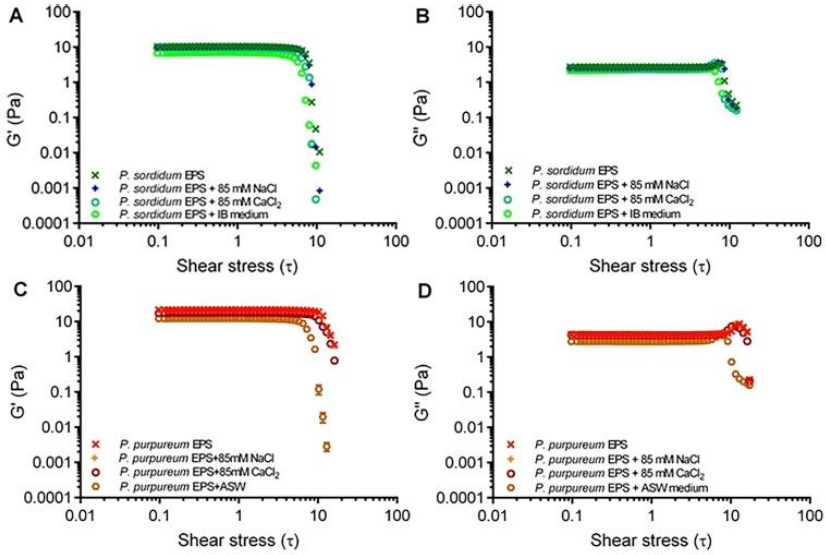

Fig. 2. Amplitude sweeps of A) *P. sordidum* EPS G′, B) *P. sordidum* EPS G″, C) *P. purpureum* EPS G′, D) *P. purpureum* EPS G″. Samples were tested without salts (x), with additional NaCl (+), with additional $CaCl_2$ (○), and high salinity media (◇), improved medium B (IB) for *P. sordidum* and artificial sea water media (ASW) for *P. purpureum*. Plots are based on the average results ($n = 3$) with standard deviation.

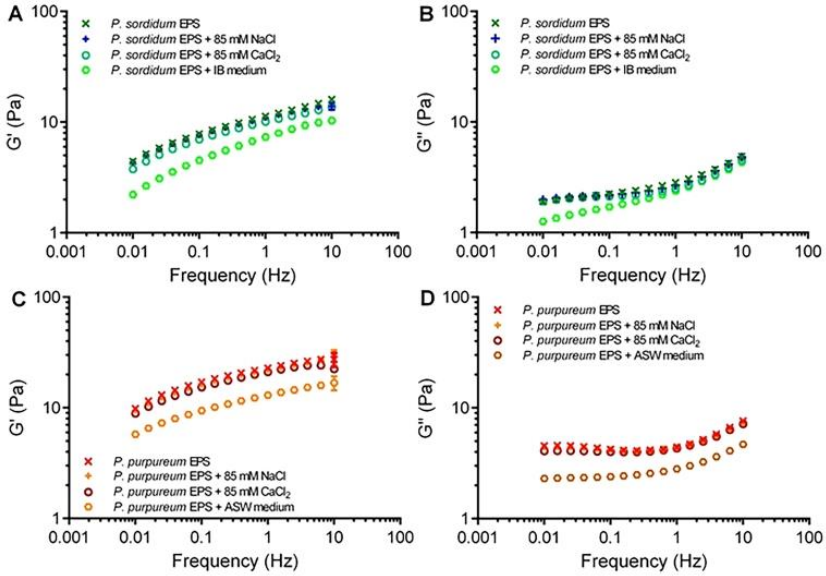

Fig. 3. Frequency sweep of 1 % solutions of A) *P. sordidum* EPS G′, B) *P. sordidum* EPS G″, C) *P. purpureum* EPS G′, D) *P. purpureum* EPS G″. Samples were tested without salts (x), with additional NaCl (+), with additional $CaCl_2$ (○), and high salinity media (◇), improved medium B (IB) for *P. sordidum* and artificial sea water media (ASW) for *P. purpureum*. Plots are based on the average results ($n = 3$) with standard deviation.

EPS concentration for the rheological characterization. In this regard, 1 % EPS solutions were used in this investigation as it was previously identified that this concentration allows to adequately determine small differences of the rheological behavior of the two individual EPS in respect to minor differences in their composition. Moreover, this EPS concentration allows a comparison of the results with a preliminary rheological study (Medina-Cabrera et al., 2020a).

Porphyridium carbohydrate polymers were rheologically investigated but the effect of salts was never tested. The addition of salts or surfactants, which may contain salts, to biopolymers is reported to alter the rheological behavior. For this, the salt effects on the rheological behavior of both *Porphyridium* EPSs was investigated (Ayyash et al., 2020; Calvo, Ferrer, Martínez-Checa, Béjar, & Quesada, 1995; Gansbiller, Schmid, & Sieber, 2019; Gansbiller, Schmid, & Sieber, 2020; Rütering et al., 2018). *Porphyridium* strains are adapted to HSM, so it was hypothesized that EPS produced by those strains are stable to the high

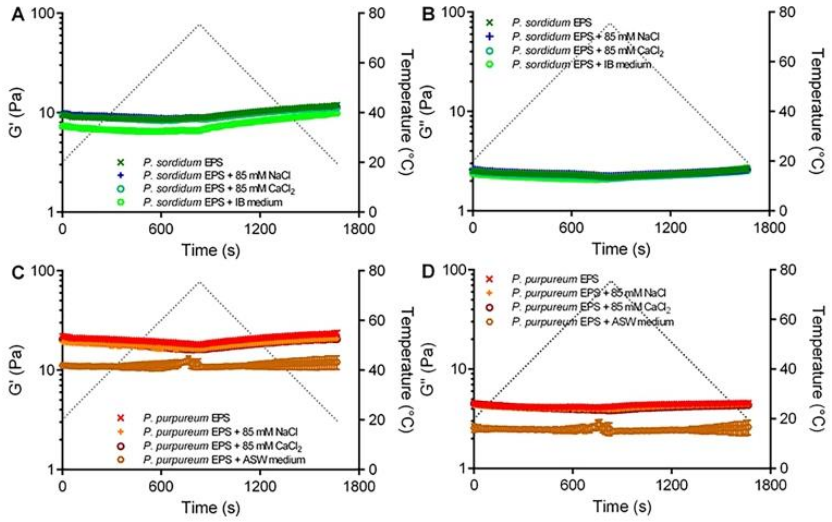

Fig. 4. Temperature sweep of 1 % solutions of A) *P. sordidum* EPS G′, B) *P. sordidum* EPS G″, C) *P. purpureum* EPS G′, D) *P. purpureum* EPS G. Samples were tested without salts (x), with additional NaCl (+), with additional $CaCl_2$ (○), and high salinity media (◇), improved medium B (IB) for *P. sordidum* and artificial sea water media (ASW) for *P. purpureum*. Plots are based on the average results ($n = 3$) with standard deviation.

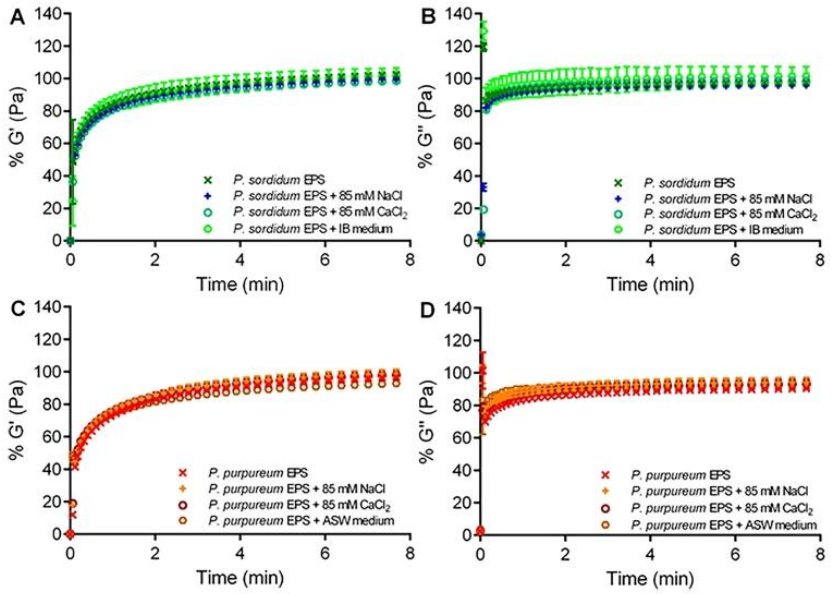

Fig. 5. Thixotropy (recovery rate) of 1 % solutions of A) *P. sordidum* EPS G′, B) *P. sordidum* EPS G″, C) *P. purpureum* EPS G′, D) *P. purpureum* EPS G″. Samples were tested without salts (x), NaCl (+), $CaCl_2$ (○), and high salinity media (◇), improved medium B (IB) for *P. sordidum* and artificial sea water media (ASW) for *P. purpureum*. Plots are based on the average results ($n = 3$) with standard deviation.

salinity environments (Medina-Cabrera et al., 2020a, 2020b; Nikolova et al., 2019; Roussel et al., 2015). The salt concentrations used in this study were selected as they are reported to be used in research on EPS applications in food, cosmetic and pharmaceutical fields (Gansbiller et al., 2019; Kiani, Mousavi, Razavi, & Morris, 2010).

A comparison of flow curves demonstrated that the shear thinning or pseudoplastic behavior of both microalgae EPSs. This behavior can be explained since viscosity decreases whereas shear rate increases; during this process, the rigid rods, which form with dimerization into double helices, are organized and subsequently decrease the intramolecular interactions, thus decreasing the viscosity. It was described that *P. purpureum* EPS showed higher viscosity (Fig. 1), storage and loss modulus (Fig. 2) than *P. sordidum* EPS. This behavior is more accentuated in this investigation compared to what was previously reported for the same EPSs (Medina-Cabrera et al., 2020a). The first explanation could be the changes in the recovery and purity of both EPSs (as indicated by the quantifiable fraction) resulting from the improved bioprocess used for obtaining each batch. Nevertheless, a better

explanation could be distilled from the rheological evaluation. *P. purpureum* EPS showed higher values of viscosity, storage and loss moduli (Tables 2 and 3) than *P. sordidum* EPS. The difference in rheological behavior is likely mediated by the carbohydrates distinct derivatization observed for each *Porphyridium* EPS (Table 1). Firstly, *P. sordidum* EPS contained higher sulfate content compared to *P. purpureum* EPS. Secondly, while both *Porphyridium* EPSs contain methyl groups, they are apparently differently attached in each EPS. It has been reported that methyl groups can alter the rheological behavior in cellulose polymers (Akinosho, Hawkins, & Wicker, 2013; Gallego, Arteaga, Valencia, & Franco, 2013). In this sense, the particular methylation pattern displayed by each EPS, in *P. sordidum* a methyl pentose and methyl hexose and in *P, purpureum* a methyl uronic acid could also mediate the different rheological response between both *Porphyridium* EPSs. The higher sulfate content and specific methyl pattern in *P. sordidum* EPS generated lower viscosity, storage and loss moduli in all rheological evaluation compared to *P. purpureum* EPS (Figs. 1–5). Moreover, the individual mono- and divalent salt mildly effect the rheological behavior. Contrary, HSM decreases more noticeably their viscosity, storage and loss moduli when they were compared after treatments with individual salts. This could be directed by the interaction between the EPS methyl and sulfate groups with the different salts that constitute HSM. The interaction mechanisms of various salts (HSM) remain unknown and need to be clarified in future investigations. Remarking that *P. purpureum* EPS showed a more pronounced change than *P. sordidum* EPS under the influence of HSM because of the cultivation medium tested for each strain. In this sense, ASW medium used for *P. purpureum* cultivation has a larger salt concentration than IB medium used to cultivate *P. sordidum* (Medina-Cabrera et al., 2020b).

This investigation corroborated *Porphyridium* EPS gel-like behavior ($G' > G''$) identified in previous investigation (Eteshola et al., 1998; Geresh et al., 2002). Furthermore, *P. sordidum* and *P. purpureum* showed no crossover point throughout the investigated frequency range. The addition of mono- and divalent salts affected the stability of the EPS. The addition of cultivation media to the microalgae EPS solutions resulted in only minor changes to their viscoelastic properties, with gel-like behavior still displayed, although at a lower gel strength but with unaltered frequency dependency. The HSM had a high concentration of NaCl and $CaCl_2$ and included other salts such as $NaNO_3$, KH_2PO_4, $MgSO_4$, $MgCl_2$, $FeSO_4$, and $EDTANa_2$, as well as microelements, which therefore could interact with the EPS. These results demonstrate that these microalgae EPS are adapted to HSM.

The polymers obtained from *P. cruentum* soluble cell wall EPS have been reported to be stable at a high temperature of up to 80 °C (Bernaerts et al., 2018; Patel et al., 2013). A similar behavior was observed for both *P. sordidum* and *P. purpureum* EPSs. The addition of NaCl and $CaCl_2$ did not modify the rheological behavior throughout the investigated temperature range. Furthermore, the addition of cultivation media leads to a decrease of both G' and G'', possibly because of interaction between microalgae EPS and other media compounds (mentioned above) including $CaCl_2$; concentration of these compounds was below 85 mM, although the NaCl concentration was 462 mM in the ASW medium.

In previous investigations it was postulated that *Porphyridium* EPS possessed thixotropic properties which was corroborated in this investigation (Eteshola et al., 1998). Moreover, the addition of salts did not affect the thixotropic behavior of either of the microalgae EPS, which had similar rheological characteristics. This result indicates that both EPSs used in this investigation would not suffer alteration in their rheological behavior if strong deformation forces are exerted to them in HSM, which could be an interesting characteristic exploited in novel applications. Although, more investigation is required, because of the behavior displayed in their rheological characteristics and similarities in the monomer composition, it could be inferred that both EPSs have a helical branched structure as they have a similar behavior to carbohydrate polymers with this structural disposition such as xanthan (Arad & Levy-Ontman, 2010; Eteshola et al., 1998; Gansbiller et al., 2019).

It was the first time in which the effect of salts is investigated on microalgae EPS rheological properties; identifying a strong stability in high salinity environments as their gel character, temperature dependence rheological stability and structural recovery rate were not significantly altered by the addition of mono- and divalent salts and mixtures thereof (HSM). The small differences in the rheological behavior that can be seen between the samples with single salts and HSM is possibly due to the latter being prepared separately. Moreover, the effect of individual salts was evaluated by adding stock solutions with high salt concentrations, minimally changing EPS concentration (less than 0.02 % for NaCl and 0.04 % for $CaCl_2$). Nevertheless, the results presented above corroborate the hypothesis, which state that *Porphyridium* EPSs are tolerant to high concentrations of individual salts and salt mixtures contained in the HSM. This is possibly an adaptive mechanism of those microalgae strains to high salinity environments.

Sulfated carbohydrate polymers obtained from *Porphyridium* strains are reported to have antiviral, anti-inflammatory, antioxidant, anticancer, immunomodulatory, hypocholesterolemic and nutraceutical activities as well as applicability as bio-lubricant (Arad et al., 2006; Dvir et al., 2000, 2009; Harden et al., 2009; Huheihel et al., 2002; Matsui, Muizzuddin, Arad, & Marenus, 2003; Nakano et al., 1998; Schiraldi et al., 2010; Tannin-Spitz et al., 2005; Wijesekara et al., 2011) All those applications will benefit from the high salinity stability described in this investigation; because for commercial formulation the addition of salts will not affect the rheological activity as determined in this investigation. Moreover, for *Porphyridium* EPS could be utilized in a variety of environments as high temperature and salt content would not affect their properties. Furthermore, high salinity stability is not a common feature of non-neutral EPS, which set them apart from the rest of those molecules giving a wide potential for novel applications. However, further investigations are required to identify the features that enable the stability of microalgae EPS and how their different chemical compositions and structure affect their rheological properties and validate possible application for these carbohydrate polymers, taking advantages of their peculiarities.

5. Conclusions

P. sordidum EPS and *P. purpureum* EPS (carbohydrate soluble fraction) were successfully produced in a controlled environment using optimal cultivation settings with sufficient yields to enable a rheological evaluation. *Porphyridium* EPSs were stable in their respective mono-, di, HSM even at high temperatures. Small differences in the rheological behavior are possibly mediated by the sulfate and methylation patterns of each EPS. However, more specific interaction between EPS and salts need to be elucidated in future investigations. The rheological evaluation of *P. sordidum* and *P. purpureum* EPSs provides a foundation for further investigation to understand the intrinsic stability of their rheological properties.

Author statement

Edilberto Vicente Medina-Cabrera conducted the investigation. Edilberto Vicente Medina-Cabrera and Moritz Gansbiller developed the methodology. Edilberto Vicente Medina-Cabrera, Moritz Gansbiller, Broder Rühmann, and Jochen Schmid analyzed the data. Broder Rühmann, Jochen Schmid and Volker Sieber supervised the project. Edilberto Vicente Medina-Cabrera, Moritz Gansbiller, Broder Rühmann, Jochen Schmid and Volker Sieber wrote the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.carbpol.2020.117237.

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Rheological characterization of *Porphyridium sordidum* and *Porphyridium purpureum* exopolysaccharides

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Appendix A: Supplementary data

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Fig. S 1. 5 L Cultivation system used in this investigation. The system was run at room temperature (RT), with a white fluorescence LED 10 m (SimpLED, 300 LED, Paulmann). The air inlet was fixed at 2000 mL min^{-1} with a flowmeter (Red-y for gas flow, Switzerland), in 4500 mL of culture volume. The air deliver system was assembled using 0.3–0.5 mm silicon tubing, with one Y polypropylene connection (Bürkle), two T polypropylene connections (Bürkle), and two 0.2 µm PTFE filters Midisart 2000 (Sartorious Stedim).

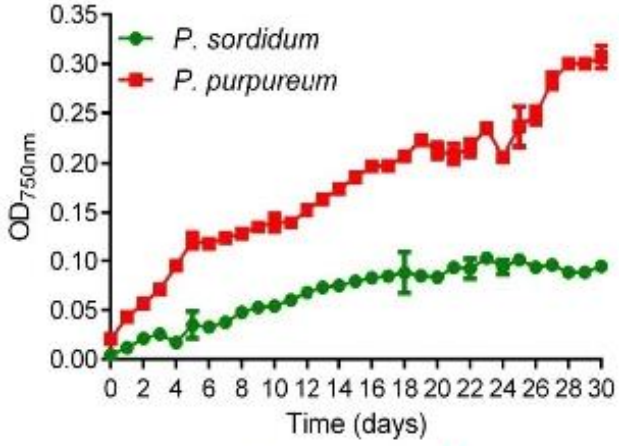

Fig. S 2. Growth behavior of the *P. sordidum* (●) and *P. purpureum* (■) in 5 L vessel (n = 4). The lower response of *P. sordidum* at 750 nm than of *P. purpureum* is clearly seen and is consistent with previously reported results (Medina-Cabrera, Rühmann, Schmid, & Sieber, 2019). The evaluation was conducted in 96-well microtiter plates. The graph is based on the average (n = 4), including the standard deviation as variability.

4.4.1. Effect of extreme pH and temperature on the flow curve of *P. purpureum* EPS

In Result 4.4, Fig. 4 both *Porphyridium* EPSs showed a stable behavior at different temperatures (20 to 75 °C), similarly to results presented for other *Porphyridium* EPSs in previous investigations [391-393]. As well, a remarkable rheological stability at high salinity environments was shown by *P. sordidum* and *P. purpureum* EPSs (Result 4.4, Fig. 1, 2, 3 and 5). In this section a 0.75 % (w/v) solution of *P. purpureum* EPS flow curve were measured at different pH (3.5, 6, 8.3). As well, *P. purpureum* EPS samples were exposed to extreme temperature (121 °C for 20 min/autoclaving process and freezing and unfreezing at –80 °C, overnight). Those treatments were compared to EPS samples that were not exposed to the extreme pHs and temperatures. *P. purpureum* EPS showed a remarkable stability under extreme temperature and pH (Figure 4.3).

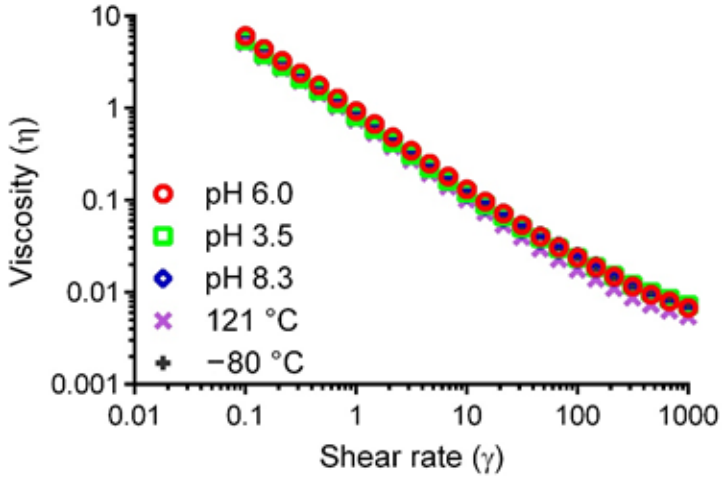

Figure 4.3. Effect of pH and temperature on the *P. purpureum* EPS flow curve. 0.75 % EPS solution were tested. Basic (8.3), acidic (3.4) pH and extreme temperatures (121 and –80 °C) did not affect the rheological behavior of *P. purpureum* EPS. The graph is based on the average including the standard deviation as variability (*n=3*). Because the graph is a in logarithmic scale the standard deviations are imperceptible.

The rheological stability detected in *P. purpureum* EPS suggested that they could be utilized in different environments, as they are not affected by extreme conditions. These preliminary data, must be endorsed with a rigorous rheological characterization of EPS exposed to extreme environments using the procedure described in this section.

Unfortunately, not enough *P. sordidum* EPS was collected to perform the same evaluation (extreme pHs and temperatures). However, similar results are expected, due to the close chemical composition and rheological behavior of both *Porphyridium* EPS [393, 394].

4.5. One-step phycobiliproteins extraction from *P. sordidum* and *P. purpureum* biomass

Microalgae EPSs are purified from the cell free supernatant, leaving microalgae biomass intact. For that reason, microalgae biomass could be used to obtain other valuable biogenic compounds. An important example are phycobiliproteins, which are extracted from various *Porphyridium* biomass, using different harvesting procedures (freeze-drying, thawing and phosphate buffer extraction); noticing that EPS was not produced in those investigations [132-135]. In this regard, a one-step phycobiliproteins extraction approach was developed for *P. sordidum* and *P. purpureum*, exploiting the results observed in the EPS abiotic induction investigation (Results 4.3, 2.5). There, it was observed that different NaCl concentration affected *Porphyridium* cells integrity releasing their phycobiliproteins into the supernatant. *P. purpureum* biomass released phycobiliproteins when it was re-suspended in ultra-pure water, contrary *P. sordidum* phycobiliproteins were released when the biomass was re-suspended in high salinity solutions (NaCl concentration higher than 27 g L^{-1}). These phenomena could be explained as follows: changes in the salinity and such in osmolarity could damage microalgae cell wall releasing phycobiliproteins. Highlighting, that buffers and solutions used for phycobiliproteins extraction contain different NaCl concentration to the ones described for their cultivation medium. *P. purpureum* better grew in high salinity environments while *P. sordidum* in low salinity medium [135, 332]. In this study, *P. purpureum* biomass was re-suspended in a 20 mM phosphate buffer pH 7 (100 mL) and *P. sordidum*, was re-suspended in 50 mL using a 50 g L^{-1} (856 mM) NaCl solution. The extracted phycobiliproteins were quantified using four different equations (Appendix 3); the results are summarized in Table 4.2. It was corroborated the results obtained by other research teams, Phycoerythrin (B-PE) is the most abundant phycobiliprotein in *P. purpureum* biomass, evidenced by the red color of the buffer (Figure 4.4) after the extraction procedure; followed by Allophycocyanin (APC) and Phycocyanin (R-PC) [96, 135]. On the other hand, it was the first time phycobiliprotein were extracted from *P. sordidum* biomass. B-PE was also the most abundant phycobiliprotein followed by APC and R-PC; however, their concentrations were different to the ones obtained from *P. purpureum* (Table 4.2). This result was firstly evidenced by *P. sordidum* extract color, which was reddish-purple (Figure 4.4). In addition, because more biomass was generated in the cultivation process more phycobiliproteins were extracted from *P. purpureum* than from *P. sordidum* biomass.

It is important to highlight although phycobiliproteins were successfully extracted using a single step procedure more investigation is required. Firstly, the purity needs to be further analysis, to determine other unit operations that are needed to generate a final product. As well, the harvesting strategy must be optimized to obtain higher yields with the minimum number of reagents and steps. And also, the

residual biomass composition should be determined to identify if other valuable compounds could be harvested from it.

Table 4.2.
Quantification of phycobiliproteins from the two *Porphyridium* strains obtained from 0.5 L of cultivation medium. The average is presented including the standard deviation as variability (*n=3*).

Phycobiliproteins (mg L^{-1})	*P. sordidum*	*P. purpureum*
Phycocyanin (R-PC)	6.01±0.36	8.28±0.36
Allophycocyanin (APC)	10.44±0.10	13.19±0.43
Phycoerythrin (B-PE)	11.82±0.16	29.19±0.87
Total (T-Ph)	28.27±0.45	50,66±1.62

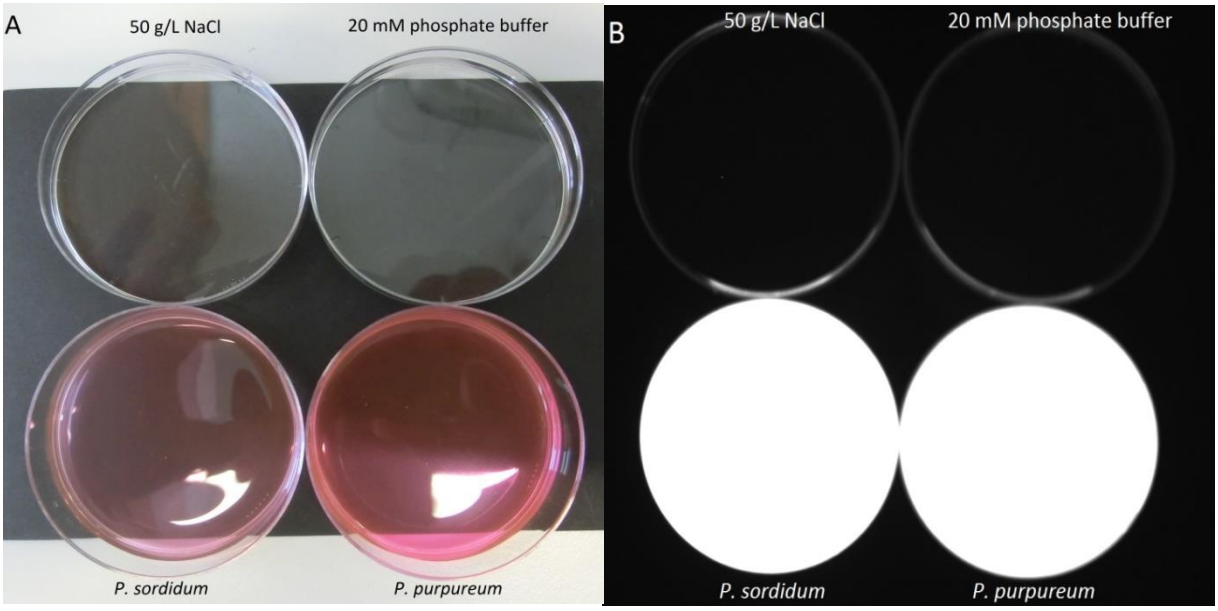

Figure 4.4. Extracted phycobiliproteins from *P. sordidum* (purple) and *P. purpureum* (pink). In the upper section the buffer used for the extraction are shown and in the lower section the crude extracts are presented. A. Buffer and phycobiliproteins solutions under white light. B. Buffer and phycobiliproteins solutions under the UV light. The comparison was done to assure that the fluorescence capacity was mediated by phycobiliproteins and not by the extraction buffers.

Also, an important feature of the extracted phycobiliproteins from macroalgae, cyanobacteria and other rhodophyta strains is the fluorescence they emitted [33, 133, 395]. The fluorescence of phycobiliproteins extracted in this investigation are presented in Figure 4.4.

Furthermore, the crude extract fluorescence spectrum was evaluated (Figure 5.5). In this test the emission and excitation from the phycobiliproteins obtained from *P. sordidum* and *P. purpureum* were investigated.

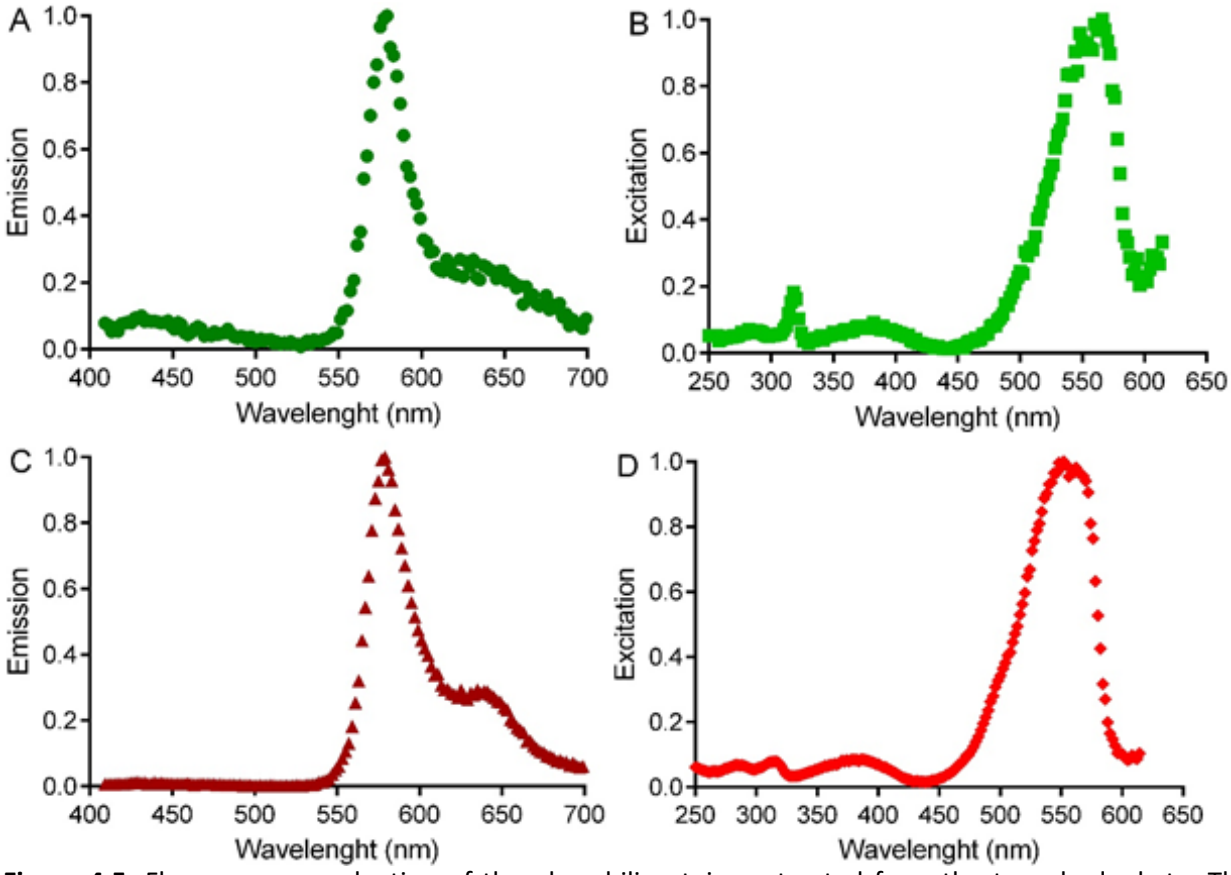

Figure 4.5. Fluorescence evaluation of the phycobiliproteins extracted from the two rhodophyta. The emission was evaluated from 409 to 700 nm and the excitation from 250 to 615 nm.

The fluorescence spectrum from both extracts were similar as the same three phycobiliproteins are part of the mixture. The small difference in evaluation could be mediated by the phycobiliproteins concentrations particular in each rhodophyta strain.

In future investigation phycobiliproteins should be separated to evaluate their individually fluorescence. Those results could give more information to postulate applications in which those proteins could be applied.

5. Discussion

Nowadays, a prominent research topic is the identification of microbes capable of producing EPS. The importance of this topic is rooted in the large number of applications in which EPS are currently used (Introduction, Table 1.3) [175-177]. Many more bacterial and fungal strains are described as EPS producer in comparison to microalgae [176, 274, 396, 397]. For that reason, *P. sordidum* a mentioned EPS producer but not fully characterized, was investigated and compared to *P. purpureum* an already described producer [332].

5.1. Medium characterization as a critical step to investigate microalgae EPS producer

Initially, both rhodophyta strains were investigated using a 0.5 L cylinder system (Result 4.2, Annex 2). ASW was used to cultivate both strains as it was reported to be suitable for various strains of the *Porphyridium* genus; other media were tested but they did not allow microalgae growth [135, 333, 398-404]. The characterization test allows to determine the effect of the initial cultivation conditions on *P. sordidum* and *P. purpureum*. In this sense, *P. purpureum* growth rate (Result 4.2. Fig. 2), nutrient uptake (Results 4.2, Fig. 3) and EPS production (Result 4.2 Table 4) was favored by the ASW medium. Contrary, the results of the characterization suggested that ASW composition need to be modified to better suit *P. sordidum* requirements to improve their growth. Furthermore, the low EPS yields generated by *P. sordidum* in comparison to *P. purpureum* confirmed that the medium should be modified. In this sense, because *P. sordidum* was isolated from a greenhouse sample (non-high salinity environment), the synthetic medium used for its cultivation should emulate those conditions [405]. After the initial characterization a deeper literature review was done, identifying the Pm and Brody Emerson media as possible candidates to cultivate *P. sordidum* [287, 332]. Unfortunately, in this investigation both media did not allow *P. sordidum* growth. However, the characterization evaluation and literature review, suggest that to better fit *P. sordidum* requirements; ASW nutrient composition should be changed to reach similar values to the ones described on the Pm and Brody Emerson media (increasing the nitrate and phosphate compounds concentration, while decreasing the sulfate and chloride ones) [332, 405].

The above discussed results demonstrated that an initial media characterization should always be used as first step in an investigation related to microalgae EPS producers. As it determines the specific requirements that each strain has, setting them apart even from similar strains. Furthermore, the media characterization identifies the most suitable conditions from the ones that should be modified in the subsequent steps to improve microalgae growth and EPS production.

5.2. Evaluation of the optimization procedure used in this investigation

An optimization procedure was designed to modify the cultivation medium and parameters, improving *P. sordidum* growth rate and EPS production (3.4 times). As well, with the same optimization procedure *P. purpureum* EPS production was increased 1.2 times. The details of the optimization procedure used in this investigation are summarized in Table 5.1. As well, other strategies used to increase EPS production in microalgae are presented in the above mentioned table. In this section, the advantages and challenges of the optimization strategies used in this investigation will be discussed.

5.2.1. Advantages

Utilizing microalgae individual features for the optimization process. In the characterization evaluation, it was determined that the cultivation medium needs to be modified to better fit *P. sordidum* needs [393]. Although, ASW was the most suitable medium for *P. purpureum*; changes in its compositions, reported by other research teams, suggested that it could be further improved to enhance growth and EPS production [287, 291, 332, 398]. Now in this investigation the individual needs of each *Porphyridium* strain were exploited to better fit their specific requirements. By that, two different media were developed; IB medium, which enhanced growth and EPS production for *P. sordidum*. The better ratio of the most important compounds of the IB medium: $CaCl_2$, NaCl, $NaNO_3$, $MgSO_4$ and microelements better fit *P. sordidum* growth requirements; as well a suitable salinity and pH allows this strain to produce more EPS (Results 4.3, Fig. 3). Else ways, I3 medium improved EPS production in *P. purpureum;* due to a suitable $NaNO_3$ and KH_2PO_4 ratio, high salinity and pH fix at 6 (Results 4.3, Fig. 3). As well, it was corroborated that ASW is the most suitable medium for *P. purpureum* cultivation [135, 398-400]. Furthermore, the importance of individual requirements was corroborated as different abiotic factors were identified as EPS inducers for each *Porphyridium* strain (Result 4.3, 3.4). In contrast, other optimization approaches used general information from other microalgae rather than the individual requirements of a specific strain to optimize the cultivation media and parameters [287, 290]. In this sense, not fully optimized medium and parameters could be determined if general information is used instead of the specific requirements that each strain has for growth and EPS production.

Utilization of DOE for the optimization process: Plackett-Burman (PB) and Taguchi design were used to develop improved cultivation media, because they need less experiments to perform the optimization in comparison to other methodologies, in which each compound is individually modified while the other compound concentrations are maintained constant [325]. Furthermore, the two DOE used in this investigation allow to test several compounds at the same time, without increasing the difficultness of

the procedure; as it occurs when the surface response methodology (RSM) is used [381, 406]. In this regard, six medium compounds were tested at the same time, which are double the number of compounds optimized with RSM in microalgae systems [407-409]. As well, four abiotic factors were optimized for EPS induction at the same time, which is double the number of abiotic factors optimized at the same in microalgae for EPS production [323, 410, 411]. Furthermore, the Taguchi design allowed to identify new abiotic factors that induce EPS production in microalgae: time that the cultivation broth are exposed to light and shifting pH of the cultivation medium [411].

Two-stage cultivation system. This procedure is used to enhance valuable biogenic compounds in microalgae biomass, such as astaxanthin, lipids and intracellular carbohydrates [412-416]. In a first-stage, an optimal cultivation medium and parameters are used to generate a highly dense cultivation broth. Then, the dense cultivation broth is induced to produce secondary metabolites in a second-stage using predetermine abiotic factors [411-416]. The two-stage cultivation strategy has the advantage to use two optimization strategies (cultivation and abiotic factor optimization) at the same time, exacerbating EPS generation in one bioprocess run. This approach was successfully used to optimize 3.4 times EPS yields in *P. sordidum* and 1.2 times in *P. purpureum*.

5.2.2. Challenges

Cultivation parameter optimization. Microalgae growth and EPS production was enhanced when cultivation parameters such as: pH, temperature, aeration, exposure time, wavelengths and light intensities were optimized (Table 5.1) [170, 290, 325, 410, 417]. Due to the limited number of units available (specifically design or customized for microalgae) for performing replications and/or runs; the effect of different wavelengths was the only parameter optimized for *P. sordidum* and *P. purpureum* (Results 4.3, Fig. 1 and Table S1). However, in order to further increases EPS production other cultivation parameters, need to be tuned as it was done in other optimization strategies [287, 290, 325, 417].

Determination of optimal values. Although, the optimization procedure used in this investigation improve microalgae growth and EPS production; an important challenge was detected. The Taguchi design only allows to identify optimal values from the predetermined fixed levels, as interpolating between them is not possible. So, if the items (medium concentration/abiotic factor) in evaluation are not thoroughly fixed, an accurate optimum would not be determined [381]. This problem was solved in this investigation by reliably setting the evaluation levels with three tools. The PB design, characterization test and literature review were used for two proposes: identify the most important compounds/factors and then determining if they should be decreased, maintained or increased.

Table 5.1.
Optimization strategy utilized for the EPS induction on microalgae. The strains, cultivation the cstrategies used for optimization, EPS yields, cultivation systems and other optimization strategies used to induce EPS production are summarized.

Reference	[325]	[290]	[310]	[287]	[417]	[411]	
Microalgae	*G. impudicum*	*R. violacea*	*C. reinhardtii*	*P. marinum*	*Arthrospira spp.*	*P. sordidum*	*P. purpureum*
				Optimization			
Parameters	Initial pH, light intensity, temperature and time expose to light.	Light intensity, temperature and pH	-	Light intensity, temperature and pH	Initial pH, light intensity, aeration, temperature, medium concentration and cyanobacterial strains.	Wavelength's optimization. Fixed cultivation parameters: temperature, pH and light intensity.	
Procedure	Evaluation of different levels	Evaluation of photosynthetic activity	-	Evaluation of photosynthetic activity	DOE optimization: Taguchi design was used for parameter optimization		
Medium optimization procedure	Tuning macroelements, microelements, hormones, vitamins and CO_2 concentrations	Stochiometric approach	DOE: PB design and surface response methodology	Stoichiometric approach and double concentration of medium compounds	-	DOE: PB design and Taguchi design	
				EPS production (mg L^{-1})			
Initial	18	133	383	1030	-	50	100
Final	134	380	628	4100	154	170	120
Folds	7.7	2.9	1.6	4.0	-	3.4	1.2
Time (days)	19	45	9	40	14	20	
Volume (mL)	2000	720	1000	5000	200	1200	
Cultivation system	Photo bioreactors	Photo bioreactors	Shaking flasks	Photo bioreactors	Cultivation tubes	DASGIP system	
Important features of optimization strategies.	Medium and parameters were individual optimized and then combined	Optimization: Medium (theoretical) parameters (In directed approach)	No growth evaluation was tested in the investigation	Optimization: Medium (theoretical) parameters (In directed approach)	The optimum was not compared to the initial conditions. Phycocyanin was also optimized	Medium and wavelength optimization, were combined with EPS abiotic induction in a two-stage cultivation system to maximize EPS production	

5.3. Comparison of system and strategies used for *Porphyridium* EPS production

Three different cultivation systems and two harvesting strategies were used in this investigation. In this section they are going to be discussed.

5.3.1. Cultivation systems

Two vertical cylindrical bubbling systems (columns), one of 0.5 L (Result 4.2, Annex 2), another of 4.5 L (Result 4.4, Fig. S 1) and 1.2 L stirred tank (DASGIP) system (Appendix 2, Figure A 1) were utilized to cultivate both *Porhyridium* strains.

Of these three systems, the stirred tank (DASGIP) was the most suitable for *P. sordidum* growth as more biomass was generated in it. This was evidenced by the highest OD_{750nm} which was measured at the end of the cultivation process (Table 5.2). Conversely, the stirred tank system generated the lowest EPS yield and productivity in contrast to the other two cultivation system. Although, similar yields and productivities were obtained in both cylinder systems; a close comparison showed a slightly higher EPS productivity in the 0.5 L system because of several reasons: Cultivation parameters are better controlled in the smaller cultivation system. Operational changes occur in the scaling up process as the column diameter increased (more than two times). In addition, the increase in diameter also affects external light availability inside a dense cultivation broth; as light is block by microalgae near the edges of the system vessel preventing them to reach microalgae deep inside the reactor, decreasing growth rate. Additionally, EPS production could be decreased by low light irradiance in a dense cultivation broth; as EPS production is induced by light exposure in *P. sordidum* (Result 4.3, 3.4). The result for *P. sordidum* suggested that cultivation could be performed in a two-stage cultivation system, each stage in a different reactor. In a first-stage performed in a stirred tank system, microalgae growth could be promoted to obtain a dense cultivation broth in a shorter period of time. Then, in a second-stage the dense cultivation broth could be transferred into a cylinder system to induce higher EPS yields. Furthermore, in the second-stage, the abiotic factor induction (Result 4.3, 3.4) could also be used to further increase EPS production on *P. sordidum*. Also, the proposed two-stage cultivation system could prevent the sparger blockage described in the stirred tank system (EPS induction phase) as the dense microalgae cultivation broth will be transferred into a bubbling system [411].

For *P. purpureum,* the most suitable system for growth and EPS generation was the 0.5 L cylinder system, as more microalgae was measured at the end of the cultivation stage (OD_{750nm}) and more EPS was also quantified. A lower growth and EPS yield was observed in 4.5 L cylinder system compared to the 0.5 L for *P. purpureum*. This could be explained similarly to *P. sordidum* (scaling up process, increase in the

diameter and reduce in the light available for microalgae growth inside the cultivation vessel). However, when the EPS volumetric productivity from the three-cultivation systems were compared, similar results were measured. The results suggested that *P. purpureum* could be cultivated in the stirred tank system to decrease the cultivation time (Table 5.2) without decreasing EPS productivity. However, because the two bubbling systems produce higher EPS yields than in the stirred tank and more biomass is produced in the small cylinder system, a more suitable approach could be the following: A rapid growth phase could be performed in the stirred tank system. And then, when the cultivation broth reaches their maximum values, it could be could be transferred into a small bubbling system to promote growth and them applied the abiotic factors induction to enhance EPS production (g L^{-1}).

The discussed two-stage, two-cultivation system for both *Porphyridium* strains could further enhance growth and EPS production. However, further analysis needs to be done as only a single run was performed in some cultivation systems (Table 5.2), which are discussed in this section.

Table 5.2.
Comparisons of the microalgae cultivation systems. The EPS yields (g L^{-1}) volumetric productivity (g L^{-1} day^{-1}) and OD_{750nm} obtained at the end of the cultivation process are summarized in this table. *P. sordidum* was cultivated in IB medium and *P. purpureum* in ASW medium. The cross-flow filtration strategy as reported in Methods 3.6.2 was used for EPS harvesting.

System	0.5 L cylinder [a]			DASGIP system [b]			4.5 L cylinder [c]		
Aeration mechanism	Bubbling			Stirred tank			Bubbling		
Cultivation time (days)	30			20			30		
Strains	EPS, g L^{-1}	Final OD_{750}	Volumetric productivity, g L^{-1} day^{-1}	EPS, g L^{-1}	Final OD_{750}	Volumetric productivity, g L^{-1} day^{-1}	EPS, g L^{-1}	Final OD_{750}	Volumetric productivity, g L^{-1} day^{-1}
P. sordidum	0.21	0.56	0.013	0.17	1.88	0.005	0.21	0.61	0.011
P. purpureum	0.22	3.04	0.002	0.10	2.70	0.002	0.17	2.11	0.003

[a] OD_{750nm} and EPS yields data were obtained from a single run in the 0.5 L system.
[b] OD_{750nm} and EPS yields data obtained from Result 4.3, Table 2.
[c] EPS data was obtained from Result 4.4, 3.1. OD_{750nm} data was measured with a 1 mL plastic cuvette.

5.3.2. Harvesting strategy

This investigation focused in the production of microalgae EPS. Because the majority of harvesting strategy focus in recovering microalgae biomass (Introduction, Table 1.2) and the EPSs are exported outside microalgae cells. This investigation developed two harvesting strategies, which focus in the obtention of cell free supernatant to then harvest microalgae EPS, while the biomass is left as a byproduct.

Both strategies used centrifugation to separate the biomass from the supernatant as it was the most suitable unit operation because of two reasons. No biological or chemical compounds are added to the cultivation medium (flocculation or coagulation) as they could contaminate EPS. And centrifugation reduce the time needed to separate the biomass from the supernatant in contrast to the long time needed if sedimentation is used, which could be increased due to EPS-biomass interaction.

The free microalgae supernatant was treated differently in each strategy. The first strategy (Result 4.2, 2.4) used three steps (precipitation, re-dissolving EPS and high-speed centrifugation) to reduce the working volume and to eliminate impurities from the EPS samples. A second strategy (Result 4.3, 2.2) used a cross-flow filtration step to reduce the working volume and to purify EPS. Remarking that a dilution step was done before the cross-flow filtration step to enhance EPS purification. The effect of the harvesting strategy on the EPS composition is summarized in Table 5.3.

Table 5.3.
Comparison of the EPS mass percentage purified with the two harvesting strategies developed in this investigation. The EPS obtained from the one-stage cultivation system (Result 4.3, Table 2) were compared to the one obtained from the characterization evaluation (Result 4.2, Table 3).

Strains		Compounds	Centrifugation strategy (Result 4.2)	Crossflow-filtration strategy (Result 4.3) **
P. sordidum	% EPS Quantifiable molecules	Glucose	14 ± 1.4	19 ± 1.7
		Galactose	22 ± 2.2	21 ± 2.5
		Xylose	31 ± 0.6	38 ± 1.2
		Glucuronic acid	2.9 ± 0.1	3.4 ± 0.9
		Sulfate	28 ± 4.1	16 ± 1.5
		Protein*	1.3 ± 0.1	2.7 ± 0.3
		Recovery	65 ± 4.9	47 ± 3.7
P. purpureum	% EPS Quantifiable molecules	Glucose	16 ± 0.9	17 ± 0.6
		Galactose	23 ± 0.8	23 ± 0.0
		Xylose	36 ± 2.4	41 ± 0.4
		Glucuronic acid	3.0 ± 0.2	1.8 ± 0.1
		Sulfate	21 ± 3.7	14 ± 0.3
		Protein*	1.3 ± 0.5	2.9 ± 0.1
		Recovery	59 ± 4.4	50 ± 0.5

* Protein is considered a contamination in the EPS.
** Data reported in g L^{-1} instead of mass %.
The EPS compared in this table were obtained from different cultivation system using all ASW medium.

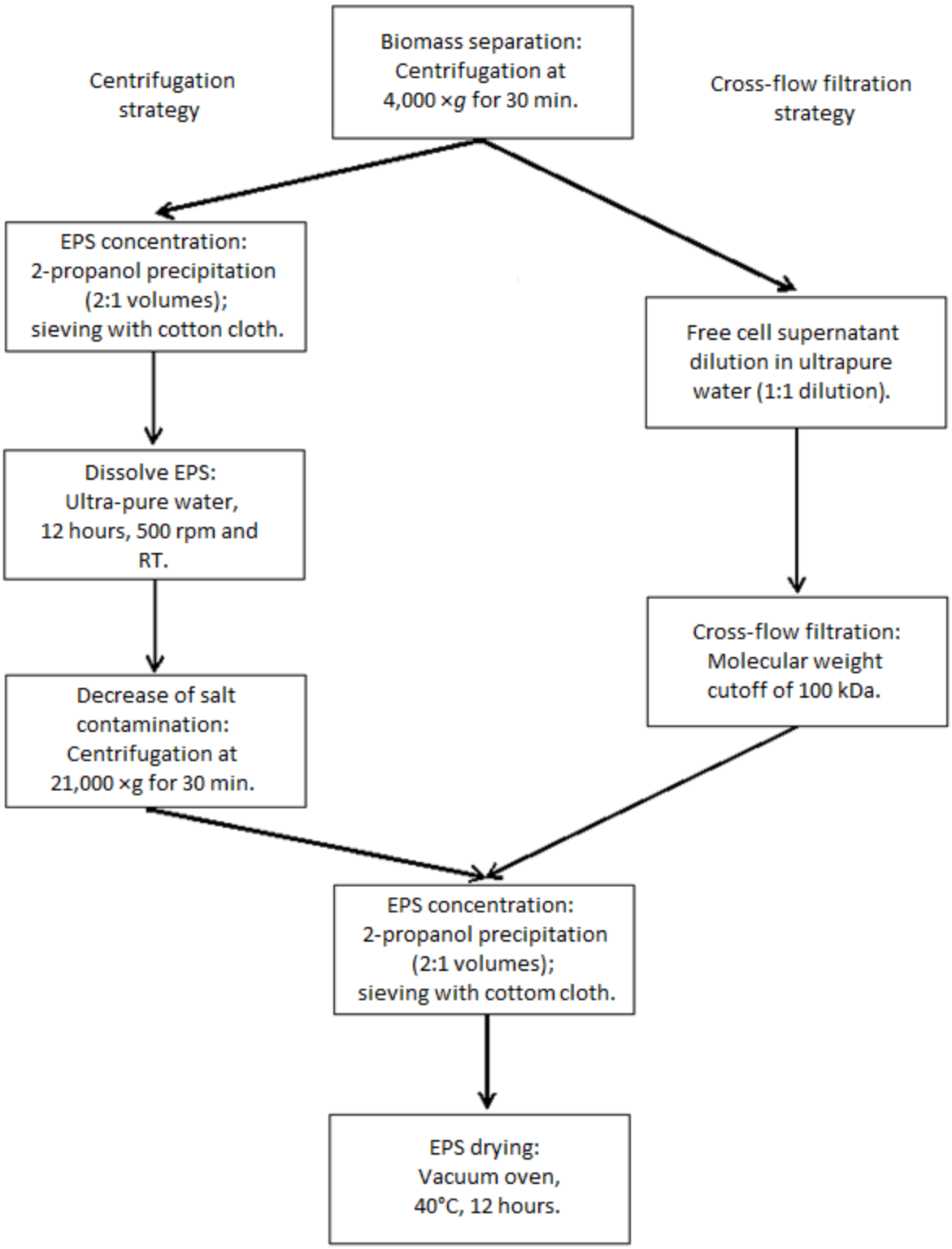

Figure 5.1. Harvesting strategies flow scheme. The centrifugation (Result 4.2, 2.4) and cross-flow filtration strategies (Result 4.3, 2.2) used for harvesting EPS in this investigation.

Despite different harvesting strategies EPS compositions were not heavily altered. A slightly higher xylose content was measured in the EPSs obtained from the cross-flow filtration strategy. This small change could be generated by factors unrelated to the harvesting strategy such as: different cultivation parameters, different shear rates in the cultivation system and time used for production of each EPS batch. Contrary, the sulfate content was higher in the EPSs obtained from the centrifugation strategy (Table 5.3). Although, the difference in EPSs sulfate content could have the same explanation as for the xylose content; it is important to remark that salts are not selectively eliminated by centrifugation as in the cross-flow filtration strategy. So, the increase in sulfate content could be driven by salt traces that were co-precipitated when the supernatant was mixed with 2-propanol in the centrifugation strategy. Despite the small chemical variations in their composition, both strategies proved to be effective for harvesting microalgae EPS. Furthermore, both harvesting strategies did not impact EPSs viscoelasticity and rheological behavior as it can be observed in Result 4.2, Fig. 5, Result 4.4, Fig. 1 and Fig 2. In addition, salts are reported to not affect *Porphyridium* EPSs rheological behavior. So, because both strategies can harvest EPS without heavily altering their composition and rheological properties, three aspects should be considered for selecting the most adequate one. The EPSs' intended application, as the cross-flow filtration strategy could generate a highly pure EPS that could be used in pharmaceutical or medical areas while the centrifugation strategy could be used to obtain EPS (possibly with salt traces) for environmental and industrial purposes. The centrifugation strategy could be used if the cost is an important variable as a it does not require expensive equipment's (like cross-flow filtration). On the other hand, if time is an important factor the cross-filtration strategy should be selected as it can be performed in one day; while the centrifugation process requires an extra day to redissolve microalgae EPS.

As well, ethanol was tested as precipitating agent but an important drawback was observed. Although, higher EPS yields were obtained when ethanol was used instead of 2-propanol in high salinity medium such as ASW medium, the carbohydrate fraction was smaller while the sulfate fraction increased. This is due to ethanol co-precipitating salts from the cultivation medium in a higher ratio. This behavior was also reported by other research groups [392]. Because of that, 2-propanol was selected as the most suitable precipitating agent for harvesting *Porphyridium* EPSs.

Developing an adequate bioprocess (upstream and downstream) for EPS production, would allow to reduce the cultivation time, increase growth rate and EPS productivity. In addition, an efficient harvesting procedure would enhance the EPS yields generate from the bioprocess.

5.4. Exploiting microalgae EPS chemical and rheological properties

Microbial EPS wide chemical diversity and rheological characteristics make them suitable to be utilized in various applications (Introduction, Table 1.3). So, if microalgae EPSs' special decoration (sulfate and methyl residues) and rheological stability under the effect of high salt concentrations, extreme temperature and pHs (Result 4.4; Fig. 1, 2, 3, 4 and 5 and Result 4.4.1, Figure 4.3) are exploited; current application could be enlarged.

Cation biosequestration: Carbohydrate polymers are used to capture and separate copper (Cu^{2+}) and lead (Pb^{2+}) cations from an aqueous environments [418, 419]. In this regard, the sulfate residues (SO_4^{-2}) from *P. sordidum* and *P. purpureum* EPSs, have the potential to interact and capture cations more efficiently than non-sulfated carbohydrate polymers [418].

Biosurfactant: Surfactants are compounds soluble in water that can interact with hydrophobic residues. Several polymers are reported to be surfactants including microalgae EPS (biopolymer with sulfate and methyl groups) [274, 420, 421]. Polymeric surfactants like *Porphyridium* EPSs have the potential to interact with oil residues (contamination) in aqueous environments. The working mechanism could be the following: As EPS solidifies, oil will be trapped in a gel matrix, making them easier to be removed from aqueous phase. In addition, microalgae EPS could be used in different salinity environments (fresh, brackish and salty) due to the strong rheological stability described in this investigation.

Anti-viral products: Sulfated carbohydrate monomers and polymers are reported to have antiviral properties [30, 153, 159]. In this sense, modified carrageenan's (sulfated carbohydrate polymers) have been reported as antiviral agent against herpes 1 and 2 (HSV-1 and HSV-2), human immunodeficiency viruses (HIV), influenza A virus (IFV-A) to mention some examples [29, 153-158]. As well, it has been suggested that sulfate carbohydrates could be used against COVID-19 [422, 423]. In this regard, *P. sordidum* and *P. purpureum* EPS (sulfated carbohydrate polymers) should also be tested as antiviral agents for different viruses including COVID-19 [154, 393, 424]. The COVID-19 pandemic, has demonstrated that the quest for novel antiviral agents, like sulfate carbohydrates (*P. sordidum* and *P. purpureum* EPSs) should be further investigated.

Diverse pharmaceutical formulations: Additional to antiviral activities, sulfated carbohydrates have other biological properties; the most prominent ones are: Antiinflammatory, antioxidant, anticancer and immunomodulatory [29, 30, 396]. Combining EPS rheology and the previously mentioned properties, novel pharmaceutical formulation could be developed against diverse illnesses. Those formulations would be stable despite of extreme environments (Result 4.4; Fig. 1, 2, 3, 4 and 5 and Result 4.4.1, Figure 4.3) setting them apart from other commercial polymers. Furthermore, microalgae EPS pharmaceutical

formulation could alleviate the high demand for sulfated carbohydrate polymers like glycosaminoglycan (GAG) and chondroitin [148, 152, 153]. In addition, because microalgae are cultivated in controlled environments, the EPS batches would have similar chemical composition in contrast to other carbohydrate polymers obtained from macroalgae and animal tissue [29, 147].

Although, *Porphyridium* EPSs have many putative applications, more investigation into their chemical composition and rheological properties need to be done, unveiling new applications.

5.5. Perspectives

5.5.1. High potential to identify new microalgae as EPS producers

There are two microalgae sources that could be investigated to identify new microalgae as EPS producer. Microalgae stored in strain collections, which have never been tested as EPS producer. In this sense, microalgae strains that are part of a genus reported as EPS producer can potentially generate those molecules [9, 10, 13]. For example; *Dixoniella grisea, Galdieria sulphuraria* and *Cyanidioschyzon merolae* could also be EPS producer as other rodophyta strains such as: *Flintiella sanguinaria, Porphyridium aerugineum, Porphyridium cruentum, Porphyridium marinum, Porphyridium purpureum, Porphyridium sordidum, Porphyridium sp., Rhodosorus marinus, Rhodella grisea, Rhodella maculate, Rhodella reticulata* and *Rhodella violaceae* [151, 287, 288, 290, 291, 331, 335, 336, 393, 425-428]. Although, many of those strains have been isolated several decades ago, their capability to produce EPS has been detected many decades after. In this sense, *P. sordidum*, which was isolated in 1931 and stored in collections; was described as EPS producer in late 2019; and its EPS was chemical and rheological characterized was detail in 2020 [332, 405, 429].

In addition to rhodophyta, other six eukaryotic phyla: charophyta, chlorophyta, cryptophyta, dinophyta (miozoa), haptophyte, heterokontophyte (diatoms) and ochrophyta and one prokaryotic (cyanophyta) are reported to have EPS producer strains [8-11]. Nevertheless, not all the strains of those phyla have been tested as EPS producer. Furthermore, there are three eukaryotic phyla (chlorarachniophyta, euglenophyta and glaucophyta) and one prokaryotic (prochlorophyta) phylum stored in strain collection, which have never been investigated as EPS producers [5, 7, 9, 287]. So, the potential to identify new EPS producers in microalgae strains stored in collection is significantly large. In this regard, there are over 1,000 different strains stored in those collections; the majority of them have never been tested as EPS producers [13].

In addition to store strains, newly isolated microalgae strains must be also tested to identify novel EPS producer. Currently, microalgae are being isolated as a source for multiple biogenic compounds such as carotenoids, lipids and antibiotics to name a few [430-433]. However, EPS production is not been actively

screened, because the investigation focusses in intracellular compounds; discarding the cultivation medium were EPS are excreted [19, 21]. Those new isolates should be tested to identify if they produce EPS, implementing detection protocols in the microalgae biogenic compounds screening process. Several methodologies are being evaluated to accelerate microalgae EPS screening process [9, 10, 434]. Although, several microalgae strains were successfully identified with the before mentioned approaches, they have two notorious disadvantages: long cultivation periods and large volumes required to identify EPS producer. To solve those challenges a 96 deep-well-plates format could be used to identify EPS producer with the following advantages. Because microalgae can be cultivated in small volumes; large inoculums could be used to shorten the cultivation time. Also, different cultivation media could be tested in the screening process, increasing the number of strains that could be identified in a single run [9]. Due to the small size of 96 deep-well-plates format, a small cultivation system with external light source, agitation platform and temperature control could be implemented to regulate the cultivation conditions and EPS production [410, 411, 416]. And finally, an automatic liquid handling system could be used to eliminate human error is couple to the system. A similar system to the one above described has been successfully used to identify bacterial EPS producer [378]. However, this system needs to be adapted for microalgae, for example, transparent breathable films need to be used to allow microalgae growth and EPS production in autotrophic cultivation mode.

5.5.2. Optimizing of compounds involve in microalgae growth and EPS production

Microalgae EPS optimization strategies focuses in the cultivation medium, leaving the carbon concentration unoptimized [287, 290, 411]. This is an important limitation as EPS have a large carbon content in their structure. They are two ways in which higher carbon concentration could be provided into microalgae cultivation system.

Mixotrophic cultivation. Several carbon sources are currently used to mixotrophically cultivate microalgae: specific organic compounds like glycerol, glucose, lactose, xylose and ribose to name a few or undefine mixture such as waste water [56, 61, 435-438]. It is reported that mixotrophic cultivation favor growth and secondary metabolites production when they are compared to autotrophic cultivation of the same microalgae strains [439-441]. Furthermore, it has been reported that exopolymeric substances were generated via mixotrophic cultivation in *Chlorella sp., Neochloris oleoabundans* and *Nostoc flagelliforme*. However, those molecules contain in addition to carbohydrates, lipids and proteins [308, 326, 442]. In order to identify if exopolymeric substances are equivalent to EPSs two evaluation must be performed. Their monomer chemical composition and their rheological properties must be elucidated. Only

temperature stability has been tested on exopolymeric substances; showing a different behavior than *Porphyridium* EPSs [186, 326, 391, 393, 394]. However this results must be corroborated with a rigorously rheological evaluation [394]. In this investigation mixotrophic cultivation was briefly evaluated. Unfortunately, bacteria and fungi overcome microalgae growth and EPS production. To solve this problem, a mixotrophic mode could be used to induce EPS production after a cultivation phase, limiting the time in which other microbes could grow faster, preventing microalgae growth and EPS production.

CO_2 inlet supplementation. A second way to increase the carbon concentration for microalgae cultivation and EPS production is to enrich the air inlet with CO_2. In this regard, *P. cruentum* and *P. purpureum* growth in ASW medium was favored when 1 to 5 % of CO_2 was supplemented in the air inlet [134, 438, 443-446]. As well, it was reported that 5 % of CO_2 enhanced *Porphyridium sp.* growth and CPS production, while lowering the initial CO_2 concentration increased the generation of EPS [447]. This give hints that *P. sordidum* and *P. purpureum* growth rate could be enhanced by supplementing different CO_2 concentrations: A high CO_2 concentration during a cultivation-stage and a lower one in an induction-stage to favor EPS production (two-stage cultivation system).

Optimization of plant hormone and vitamins concentration in the cultivation medium. In addition to the optimization of the carbon content there are other compounds that could also be tested to improve EPS production in microalgae. Plant hormones like auxins and vitamins affect plant and microalgae physiology and metabolism [448]. In this sense, EPS production in *G. impudicum* was increased 4.1 times using auxin (7.5 µg L^{-1}) and vitamin B_{12} (0.75 µg L^{-1}) [298, 325]. In a similar way, *P. sordidum* and *P. purpureum* growth and EPS production could be enhanced with an appropriate concentration of auxins and vitamins.

5.5.3. Pending characterization analysis to unveil microalgae EPS properties

It was determined that *P. sordidum* EPS contained a 4-methyl galactose and 3-methyl xylose, (Result 4.4.1) while *P. purpureum* EPS have a methyl hexose glucuronic acid dimer (Section 4.2, Table 3) in their structures. Also, it was identified that *P. sordidum* EPS contain a higher sulfate concentration than *P. purpureum* EPS [393]. However, to fully understand *Porphyridium* EPSs properties their chemical properties and molecular weight must be fully unveiled in future investigations.

Molecular weight determination. Attempts to measure *P. cruentum* EPS molecular weight has been reported using a size exclusion chromatography and light scattering [391]. However, it is possible that an accurate determination could not be achieved because EPS sulfate and methyl residues can interact with the chromatography column changing their elution time. Furthermore, the lack of molecular weight standards with a similar chemical composition to *Porphyridium* EPSs, prevents to precisely determine the

molecular weight. Pullulan standards could be used to determine their molecular weight, but in relative terms because of the difference in their chemical composition, which can lead to different interactions with the size exclusion chromatography matrix [181, 207]. To overcome those challenges various size exclusion matrices should be tested, to select the one that minimizes or suppress the interactions with *Porphyridium* EPS and standards, to accurately determine the relative molecular weight.

5.5.4. Strategies to enable microalgae EPS to commercial production

Microalgae low EPS productivity and long cultivation, set them apart from the requirements for industrial production [263, 271]. To overcome those drawbacks two approaches will be discussed in this section.

Production of multiple biogenic products from a single bioprocess. After the production EPS, *Porphyridium* biomass is left as by-product, so it could be used to obtain other valuable biogenic compounds. To achieve this goal an effective harvesting strategy should be developed to obtain multiple products from the biomass. The proposed harvesting strategy must have the following characteristics: Use of low-cost harvesting methodologies, utilizing a minimum number of steps to obtain the target molecules and it should be ecofriendly. Following those premises, first a single step phycobiliproteins extraction (Result 4.5) could be used. Then, valuable lipids could be extracted from *Porphyridium* biomass, utilizing a biobased solvent [437, 444, 449, 450]. Remarking, that high lipid concentration could be found in *Porphyridium* biomasses due to nitrate and phosphate consumption, which could be achieved during the cultivation-stage [446]. Finally, the residual biomass would be dried to be used as a food supplement for human and animal nutrition or as fertilizer for different agricultural crops [112-118].

Metabolic engineering: Lipids and biohydrogen production has been increased in microalgae utilizing metabolic engineering [451, 452]. Furthermore, metabolic engineering is been currently investigated on bacterial strains to increase EPS production [453]. However, metabolic engineering has three challenges to overcome in rodophyta strains. Firstly, it is difficult to obtain pure genomes as samples are usually infected with bacteria. So, when microalgae DNA is extracted also bacterial traces are obtained, making sequencing unviable or difficult to perform. In second place although, two rhodophytas genomes have been sequenced (*P. purpureum* and *Cyanidioschyzon merolae)*, the gene clusters and metabolic pathways related to EPS production are still unknown [454]. And thirdly, genetic engineering techniques for transforming rhodophytes need to be developed [455]. In this sense, so far only a biolistic microparticle bombardment was reported for transforming *Porphyridium sp.* chloroplast [456].

If the before mentioned approaches are investigated and developed, *Porphyridium* EPS cost might be reduced while their yields increase closing the gap to reach commercial production.

6. Appendix

6.1. Appendix 1. Compositions of microalgae cultivation medium

Table A 1.
CHU medium composition.

Macroelements		
Components	Concentration	
	g L^{-1}	mM
$CaCl_2$ x $2H_2O$	0.368	2.50
$MgSO_4$ x $7H_2O$	0.370	2.12
K_2HPO_4	0.087	0.35
$NaNO_3$	0.085	1.00
$EDTANa_2$	0.080	0.48
$FeSO_4$ x $7H_2O$	0.010	0.24
Na_2SiO_3 x $9H_2O$	0.284	1.00
Microelements*	1 mL	

* The microelements solution was the same used in the ASW medium.

Table A 2.
ASN III medium composition.

Macroelements		
Components	Concentration	
	g L^{-1}	mM
NaCl	25	427.79
$MgSO_4$ x $7H_2O$	3.5	14.20
$MgCl_2$ x $6H_2O$	2.0	9.84
$NaNO_3$	0.75	8.82
KH_2PO_4	0.75	5.51
$CaCl_2$ x $2H_2O$	0.50	3.40
KCl	0.50	6.71
Na_2CO_3	0.02	0.19
$EDTANa_2$	0.0005	0.001
Citric Acid	0.003	0.02
Ferric Ammonium Citrate	0.003	0.01
Microelements*	1 mL	

* The microelements solution was the same used in the ASW medium.

6.2. Appendix 2. Complementary information of the optimization procedure

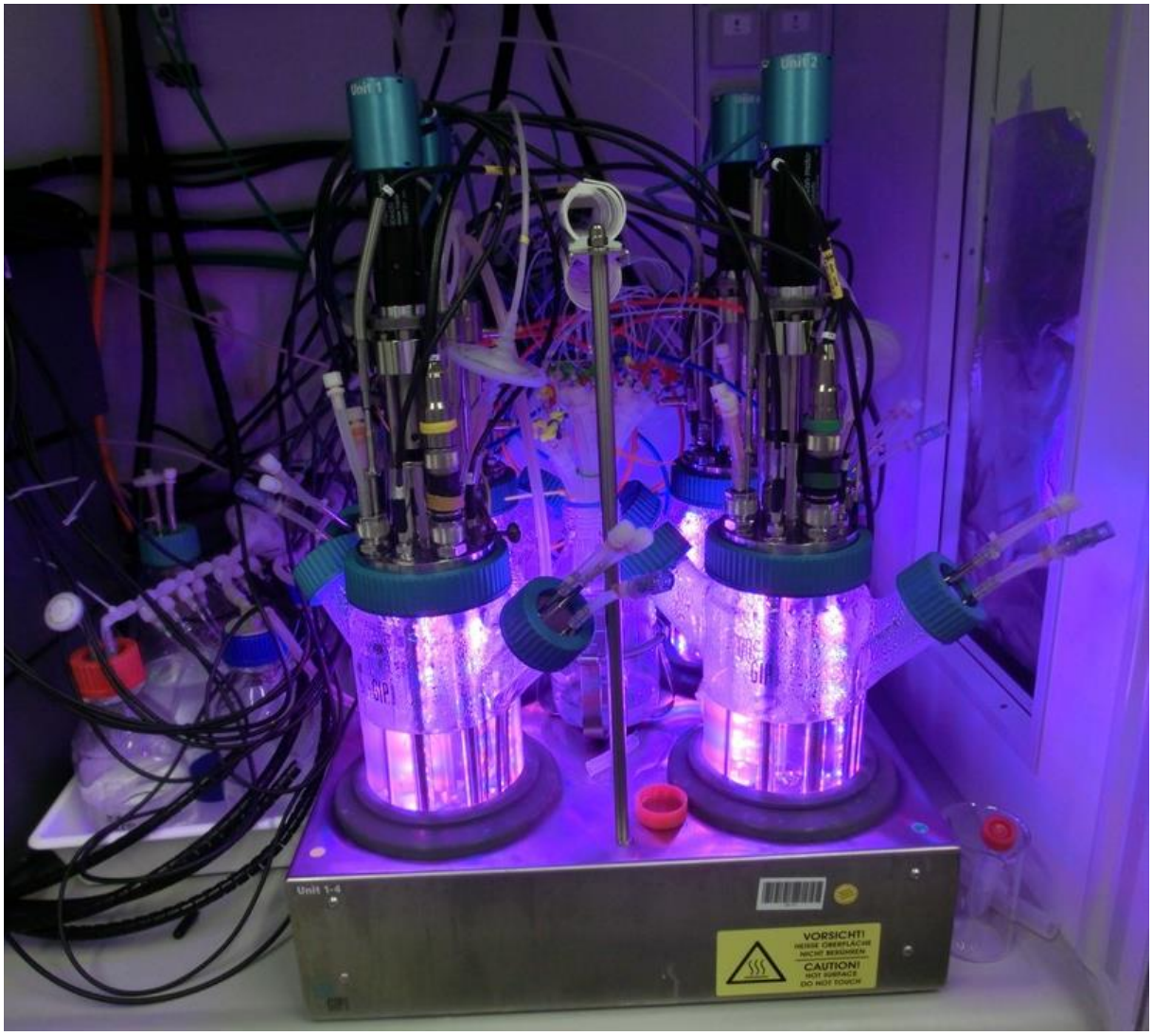

Figure A 1. DASGIP (Stirred tank) system. Features: 1.5 L vessel, three DASGIP LEDS (module A: 430 nm, blue light; module B: 572 nm, green light/625 nm, yellow light/640 nm, orange light and module C: 660nm, orange light and 780 nm, red light) per vessel, propeller, pH electrode, dissolved oxygen electrode, temperature probe, three-port. General settings for cultivation: 1.2 L final cultivation volume, maximum irradiance 1.87 µE s^{-1}, irradiance increase following a cosine function in 12/12 hours cycle and temperature 25 °C.

Table A 3.
Plackett-Burman (PB) design. This evaluation determined the effects and significance of the ASW medium compounds on growth and EPS production. +, when the compound was presented and –, when the compound was absent from the evaluation.

Evaluations	NaCl	$MgSO_4$	$MgCl_2$	$CaCl_2$	$NaNO_3$	KH_2PO_4	$NaHCO_3$	Microelements	EDTA-$FeSO_4$
1	+	–	+	–	–	–	+	+	+
2	+	+	–	+	–	–	–	+	+
3	–	+	+	–	+	–	–	–	+
4	+	–	+	+	–	+	–	–	–
5	+	+	–	+	+	–	+	–	–
6	+	+	+	–	+	+	–	+	–
7	–	+	+	+	–	+	+	–	+
8	–	–	+	+	+	–	+	+	–
9	–	–	–	+	+	+	–	+	+
10	+	–	–	–	+	+	+	–	+
11	–	+	–	–	–	+	+	+	–
12	–	–	–	–	–	–	–	–	–

Equations A 1.

Effect of the factors (PB design) [380].

$$\text{Effect} = \frac{2[(\sum y^{+}) - (\sum y^{-})]}{N}$$

y^{+}: Responses when the factor is at its higher level.

y^{-}: Responses when the factor is at its lower level.

N: Number of evaluations.

Equations A 2.

Significance of a factor (PB design) [380].

$$SS = \frac{N \text{ x } (\text{Effect})^2}{4}$$

Table A 4.
Taguchi design used for the media optimization. The aim of this test was to obtain higher amounts of EPS, so the larger the better definition was used to identify the optimal levels of each medium compound. The five most important compounds were evaluated using three levels and two levels were used for the sixth one.

Evaluations	*P. sordidum*					
	KH_2PO_4	NaCl	$MgSO_4$	$CaCl_2$	$NaNO_3$	Microelements
1	1	1	1	1	1	1
2	1	1	2	2	2	2
3	1	1	3	3	3	3
4	1	2	1	1	2	2
5	1	2	2	2	3	3
6	1	2	3	3	1	1
7	1	3	1	2	1	3
8	1	3	2	3	2	1
9	1	3	3	1	3	2
10	2	1	1	3	3	2
11	2	1	2	1	1	3
12	2	1	3	2	2	1
13	2	2	1	2	3	1
14	2	2	2	3	1	2
15	2	2	3	1	2	3
16	2	3	1	3	2	3
17	2	3	2	1	3	1
18	2	3	3	2	1	2
Evaluations	*P. purpureum*					
	$CaCl_2$	NaCl	$MgSO_4$	$NaNO_3$	KH_2PO_4	$NaHCO_3$
1	1	1	1	1	1	1
2	1	1	2	2	2	2
3	1	1	3	3	3	3
4	1	2	1	1	2	2
5	1	2	2	2	3	3
6	1	2	3	3	1	1
7	1	3	1	2	1	3
8	1	3	2	3	2	1
9	1	3	3	1	3	2
10	2	1	1	3	3	2
11	2	1	2	1	1	3
12	2	1	3	2	2	1
13	2	2	1	2	3	1
14	2	2	2	3	1	2
15	2	2	3	1	2	3
16	2	3	1	3	2	3
17	2	3	2	1	3	1
18	2	3	3	2	1	2

Table A 5.
Taguchi design used to optimize abiotic factors for EPS induction. The Taguchi larger the better definition aiming for larger amounts of EPS for both *Porphyridium* strains.

Evaluations	NaCl (g L^{-1})	pH	Light exposure (h)	Temperature (°C)
1	1	1	1	1
2	1	2	2	2
3	1	3	3	3
4	2	1	2	3
5	2	2	3	1
6	2	3	1	2
7	3	1	3	2
8	3	2	1	3
9	3	3	2	1

6.3. Appendix 3. Phycobiliproteins evaluation

Equations used to calculate Phycobiliproteins (mg mL^{-1}).

The equations were obtained from different investigations [135, 385, 457]

Equations A 3.

Phycocyanin (R-PC)

$$R - PC = \frac{OD_{620nm} - 0.7OD_{650nm}}{7.38}$$

Equations A 4.

Allophycocyanin (APC)

$$APC = \frac{OD_{650nm} - 0.19OD_{620nm}}{5.65}$$

Equations A 5.

Phycoerythrin (B-PE)

$$B - PE = \frac{OD_{565nm} - 2.8[R - PC] - 1.34[APC]}{12.7}$$

Equations A 6.

Total Phycobiliproteins (T-Ph)

$$T - Ph = (R - PC) + (APC) + (B - PE)$$

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8. Abbreviations

%	Percentage
°C	Degree Celsius
AA	Arachidonic acid
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ADP	Adenosine diphosphate
APC	Allophycocyanin
ASW	Artificial seawater
ATP	Adenosine triphosphate
BBM	Bold basal medium
BGP	Biogenic Polymers
bp	Base pair
B-PE	Phycoerythrin
BSA	Bovine serum albumin
CBR	Chemistry of Biogenic Resources
$CaCl_2$	Calcium chloride
CO_2	Carbon dioxide
Cu^{2+}	Cooper divalent cation
CPS	Capsular polysaccharides
CS	Campus Straubing
d	Diameter
Da	Dalton
DA-64	N-(Carboxymethylaminocarbonyl)-4, 4'-bis(dimethylamino)diphenylamine Sodium Salt
DHA	Docosahexaenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DOE	Design of experiments
EDTA	Ethylene diamine tetraacetic acid
EDTA Na_2	Ethylene diamine tetraacetic acid disodium
EPA	Eicosapentaenoic acid
EPS	Exopolysaccharides
$FeSO_4$	Iron (II) sulfate
G’	Storage modulus
G”	Loss modulus
g L^{-1}	Gram per liter
GLA	γ -linolenic acid
H_2O	Water
HIV	Human immunodeficiency viruses
HPLC	High-performance liquid chromatography
HSM	High salinity media
HSV-1, -2	Herpes virus 1 and 2
HT	High throughput
Hz	Hertz
I	Iota
i.d.	inner diameter

I1	Improve media 1
I2	Improve media 2
I3	Improve media 3
IA	Improve media A
IB	Improve media B
IC	Improve media C
IFV-A	Influenza A virus
K	Consistency
K_2HPO_4	Dipotassium phosphate
KCl	Potassium chloride
KDa	Kilodalton
KH_2PO_4	Monopotassium phosphate
L	Liter
LVE	Linear viscoelastic region
M	Molar
mA	Milliampere
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulfate
min	Minutes
mL	Milliliter
mM	Millimolar
mm	Millimeter
mV	Millivolts
mt	Metric tons
NMR	Nuclear magnetic resonance
MS	Mass-spectrometer
MTP	Micro titer plate
m/z	Mass to charge ratio
N	Number of factors
N	Flow index
Na_2SiO_3	Sodium silicate
Na_2CO_3	Sodium carbonate
NaCl	Sodium chloride
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduce Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium hydrogen carbonate
$NaNO_3$	Sodium nitrate
NCBI	National Center for Biotechnology Information
NH_2^{-1}	Amino ion
NO_3^-	Nitrate ion
nm	Nanometer
n.p.	Not possible peak
O_2	Oxygen molecule
OD	Optical density
OH^{-1}	Hydroxyl ion
Pa	Pascal
PB	Plackett-Burman
PCR	Polymerase chain reaction

Pb^{2+}	Lead divalent cation
PEG	Polyethylene glycol
pH	Decimal logarithm of the reciprocal of the hydrogen ion activity
Pm	Provasoli medium
PMP	1-phenyl-3-methyl-pyrazolone
PO_4^{3-}	Phosphate ion
POX	Pyruvate oxidase
PP	Polypropylene
PSI	Photosystems I
PSII	Photosystems II
PUFAs	Polyunsaturated fatty acids
R-PC	Phycocyanin
rpm	Revolutions per minute
rps	Revolutions per second
RSM	Response surface methodology
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
s	Seconds
SAG	Göttingen University Culture Collection of Algae
SO_4^{-2}	Sulfate divalent cation
sp.	Specie
spp.	Species
T-Ph	Total Phycobiliproteins
t_d	Doubling time
TFA	Trifluoroacetic acid
TRIS	Tris(hydroxymethyl)aminomethane
TRIS-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TUM	Technical University of Munich
US$	United states dollars
UV	Ultraviolet
w/v	Weight per volume
$\dot{\gamma}$	Shear rate
μ	Viscosity
µL	Microliter
µm	Micrometer
μ_{max}	Specific growth rate
κ	Kappa
λ	Lambda

9. List of Figures

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11. Curriculum vitae

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