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Production of exopolymer substances from the thermophilic chlorophyte *Graesiella*: industrial and ecological applications

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Abstract

Microalgal extracellular polymeric substances (EPSs) are heteropolysaccharides that have characteristics suitable for industrial and biotechnological applications. Notably, they have strong anionic nature and high hydrophobicity. Nevertheless, systematic studies to demonstrate the viability of the production of EPSs on an industrial scale are still crucial. In this research, the chlorophyte *Graesiella* was grown on a raceway pond to view its EPS valorization. The biomass production achieved a maximum of 1.98 g L^{-1} and an EPS production of 1.6 g L^{-1} after six production days. The *Graesiella* EPSs with a molecular weight above 100 kDa are sulfated exopolymers containing mainly polysaccharide (70%) and protein (16%). The EPSs produced more stable emulsions with hydrocarbons and oils than Tween-20. The emulsification indices with *n*-hexane (88%) and maize oil (28%) indicate the EPSs' strong emulsion-stabilizing capacity. The EPSs showed a peak flocculating percentage of 95% to kaolin suspension, with better flocculation performance than $Al_2(SO_4)_3$ and alginate. Moreover, *Graesiella* EPSs had a significant effect on antimicrobial activity, significantly inhibiting fungal growth (71% for *Botytis cinerea* and 87% for *Fusarium oxysporum*), spore germination (100% of inhibition at a concentration of 1.8 g L^{-1}), and mycelium growth (68% of inhibition). Also, *Graesiella* EPSs acted as a bactericide against *Vibrio anguilaruim* and *Listonella anguilaruim* (100% inhibition). EPSs were also found to have potent antioxidant activity compared with L-ascorbic acid. The obtained results open new perspectives to the further exploration of *Graesiella* sp. as a potential EPS producer, making it a promising candidate for numerous industrial applications.

Keywords Chlorophyceae · *Graesiella* EPSs · Industrial applications · Ecological applications · Biological activities

Introduction

International demand for polymers continues to climb as the world population increases. Nearly all polymers are formed from fossil resources, which cover about 8% of total fossil oils. It is predicted that this quantity could rise to 20% by 2050 (Hopewell et al. 2009; Schneiderman and Hillmyer 2017). Amidst the increasing worry regarding harmful environmental damage and exhaustible fossil resources, there is significant interest in using natural resources for polymeric materials (Hillmyer 2017; Hong and Chen 2017). Various sustainable polymers extracted from macroalgae (agar, carrageenan, and alginate) and bacteria (dextran and xanthan gum) have been found to have biodegradability, biocompatibility, thermo-stability, and even biological activities (Brunchi et al. 2016; Martău et al. 2019; Wang et al. 2020). These properties have targeted applications in the packaging, agricultural, textile, pharmaceutical, and medical domains (Nwodo et al. 2012). Generally, biopolymers extracted from macroalgae and bacteria are obtained following chemical or enzymatic

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transformations and by hydrolysis, polymerization, or condensation (Wang et al. 2020). These traditional methods are time-consuming, are expensive, and require large sample volumes, chemical agents, and numerous manipulation steps. They can also negatively impact the functional and structural properties of these polymers (Abdul Khalil et al. 2018).

In recent years, the potential uses of microalgae in industrial applications have received increased attention, not only because of their biomass but also because of the existence of a large quantity of polymers in cell-free medium available from biomass cultivation, known as exopolysaccharides or extracellular polymeric substances (EPSs). These polymeric substances are metabolic products reported as heterogeneous groups with high molecular weight. Chemically, EPSs are composed essentially of polysaccharides, proteins, a small quantity of lipids, and humic substances (Delattre et al. 2016). They could be used as natural food additives, thickeners, flocculants, emulsifiers, gelling agents, and stabilizers (Xiao and Zheng 2016). For instance, EPSs produced by *Dunalliela salina* showed important emulsifying activity (Mishra and Jha 2009). Apart from this, EPSs are also known for their antioxidant, anti-inflammatory, antiviral, antifungal, and antibacterial properties (Xiao and Zheng 2016). These characteristics make microalgal EPSs extremely attractive for industrial and biotechnological applications. Indeed, in an industrial context, the exploitation of microalgae is more profitable compared with the use of plants or macroalgae, as their growth rates are higher, their minimal nutrition requirements are lower, their cultivation does not pose any ethical problems and their growth conditions are easier to manipulate (Parikh and Madamwar 2006; Pereira et al. 2009; Arad and van Moppes 2013). Despite all these advantages, EPSs remain mostly unexploited and there are few systematic studies that focus on the optimization of EPSs on a laboratory scale, let alone on a large scale. To date, only a few researchers (Bafana 2013; Ekelhof and Melkonian 2017; Zhang et al. 2019) have reported about the production and characterization of EPSs from green algae, which limits the successful implementation of a biotechnological system.

Presently, the microalgae market is limited to only a few exploited microalgae strains. The research of new species makes it possible to envisage new contributions in various sectors using microalgae and/or their bioactive compounds, either directly or indirectly through the incorporation in new products. Thermophilic and thermotolerant microalgae, like *Graesiella*, have advantages for industrial production, especially in summer outdoor climatic conditions, where high temperatures often inhibit growth of common microalgae species.

Therefore, this study was focused on the EPSs produced by the thermophilic green microalga, *Graesiella* sp., on a large scale in a raceway pond. The physicochemical properties of *Graesiella* EPSs were investigated—namely the emulsifying activity, kinematic viscosity, and flocculating properties—and compared with commercial surfactants and flocculants. Moreover, the antifungal, antibacterial, and antioxidant activities of the aqueous EPSs were evaluated.

Materials and methods

Strain and growth condition culture

The *Graesiella* strain was isolated from natural hot spring water located in Ain Echafa, Tunisia (36° 490 N, 10° 340 E) and is in the algal collection of the Tunisian National Institute of Marine Sciences and Technologies, Laboratory of Blue Biotechnology and Aquatic Bioproducts. Growth phase cultures (200 L volume) were placed under conditions that optimize the algal growth: BBM medium (Bischoff and Bold 1963); 30 ± 1 °C; 120 μmol photons m^{-2} s^{-1} and performed in a 250 L capacity closed raceway pond for 10 days. The photobioreactor (Fig. 1) consisted of a channel-shaped basin (2 m × 1 m × 0.15 m) traversed by a network of chicanes (15 cm edge), creating a route for the culture medium. Two large paddle wheels were installed separately within the raceway for mixing and were driven by a speed-adjustable pulley motor that allows the agitation of the crop motors and provides a flow velocity of 30 cm s^{-1}. This structure was placed in an isothermal enclosure with double-glazed walls and temperature regulation controlled by a temperature probe. The working culture depth was 25 cm. Experimental cultures were exposed to a series of LED tubes (type C-LED tubes, Cefla, Italy) with a light/dark photoperiod of 16:8 h. The light intensity of the culture (120 μmol photons m^{-2} s^{-1}) was ensured by adjusting the distance between the light source and the surface of the culture. The strain was inoculated with an initial optical density (680 nm) of 0.07. The growth was monitored by a daily absorbance measurement at 680 nm using a spectrophotometer (Beckman Coulter DU 640B). The algal cell growth was measured daily via the dry biomass to determine the biomass density (g L^{-1}). The samples (1 L), in triplicate, were

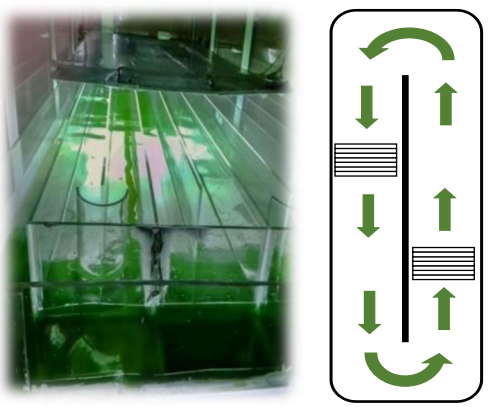

Fig. 1 Configuration of the used photobioreactor (250 L)

filtered through prewashed 0.2 μm microfiber filters (GF/F filter, Whatman Plc., UK) and desiccated overnight at 80 °C. The filters were cooled to room temperature in a desiccator before the weighing.

EPS extraction

The EPSs were extracted from the *Graesiella* cultures by stirring the algal culture for 30 min, followed by centrifugation (3985 *g* for 10 min, at 4 °C) to separate the algal cells from the culture medium containing the released EPSs. The total recovered supernatant (culture medium and soluble EPSs) was concentrated (10-fold) via tangential ultrafiltration (Vivaflow 50, Sartorius) using Millipore membranes with a cut-off of 100 kDa. The removal of low molecular weight substances and inorganics was ensured by a cycle of consecutive concentrations and dilutions with ultrapure water until constant conductivity was reached (0.017 mS^{-1}). The recovered filtrate, rich in EPSs, was freeze-dried and weighed for gravimetric EPS determination.

The biomass and EPS productivities (g L^{-1} day^{-1}) at any culture time (t) were calculated using Eq. 1, with C_0 and C_t the concentration of dry biomass (g L^{-1}) at the start and at time t (day), respectively.

$$\mathrm{Pt} = \frac{C_t - C_0}{t} \tag{1}$$

Chemical analysis

The EPSs produced by *Graesiella* sp. were characterized by estimating the total sugar (Dubois et al. 1956), protein (Lowry et al. 1951), DNA (Desjardins et al. 2009), ester sulfate (Craigie et al. 1984), and lipid (Folch et al. 1957) contents using gravimetric and spectrophotometric methods. Each test was performed in triplicate.

Physicochemical characterization of EPSs

EPS solubility

The solubility of EPSs (0.5%; *w*/*v*) in water and in different organic solvents (≥ 99%)—chloroform (372978, Sigma-Aldrich), methanol (67-56-1, Sigma-Aldrich), formic acid (64-18-6, Sigma-Aldrich), ammonia (7664-41-7, Sigma-Aldrich), tetrahydrofuran (109-99-9, Sigma-Aldrich), benzene (71-43-2, Sigma-Aldrich), carbon tetrachloride (56-23-5, Sigma-Aldrich), ethanol (64-17-5, Sigma-Aldrich), and toluene (108-88-3, Sigma-Aldrich)—was evaluated in triplicate by mixing dry EPSs and water or solvents in 2 mL tubes. Samples were vortexed for 0.5 min and kept for 24 h at 25 ± 1 °C. After that, the insoluble portion of the EPSs, recovered after centrifugation for 10 min at 3000×*g* at 25 ± 1 °C, was subsequently dried at 105 ± 1 °C for 24 h and weighed again. The solubility (S) was calculated by Eq. 2, where w_i and w_f represent the initial and insoluble weight of the EPSs, respectively.

$$\text{Solubility}\ (\%) = \frac{w_i - w_f}{w_i} \times 100 \tag{2}$$

Structural analysis

The UV-visible spectrum of the aqueous solution of EPSs (30 g L^{-1}) was recorded at between 200 and 800 nm on a spectrophotometer (Beckman Coulter DU 640B).

The Fourier transformed infrared spectroscopy (FTIR) of the EPS was recorded to be in the region of 3900–800 cm^{-1} by grinding in KBr disks, followed by pressing into a 16-mm-diameter mold with a Perkin–Elmer spectrum GX FTIR system (Perkin–Elmer, USA). The spectrum was evaluated using the Agilent software.

Emulsifying activity

The EPS emulsifying activity to commercial maize oil, biodiesel, and *n*-hexane was determined based on Castellane et al. (2015). Briefly, oil or hydrocarbon (10 mL) was added to 5 mL of aqueous solution of EPSs (concentration ranged from 0.05 to 100 g L^{-1}) in a glass tube and stirred in a vortex for 5 min. After 24 h, the emulsion index (E_{24}) was determined using Eq. 3, where h_i is the height of the emulsion layer and h_t is the overall height of the mixture, expressed in millimeters.

$$E_{24} = \frac{h_i}{h_t} \times 100 \tag{3}$$

All samples were stored at room temperature and the tests were performed in six replicates. The emulsifying activity to the industrial surfactant, Tween-20 (9005-64-5, Sigma-Aldrich), was also determined, in the same conditions. A control was conducted in the same manner, except that distilled water was used instead of aqueous solution of EPSs.

The size of droplets formed during the emulsification process was estimated after 24 h incubation at 25 ± 1 °C. The size of the emulsion droplets was determined by photography using a light microscope (Olympus CX40, Olympus, Japan) equipped with a Leica DPD 250 camera (40×). The emulsion was placed into a measuring unit with deionized water as a dispersant.

Flocculating activity

The flocculating activity was calculated using the methodology described by Li et al. (2008), with the slight modification of using kaolin clay (400 mesh, Sinopharm Chemical Reagent Company, China) as the suspended solid. In short, 99 mL of 2.0 g L^{-1} kaolin suspension, 0.5 mL of aqueous solution of EPSs (concentration ranged from 0.05 to 100 g L^{-1}), and 0.5 mL of 10% $CaCl_2$ solution were mixed. The prepared mixture was vortexed at 150 rpm for 30 min and rested for 30 min. Two milliliters of supernatant was removed from the upper layer and its absorbance $(OD_{550})_s$ was measured at 550 nm. The absorbance of a blank sample, where the fraction of EPSs was replaced by deionized water, was also measured $(OD_{550})_b$. The flocculating activity and flocculating percentage were calculated using Eqs. 4 and 5, respectively.

$$\text{Flocculating activity} = \frac{1}{(OD_{550})_s} - \frac{1}{(OD_{550})_b} \quad (4)$$

$$\text{Flocculating percentage} = \frac{(OD_{550})_b - (OD_{550})_s}{(OD_{550})_s} \times 100 \quad (5)$$

The flocculating activity was also measured for commercial flocculants—the polysaccharide sodium alginate (9005-38-3, Sigma-Aldrich) and the inorganic flocculant $Al_2(SO_4)_3$ (368458, Sigma-Aldrich)—in the same experimental conditions. The test was done at a neutral pH and room temperature (around 25 ± 2 °C). All tests were performed in six replicates.

Kinematic viscosity

The kinematic viscosity of the aqueous solution of *Graesiella* EPSs (0.1, 0.2, 0.3, 0.4, and 0.5 g L^{-1}) was measured using an automatic viscosity unit SI Analytic (Thermo Fisher Scientific) with an Ubbelhode capillary viscosimeter (DIN 51562) immersed in a water bath at a constant temperature (25 ± 1 °C). The kinematic viscosity was calculated using Eq. 6, where k is the instrument constant (0.5129 mm^2 s^{-1}) and t is the flow time in seconds.

$$\text{Kinematic viscosity} = k\,t \quad (6)$$

The kinematic viscosity of the aqueous EPSs at a concentration of 0.2 g L^{-1} was studied at different temperatures, from 10 to 60 °C.

The experiment was also carried out on the aqueous extract of the commercial polysaccharide xanthan gum (42663, Sigma-Aldrich). All the measurements were made in six replicates.

In vitro biological activities

In this research, the aqueous extract of EPSs (99% EPSs of solubility) was selected, with the aim of avoiding the use of substances that are hazardous for humans and the environment.

Antifungal activity

The tested fungi *Botrytis cinerea* and *Fusarium oxysporum* (obtained from Laboratory of Fungal Biology and Biotechnology, University of Bologna, Italy) were grown on potato dextrose agar (PDA) medium, for 7 days. Different concentrations (15, 30, and 60 g L^{-1}) were used. The procedure had two steps: in the first step, the fungi were kept in contact with the solution of EPSs in the dark for 4 h in Eppendorf tubes; in the second step, the fungi were inoculated (10^5 colony forming units (CFU) mL^{-1}) in PDA Petri dishes. The Petri dishes were incubated at 25 ± 1 °C for 20 days to evaluate the growth inhibition.

In the Petri dishes, 200 μL of each concentration of the aqueous extract of EPSs was distributed independently on agarized boxes and then homogenized, covering the entire surface of the gelled box. A mycelia disc, 4 mm in diameter, taken from the active growth zone of a week-long crop, was placed in the middle of a Petri dish. Incubation took place in the dark for 7 days at 25 ± 1 °C. The daily filament growth on each box was measured by the diameters of different colonies of filamentous fungi to calculate the inhibition percentage (I; %) (Fletcher et al. 2019). The effect of the EPS aqueous solution on spore germination of *F. oxysporum* and *B. cinerea* was analyzed using the method of Rana et al. (1997), with minor modifications. Ten microliters of fungal spore suspension (10^6 spores mL^{-1}) was inoculated on Sabouraud dextrose agar (SDA) slides containing different concentrations of EPSs (0.02–2.0 g L^{-1}) and incubated at 28 ± 1 °C for 24 h. The SDA slides alone, with fungal spores and without EPSs, were considered as a control. Following the incubation period, each slide was fixed with lactophenol cotton blue and observed under a microscope to view the spore germination. About 200 spores were examined from each slide and the percentage of spore germination was calculated using Eq. 7, where C_0 is the number of spores germinated in the control and C_T is the number of spores germinated in the test. Each assay was performed in six replicates and then averaged.

$$\text{Spore germination (\%)} = \frac{C_T}{C_0} \times 100 \quad (7)$$

Antibacterial activity

The antibacterial activity was evaluated against four bacteria: *Streptococcus pneumoniae*, *Vibrio harveyi*, *Listonella*

anguillarum, and *Vibrio anguillarum* (obtained from Marine Scientific and Technological Park of the ULPGC). Culture suspensions of each studied bacterium (10^6 CFU mL^{-1}) were inoculated onto the surface of spread on Luria–Bertani (LB) agar medium according to Valgas et al. (2007). EPSs were dissolved in distilled water to obtain concentrations of 15, 30, and 60 g L^{-1} and were loaded into wells (6 mm diameter) punched into the agar layer. Thereafter, all tested plates were kept for 1 h at 4 ± 1 °C, before incubation for 48 h at 28 ± 1 °C. The antibacterial activity was assessed by determining the diameter of the area (expressed in mm) in which bacterial growth was inhibited around the wells' film of EPSs. Each assay was performed in six replicates.

Radical scavenging ability

1,1-Diphenyl-2-picrylhydrazyl free radical (DPPH) scavenging activity The DPPH scavenging activity was studied as described by Shimada et al. (2002), with slight modifications. Aqueous solutions of EPSs in different concentrations (0.02–2.0 g L^{-1}) were mixed with a methanolic solution containing DPPH (0.02%). The mixture was then incubated for 1 h in the dark at room temperature. A control containing DPPH without EPSs was also prepared. The absorbance was measured at 517 nm (A_{sample}) and normalized to a blank sample consisting of DPPH solution ($A_{control}$). The scavenging activity of DPPH was determined using Eq. 8. The IC_{50}, defining the concentration that scavenges 50% of DPPH radical, was evaluated. The same process was done with L-ascorbic (Sigma-Aldrich-A4544 MSDS) for the purpose of comparing the scavenging activity.

$$\text{Scavenging activity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad (8)$$

Superoxide radical scavenging activity Superoxide radical scavenging activity assay was evaluated following the method used by Marklund and Marklund (1974). A mixture of 1 mL of aqueous solution of EPSs at different concentrations (0.02–2.0 g L^{-1}) and 3 mL of trishydrochloride buffer pH 8.2 (0.05 mol L^{-1}) was incubated at 25 °C for 10 min. At the same temperature, 200 μL of pyrogallol was added to the mixture, the reaction was continued for 4 min, and 0.5 mL of HCl was further added. The absorbance of the sample (A_{sample}) was measured at 320 nm against a control containing the mixture without EPSs ($A_{control}$). The superoxide radical scavenging activity (%) was calculated using Eq. 8. The same process was done with L-ascorbic acid (Sigma-Aldrich-A4544 MSDS), for the purpose of comparing the scavenging activity. The IC_{50} was determined.

Statistical analysis

The results were expressed as means ± standard deviation (SD) over experimental replicates. Statistical analysis of the data was carried out using the software SPSS Statistics 20. Differences between treatments were assessed with a one-sided Student's *t* test, and *p*-values < 0.05 were considered to be statistically significant.

Results and discussion

Microalgae growth kinetic and EPS production

The optimal growth curve of *Graesiella* sp. on an industrial scale for 10 days is shown in Fig. 2a. Under this condition, *Graesiella* displays a typical growth curve with a clear exponential phase observed from day 3 to day 5 of culture time and a maximum biomass yield of 2.10 g L^{-1}. This result is far higher than that obtained in the case of *Chlorella vulgaris* (1.1 g L^{-1}) and *Dunalilella salina* (0.997 g L^{-1}), which are considered the main chlorophytes used in industrial production (Lv et al. 2010; Morowvat and Ghasemi 2016).

EPS production by *Graesiella* sp. increased during the exponential growth phase and stabilized during the stationary phase (Fig. 2a). The maximum yield was 1.62 g $L^{-1}$of EPSs, which occurred at the beginning of the exponential growth phase. This value was higher than that of EPSs from *D. salina* (0.944 g L^{-1}) (Mishra and Jha 2009) and *Chlamydomonas reinhardtii* (0.628 g L^{-1}) (Bafana 2013) and within the highest recorded for other EPS-producing cyanobacteria and some representative EPS-producing lactic acid bacteria (Ruas-Madiedo and de los Reyes-Gavilan 2005).

Moreover, as shown in Fig. 2b, there is a strong correlation linking the kinetics of cellular growth and EPS production, with a maximum daily productivity of up to 0.5 g L^{-1} day^{-1} for both biomass and EPSs. These values are higher than those obtained in optimized *Graesiella* laboratory cultures conditions, which were 0.17 g L^{-1} day^{-1} for biomass (Zili et al. 2017) and 0.1 g L^{-1} day^{-1} for EPSs' maximum daily production (Mezhoud et al. 2014).

This phenomenon is not usually observed (Delattre et al. 2016), which could be justified by the fact that the *Graesiella* sp. has been isolated from an extreme environment (hot spring water) which may have effective mechanisms to produce EPSs to protect the cells from desiccation and therefore from death.

However, due to the fact that the EPS extraction method is not standardized, comparison between EPS strain producers is not straightforward. Indeed, several studies use a dialysis membrane to extract EPSs (Mota et al. 2013), which is well-known for its low-salt elimination, limited lifetime, and generation of a large amount of plastic waste, and therefore

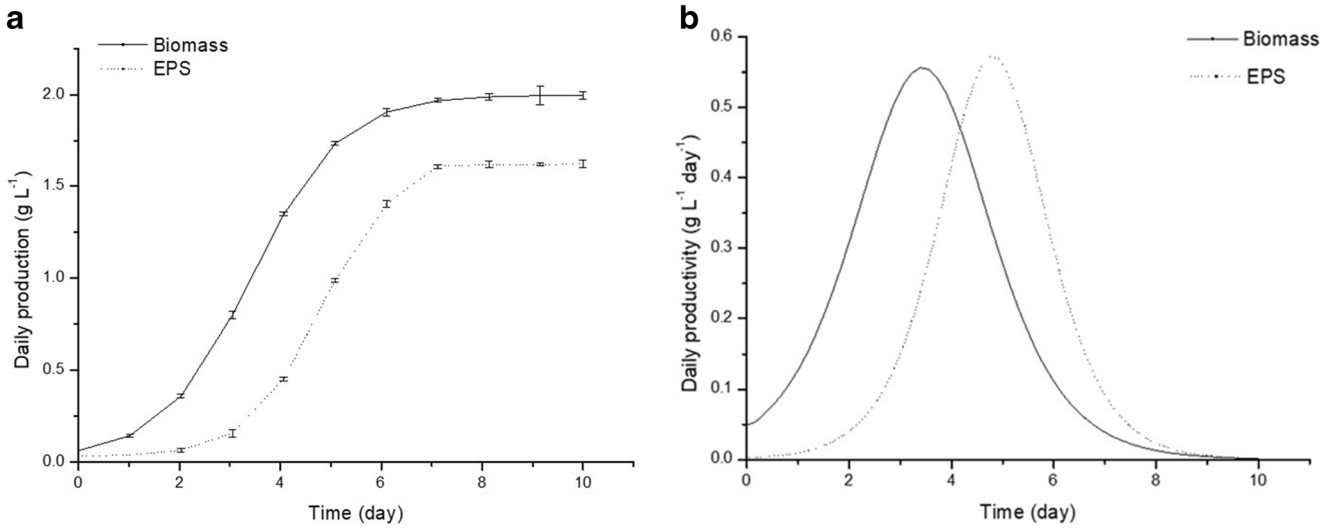

Fig. 2 Daily production (g L^{-1}) (**a**) and productivity (g L^{-1} day^{-1}) (**b**) of biomass and EPSs during 10 days of *Graesiella* sp. growth. Each value is the mean of three replicate measurements

cannot be applied at an industrial level. In contrast, Li et al. (2011) reported the efficiency of ultrafiltration for large-scale EPS extraction due its feasibility, viability, facility to manipulate, and ease to be cleaned.

Thus, the obtained results emphasize the ability of the thermophilic chlorophyte *Graesiella* sp. to be industrially cultivated for the joint production of biomass and EPSs and that the use of ultrafiltration as adopted in this study could be the best method for industrial large-scale EPS extraction.

EPS composition

Several microorganisms synthesize EPSs, which occur in two forms depending on their location. Specifically, they arise as a capsule (capsular EPSs—CPSs) when they are closely associated with the cell surface or as slime polysaccharides (released EPSs—RPSs) that are loosely associated with the cell surface. However, it can be difficult to differentiate these forms of EPSs since some strains discharge capsular polysaccharidic material on their periphery. Also, the centrifugation used to isolate the biomass partially extracted the CPSs (unpublished data), leaving the CPSs and RPSs in the supernatant. Thus, in this work, they are called EPSs.

This study analyzes the EPSs released in the medium with molecular weight above 100 kDa. The results show that the *Graesiella* EPSs are exopolymers predominantly organically composed by high levels of carbohydrates (70.20 ± 0.12%), as well as protein (16.12 ± 0.03%), lipids (0.61 ± 0.01%), and nucleic acids (0.02 ± 0.01%) with sulfate groups (12.21 ± 0.3%). The present results are in accordance with the available literature, where the heteropolymeric nature is also found in most microalgae and chlorophyte strains (Xiao and Zheng 2016) and where the carbohydrate content can reach 80%, the protein content up to 16%, and the sulfate residues up to 13% (Raposo et al. 2013; Delattre et al. 2016; Xiao and Zheng 2016). The DNA and lipid contents are rarely studied in chlorophyte EPSs; however, some studies reported the presence of extracellular DNA from 0.01 to 10.9% and low amounts of lipids ranging from 2 to 8.7% (Conrad et al. 2003; Zhu et al. 2012). All these compounds (proteins, lipids, organic and inorganic compounds, and DNA) are chemically linked, presenting a complex matrix structure which constitutes a "reinforcing grid," as was described by Mazeau and Rinaudo (2004).

Physicochemical properties

Solubility

EPSs were totally soluble in water (S = 99.12%) and had a solubility lower than 3% in all tested organic solvents: chloroform (S = 1.25%), methanol (S = 0.53%), formic acid (S = 0.25%), ammonia (S = 0.54%), tetrahydrofuran (S = 0.52%), benzene (S = 0.56%), carbon tetrachloride (S = 0.41%), ethanol (S = 2.51%), and toluene (S = 1.52%). Our results are in accordance with those found in the literature, which proved a high solubility of bacterial and cyanobacterial EPSs in water when compared with in organic solvents (Challouf et al. 2011; Kielak et al. 2017). This led us to assume that *Graesiella* EPSs have numerous hydrophilic residues in their structure. However, further structural analyses are needed to confirm this hypothesis.

Structural properties

The aqueous solution obtained by the EPSs' dissolution (30 g L^{-1} and with a water solubility of 99%) was a light yellow color. The ultraviolet scan spectrum (Fig. 3) analysis of the solution of EPSs showed a maximum absorption peak at

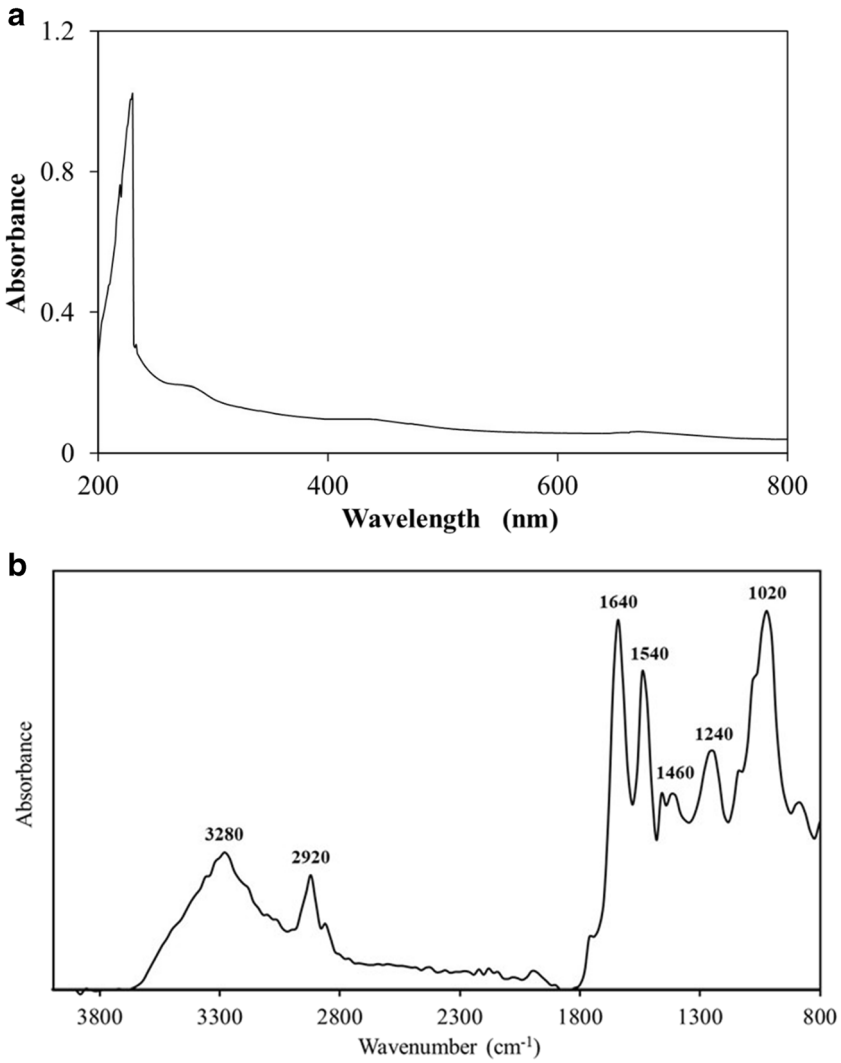

Fig. 3 *Graesiella* EPS spectral analysis: ultraviolet-visible spectrum (30 g L^{-1}) (**a**) and Fourier transformed infrared spectroscopy (FTIR) (**b**)

230 nm, attributed to $n - \sigma^*$ and/or $\pi - \pi^*$ transitions, typically from amine, carboxyl, carbonyl, and ester functional groups (Yun and Park 2003), which can be from the proteins, lipids, and nucleic acids (Okajima-Kaneko et al. 2007). These observations were confirmed by the FTIR spectrum (Fig. 3b). A strong absorption band was observed in the region around 3200 and 2900 cm^{-1}, which is attributed to the stretching of O–H and C–H typical of alkyl and hydroxyl functionality of a carbohydrate. Amide I and amide II were detected at 1540 and 1640 cm^{-1} (Mansour et al. 2010). A strong peak at 1020 cm^{-1}, corresponding to the stretching vibration of ester sulfate groups as S=O (Parikh and Madamwar 2006), was also observed, which confirm the heterosulfated nature of the *Graesiella* EPSs.

Emulsifying activity

Natural emulsifiers (surfactants that enable the combination of an organic phase and an aqueous phase), including polysaccharides, attract interest for their biodegradability and their promising production from renewable resources (McClements et al. 2017). Some exopolysaccharides, extracted from cyanobacteria and chlorophyte strains, have revealed more emulsifying efficiency than other commercial gums (Mishra and Jha 2009; Han et al. 2014). As the EPSs' ability to create emulsions with lipids is a desired property for industrial (e.g., food industry) and ecological applications (e.g., oil-polluted water bioremediation). In this work, the emulsifying capacity was studied on EPSs in solution or on EPS-hydrocarbons (n-hexane and biodiesel) and EPSs-maiz oil mixtures. The EPSs' emulsifying activity obtained for each one was compared with the commercial synthetic surfactant Tween-20.

EPSs from *Graesiella* sp. at different concentrations were used and the emulsion indices (E_{24}) were determined after 24 h of storage at ambient temperature. The high E_{24} reveals high stability of the formed emulsions. As shown in Fig. 4 and Table S1, the *Graesiella* EPSs might emulsify maize oil, *n*-

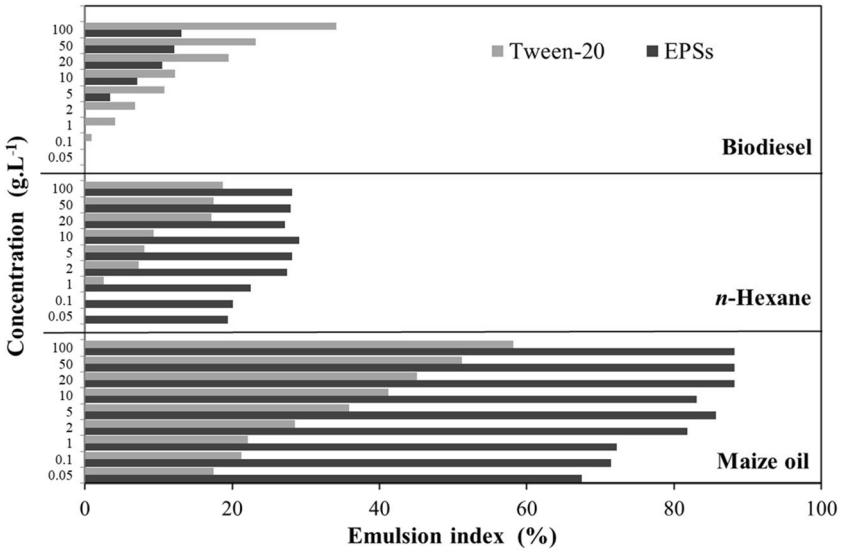

Fig. 4 Emulsifying activities of aqueous solutions of *Graesiella* EPSs and Tween-20 (concentrations between 0.05 and 100 g L^{-1}) with maize oil, *n*-hexane, and biodiesel. Each value is the mean of six replicate measurements

hexane, and biodiesel. The emulsifying activities increased with the increase of the EPSs' concentration and were higher with maize oil than with *n*-hexane. The lowest emulsifying activity was observed with biodiesel. The highest emulsion index was observed with the maize oil, which was 88% at a concentration of 100 g L^{-1}. A similar trend was observed for EPSs derived from bacteria and cyanobacteria, with more efficiency with oils than hydrocarbons (Han et al. 2014; Kielak et al. 2017).

Graesiella EPSs emulsifying activity was compared with the commercial synthetic surfactant Tween-20 (Fig. 4). It was revealed that the emulsifying activity of *Graesiella* EPSs with the maize oil and *n*-hexane was greater than with Tween-20 in all tested concentrations. EPS–maize oil emulsions were stable at 4 °C during 1 month of incubation. No sedimentation, flocculation, or coalescence of emulsions was observed. For the *n*-hexane, the emulsifying activity values were between 19 and 28%, higher than with Tween-20 (0 and 18%), forming stable emulsions that did not break up to 20 days after their preparation. However, compared to biodiesel, Tween-20 had more effect than *Graesiella* EPSs, with emulsifying activity of 34% for Tween-20 and 13% for EPSs at the same tested concentration (100 g L^{-1}). A similar trend was observed in the case of *Nostoc flagelliforme* EPSs where the emulsifying activity increased with the concentration, and the emulsion-stabilizing capacity for several compounds was higher than 50% (Han et al. 2014). The good emulsifying activity of *Graesiella* EPSs for maize oil was probably due to their hydrophobic nature, owing to the presence of proteins and anionic sulfate groups (Gutiérrez et al. 2009).

An emulsion is a macroscopic diffusion of two liquids, in which one compound is a continuous part dispersed all over small droplets of the other. Among other characteristics, rheological properties, physical stability, coalescence, sedimentation, and emulsion depend on the size and distribution of droplets. The emulsion droplet size and homogeneity are important parameters for EPS industrial applications, where smaller droplets can result in more stable emulsions (Kim and Morr 1996; Horozov et al. 2007). The results illustrated that *Graesiella* EPSs produced emulsions with *n*-hexane and maize oil with quite small and uniform droplets of lipids in the range of 0.2–10 μm (Fig. 5). On the other hand, in the presence of biodiesel, droplets were bigger and more dispersed, with a maximum diameter of 30 μm. The observed bioemulsifying ability of forming stable emulsions with vegetable oil (maize oil), hydrocarbon mixtures (biodiesel), and aliphatic (*n*-hexane) hydrocarbons shows that *Graesiella* EPSs are useful as an emulsion-forming agent for the bioremediation of pollutant oil, thus being a safe alternative to chemical emulsifiers.

Flocculating activity

Flocculants, divided into synthetic organic flocculants and bioflocculants, are extensively used in the chemical and mineral industries, in wastewater treatment and in food processing. Bioflocculants have greater advantages than the synthetic ones, according to biodegradability and safety. Several exopolysaccharides produced by bacteria and algae have shown flocculating ability and are considered as unconventional flocculants (Zhu et al. 2012; Ben Rebah et al. 2018).

In this research, flocculation reactions were performed at different concentrations of EPSs in the range 0.05–100 g L^{-1} and compared with alginate and aluminum sulfate $Al_2(SO_4)_3$, commercial flocculants. As shown in Fig. 6 and Table S2, with kaolin clay as the suspended solid, *Graesiella* EPSs had the highest flocculating activity (9.5), at a concentration of 2.0 g L^{-1}, with a corresponding flocculating percentage of 95%, which is significantly higher than the flocculating activity (7.6) and flocculating percentage (87%) to $Al_2(SO_4)_3$ and

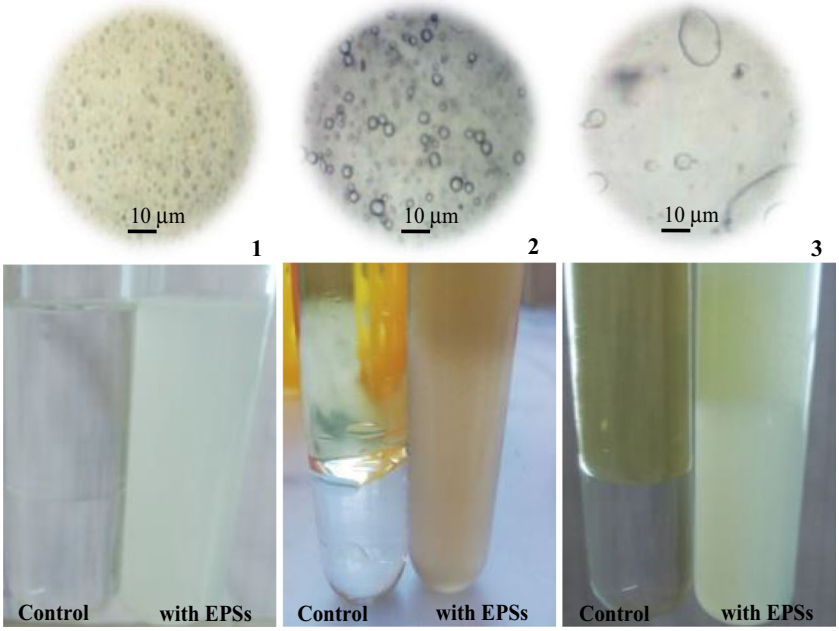

Fig. 5 Emulsions and droplets present in the solution of *Graesiella* EPSs of 100 g L^{-1} with (1) *n*-hexane, (2) maize oil, and (3) biodiesel, after 24 h at room temperature. Control is the solution without *Graesiella* EPSs

to alginate (flocculating activity of 0.92 and flocculating percentage of 15%). Flocculating activities higher than 55% are often observed in the case of some cyanobacteria and chlorophyte EPSs (Zhu et al. 2012; Han et al. 2014) but commonly bacterial EPSs have a flocculating activity lower than 50% (Bala Subramanian et al. 2010; Li et al. 2014).

Bridging may be used to explain the mechanism of flocculation to neutral or like-charged bioflocculants (Sobeck and Higgins 2002). Indeed, polysaccharides play a main role in sludge flocculation, due to their aptitude in forming bridges between their negatively charged groups and the divalent cations existing in sludge. Moreover, many studies reported that proteins are the major components for sludge floc formation by hydrophobic interactions and polyvalent cation bridging (Deng et al. 2003; Aljuboori et al. 2013). The advantage of the flocculating activity of *Graesiella* EPSs (concentrations up to 2.0 g L^{-1}) over

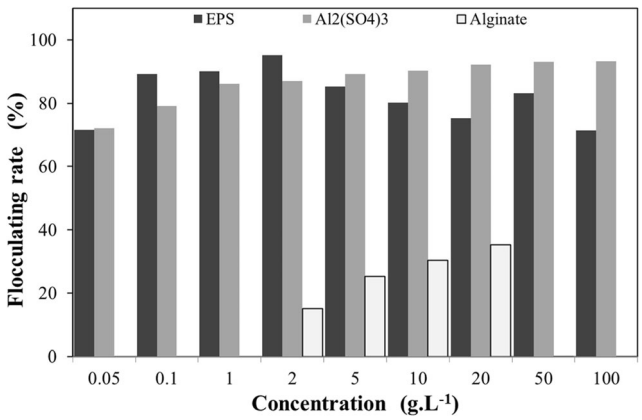

Fig. 6 Kaolin flocculating activity and flocculation percentage (%) with *Graesiella* EPSs, alginate, and $Al_2(SO_4)_3$, at different concentrations (0.05–100 g L^{-1}). Each value is the mean of six replicate measurements

$Al_2(SO_4)_3$ and alginate is a promising result. Thus, *Graesiella* EPSs could be used as a safe bioflocculant for colloid and cell aggregation in several applications, such as water treatment, food manufacturing, and industrial downstream processing.

Kinematic viscosity

Viscosity is one of the most essential properties of fluids and plays a significant role in applications for which the flow is a crucial trait. For diluted polymer solutions, viscometry offers information on the flow behavior under gravity and shear through a calibrated glass capillary viscometer.

The aqueous solution of *Graesiella* EPSs exhibits a shear thinning behavior even for low polymer concentrations. EPSs presented a viscosity of 1.49 mm^2 s^{-1}, significantly higher ($p < 0.05$) than that recorded of xanthan gum, where the viscosity was barely 1.10 mm^2 s^{-1} at the same concentration (0.1 g L^{-1}). The kinematic viscosity rose with an increase in the amount of EPSs, with a maximum of 1.65 mm^2 s^{-1} at 0.5 g L^{-1} (Table 1). To the best of our knowledge, there is no study that investigates the kinematic viscosity of microalgal EPSs. However, our result highlights the high kinematic viscosity of aqueous solution of EPSs when compared with xanthan gum (Brunchi et al. 2016). This result could be explained by the ability of the long EPS macromolecules to form intra- and intermolecular hydrogen bonding, which results in a consequent increase in the viscosity. Indeed, the increase of the EPS concentration led to a rigidification of the polymer network through the enhancement of the molecular contact. EPSs demonstrate a viscoelastic gel-like behavior and/or polymerization in water.

Table 1 Kinematic viscosity ($mm^2\ s^{-1}$) as a function of *Graesiella* EPSs and xanthan gum concentration, at a temperature of 25 °C

Concentration ($g\ L^{-1}$)	*Graesiella* EPSs	Xanthan gum
0.1	1.49 ± 0.05^a	1.10 ± 0.01^b
0.2	1.51 ± 0.02^a	1.21 ± 0.02^b
0.3	1.55 ± 0.08^a	1.32 ± 0.06^b
0.4	1.60 ± 0.04^a	1.42 ± 0.03^b
0.5	1.65 ± 0.01^a	1.51 ± 0.02^b

Each value is the mean of six replicates measurements (± standard deviation). Different letters in the same column indicate significant differences ($p < 0.05$)

To know the impact of temperature on the kinematic viscosity of *Graesiella* EPSs, aqueous solutions with concentrations of 0.1 to 0.5 g L^{-1} were subjected to different temperatures (10 to 60 °C). Similar results were found for all concentrations. Initially, a higher decrease (higher slope = − 0.061) of viscosity with temperature was observed (Fig. 7) and above 30 °C a smaller decrease (slope = − 0.020) occurred. Xanthan gum aqueous solution showed a decrease (slope = − 0.030) in the kinematic viscosity until 30 °C, like EPSs. However, the viscosity to xanthan gum increased (slope = + 0.010) when the temperature was higher than 30 °C. These results are in accordance with the result reported for some cyanobacterial EPSs (Chentir et al. 2017), and the results suggest that the increase in temperature induces a conformational change of EPS chains from a flexible state conformation in a disordered state (with a higher viscosity) to more a rigid shape (with a lower viscosity) in aqueous solution.

The high viscosity is important from the point of view of food product development, to yield good sensory properties such as mouthfeel and flavor release properties of foods. The rigid shape state could be used in the treatment and purification of polluted industrial water.

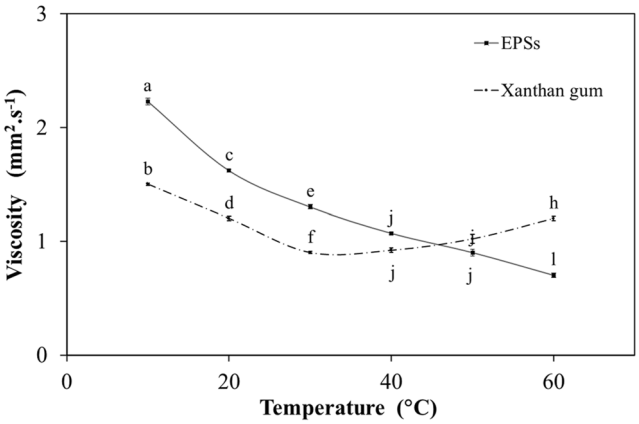

Fig. 7 Effect of temperature on the kinematic viscosity of the aqueous solution (0.2 g L^{-1}) of *Graesiella* EPSs and the commercial polysaccharide xanthan gum. Each value is the mean of six replicate measurements. Different letters indicate significant differences ($p < 0.05$) between EPSs and xanthan gum

Antimicrobial activity

The antimicrobial activity of aqueous extracts of *Graesiella* EPSs was studied against two fungal cells and four bacterial strains.

Antifungal activity

The antifungal activity of EPSs against *B. cinerea* and *F. oxysporum* was studied by measuring the inhibition percentage. As shown in Table 2, the antifungal activity rises significantly with increased EPS concentration. The growth of both *B. cinerea* and *F. oxysporum* was inhibited, reaching respectively 71 and 87%. The results obtained in the control plates showed growth equivalent to 2/3 ($I = 33\%$) of the total plate surface after 24 h, while after 72 h, the entire surface was covered by the fungus ($I = 0\%$). Compared with other tested fungicides, the antifungal activity of EPSs was higher against *F. oxysporum*, even with the use of dithiocarbamates, which are known as the most heavily used organic fungicide ($I = 31\%$), than against *B. cinerea*, which was completely inhibited (Rafin et al. 2000; Qin et al. 2012).

In the current research, the mycelium growth of *B. cinerea* and *F. oxysporum* was subjected to different concentrations of EPSs (Table 2). It is noted that the aqueous extract of EPSs exerts an inhibiting action on mycelium growth and spore germination. An increase in the concentration of EPSs results in a decrease in the growth of the mycelia. The inhibition percentage of the mycelium growth of *B. cinerea* ranged from 31 to 68%. It was between 10 and 68% for *F. oxysporuim*.

To support the antifungal activity of EPSs, the fungal spore germination assay was also done. The results of the spore germination experiment revealed that EPSs show considerable inhibition of spore germination of *F. oxysporum* and *B. cinerea* compared with the untreated cultures (Fig. 8). A decrease in spore germination was observed with the increase in the EPS concentration and a 100% inhibition of spore germination was observed at a concentration of 1.2 g L^{-1} for *F. oxysporum* and 1.8 g L^{-1} for *B. cinerea*.

Although there are several studies reporting antibacterial and antifungal activity, mainly from chlorophyte species (e.g., Debro and Ward 1979; Xiao and Zheng 2016), to the best of our knowledge, no one investigated the antifungal activity of aqueous extracts of EPSs against *B. cinerea* and *F. oxysporum*. The present results show the vigorous ability of the *Graesiella* EPSs as a fungicide agent.

Antibacterial activity

The antibacterial activity of *Graesiella* EPSs assessed at 15, 30, and 60 g L^{-1} using the agar diffusion was evaluated via the inhibitory zone against bacteria strains. Results showed (Table 3) that aqueous extracts of EPSs have a bactericide

Table 2 Growth inhibition (*I*; %) of fungal cells and mycelia of the fungal cells as a function of *Graesiella* EPS concentration after 20 days of cultivation

	EPS concentration		
	15 g L^{-1}	30 g L^{-1}	60 g L^{-1}
Fungal cells			
Botrytis cinerea	33.12 ± 1.28^{a}	67.32 ± 1.08^{c}	71.12 ± 1.14^{e}
Fusarium oxysporum	16.67 ± 1.17^{b}	73.31 ± 1.18^{d}	87.10 ± 1.23^{f}
Mycelia of the fungal cells			
Botrytis cinerea	31.19 ± 1.78^{a}	40.21 ± 1.58^{b}	68.34 ± 2.28^{d}
Fusarium oxysporum	10.21 ± 1.89^{c}	37.15 ± 2.08^{b}	68.02 ± 1.18^{d}

Each value is the mean of six replicates measurements (± standard deviation). Different letters in the same column indicate significant differences ($p < 0.05$)

Table 3 Antibacterial activity (zone inhibition expressed in mm) of *Graesiella* EPSs in aqueous solution against different organisms tested by agar well diffusion method

Strain	EPS concentration		
	15 g L^{-1}	30 g L^{-1}	60 g L^{-1}
Streptococcus pneumoniae	10.12 ± 1.50^{a}	15.61 ± 3.81^{b}	15.91 ± 0.36^{b}
Vibrio harvaeyi	2.67 ± 0.68^{a}	5.54 ± 0.15^{b}	18.24 ± 0.25^{c}
Vibrio anguillarum	–	–	–
Listonella anguillarum	–	–	–

Each value is the mean of six replicates measurements (± standard deviation). Different letters in the same column indicate significant differences ($p < 0.05$)

–, no growth was detected

effect against both *Vibrio anguillarum* and *Listonella anguillarum* even at the lowest tested concentration (15 g L^{-1}). A higher inhibition zone was also found against *Vibrio harveyi*, at 60 g L^{-1} of EPSs, but *Streptococcus pneumoniae* was shown to be more susceptible to the aqueous extracts of EPSs at lower concentrations. The few published studies show that EPSs from microalgae exhibited varying degrees of antimicrobial activity against Gram-positive and Gram-negative bacteria and proposed several possible antibacterial mechanisms of EPSs, such as antibiofilm formation, disrupting the cell wall, and blocking adhesion of pathogens to host cells (Raposo et al. 2014). The antifungal and antibacterial activities could be correlated to the sulfated nature of EPSs (Ghasemi et al. 2007; Raposo et al. 2014) and/or the presence of extracellular DNA, which acts as a chelator of cationic antimicrobials through its negative charge (Mulcahy et al. 2008).

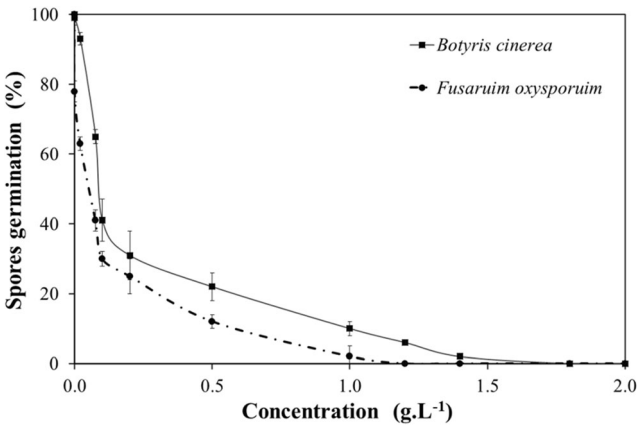

Fig. 8 Impact of the aqueous extract of *Graesiella* EPSs on spore germination in *Botytiscinerea* and *Fusarium oxysporum* at different concentrations (0.02–2.0 g L^{-1}). Each value is the mean of six replicate measurements

Radical scavenging ability

DPPH (1,1-diphenyl-2-picrylhydrazyl) is the most known stable, free, and radical species used for the evaluation of the radical scavenging potential of a range of antioxidants. The free radical scavenging activity of EPSs demonstrated that EPSs exhibit a similar curve of antioxidant activity compared with L-ascorbic acid (Fig. 9). All tested concentrations of EPSs showed a higher ($p < 0.05$) DPPH scavenging ability than L-ascorbic acid, reaching a maximum at 2.0 g L^{-1} with a maximum of 70%, compared with 51% for L-ascorbic acid. The IC_{50} value (0.21 g L^{-1}) to EPSs was significantly lower than that in L-ascorbic acid (1.07 g L^{-1}) (Fig. 9). *Graesiella* EPSs displayed evident scavenging activities on DPPH compared with *Chlorella pyrenoidosa* EPSs ($IC_{50} = 2.14$ g L^{-1}) and comparable with the EPSs isolated from *Chlorella vulgaris* ($IC_{50} = 1.86$ g L^{-1}) (Zhang et al. 2019). This result indicates that *Graesiella* EPSs have a marked inhibitory effect on the DPPH radical.

Superoxide anions are a precursor of more reactive oxidative species, such as singlet oxygen and hydroxyl radical, and are implicated in the process of lipid peroxidation (Michelson et al. 1977). The superoxide scavenging activity of EPSs increased clearly with the increase in concentration. Thus, at all tested concentrations, EPSs displayed a higher inhibitory effect than L-ascorbic acid on superoxide anion formation and achieved 83% at a concentration of 2.0 g L^{-1}, compared with 70% for L-ascorbic acid. The IC_{50} value of *Graesiella* EPSs was estimated to be 0.22 g L^{-1}, a value lower than the IC_{50} of L-ascorbic acid which was 0.53 g L^{-1}. *Graesiella* EPSs showed a significant ($p < 0.05$) superoxide radical scavenging ability which has already been reported for bacterial EPSs (El-Newary et al. 2017). Previous reports on the scavenging capacity of chlorophyte EPSs have shown the possibility of sulfate content being correlated with the potent activity (Ghasemi et al. 2007; Raposo et al. 2014). *Graesiella* EPSs carry negative charge (data not shown), which could increase the intramolecular repulsive

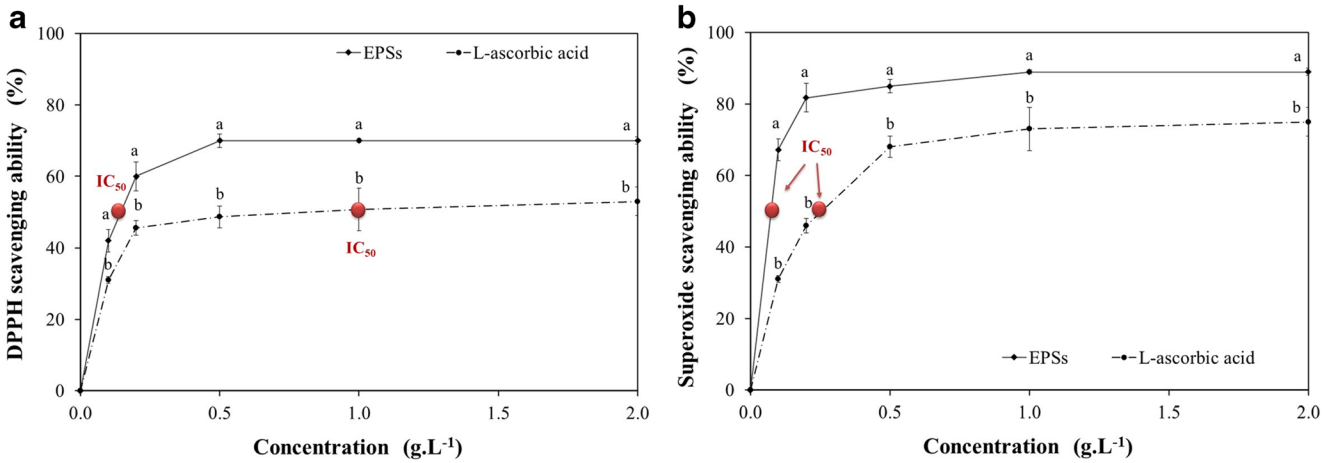

Fig. 9 DPPH (**a**) and superoxide radical (**b**) scavenging abilities of the aqueous extract of *Graesiella* EPSs at different concentrations compared with the commercial L-ascorbic acid antioxidant. Each value is the mean of three replicate measurements. Different letters indicate significant differences ($p < 0.05$) between concentrations

force and make the molecule extended. This reduces the steric hindrance for free radicals to attack (Duh et al. 1999; Kishk and Al-Sayed 2007). Thus, the free radicals become more likely to be scavenged, and EPSs exert excellent antioxidant activity.

Hence, *Graesiella* EPSs produced on a raceway pond present radical scavenging ability, which could be used as natural antioxidants in food industry and as an alternative to synthetic anti-aging reagent.

Conclusion

The aim of this research work was to investigate various applications of the thermophilic chlorophyte *Graesiella* EPSs in view of their integral (biomass and EPSs in the culture medium) industrial valorization. *Graesiella* sp. cultivated in a raceway pond showed high biomass and large amounts of EPSs produced from the start of the exponential growth phase. *Graesiella* EPSs are composed mainly of polysaccharides and proteins, with a high presence of sulfate groups. The biochemical composition and physicochemical properties give the following to *Graesiella* sp. EPSs a:

- high kinematic viscosity compared with xanthan gum, which may be useful in various fields including food and medicine to provide stability to the emulsion for long periods and/or increase the medium viscosity
- higher emulsifying and flocculating activity than Tween-20, alginate, and Al_2SO_3, which may be useful as an emulsion-forming agent for the bioremediation of oil-polluted soil and water, and for food and cosmetic applications, as a safe bioflocculant and bioemulsifier
- strong antibacterial and antifungal activity, which may be useful in the field of agriculture for bacterial and fungal infection treatments
- potent scavenging ability, higher than L-ascorbic acid, which may be useful as a dietary supplement in food products

This study shows *Graesiella* sp. to be a successful microalgal EPS producer for biotechnological and industrial exploitation.

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