Extracellular polymeric substances with high radical scavenging ability produced in outdoor cultivation of the thermotolerant chlorophyte *Graesiella* sp.

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Abstract

The present study developed a two-step strategy to enhance the production of extracellular polymeric substances (EPSs) by a thermotolerant chlorophyte, *Graesiella* sp., in view to their industrial valorisation. In the first step, *Graesiella* sp. was grown in outdoor conditions in pilot-scale photobioreactors of 100 L culture volumes. In the second step, the biomass collected in the exponential growth phase was submitted to heat stress (50 °C). A joint production of biomass reaching 0.50 g dw L⁻¹ day⁻¹ and of EPS production reaching 1.30 gdw L⁻¹ in 2 days was obtained. EPSs mainly contained polysaccharides (80%) and proteins (14%). FTIR and 1HNMR revealed the presence of primary amine and sulfated groups. The EPSs contained antioxidant enzymes (SOD, CAT, and APX) maintained in an active state by the microenvironment offered by the EPSs. The EPSs were found to have a potent antioxidant activity via directly scavenging free radicals when compared to L-ascorbic acid.

Keywords Microalgae · *Graesiella* · Chlorophyta · EPSs · Antioxidant enzymes · Characterization · Radical scavenging · Outdoor cultivation · Two-step strategy

Introduction

In various sectors such as food, cosmetic, and health, the market is growing rapidly in the support of the development of “bio” products and in a global context of demand for reduction of chemical additives. Recently, it has been shown that the microalgal field has significant potential (Vigani et al. 2015) supporting the transition from a fossil fuel-based economy and the global mass-market industry to a “circular bioeconomy” responding to a growing demand of civil society. Although the total production volumes and market size of products derived from microalgae increased 5-fold since the beginning of the century, they are still relatively small and little diversified with respect to alternative sources (Enzing et al. 2014). In this context, the mobilization of new microalgal resources with the versatility of applications (human and animal food and bioactives) would make original contributions to improve the potential of microalgae as a future source of renewable bioproducts.

Among these resources, thermotolerant microalgal strains have exceptional potential. Several authors have found that these microalgal species, with high optimal growth temperature, have higher productivity (Bleeke et al. 2015; Xiao and Zheng 2016), especially in summer outdoor climatic conditions where high temperatures often result in an inhibition of the growth of common microalgal species. Furthermore, as a consequence of growth at high temperatures, thermotolerant microalgae can possess wide possibilities for physiological adaptation and genetic modifications that make them potential producers of high-value thermo-stable bioactive compounds (Haki and Rakshit 2003).

Almost all of today’s industrial microalgae products of interest are derived from their intracellular biomass. However, microalgae can also release various extracellular polymeric substances.
substances (EPSs) into the medium (Xiao and Zheng 2016; Ekelhof and Melkonian 2017; Gaignard et al. 2018) a resource which is still largely untapped. They can be the key solution for the effective economic sustainability of the microalgae industry.

Extracellular polymeric substances (EPSs), also called exopolysaccharides, represent a class of valuable polymers. The common EPSs of most microalgae are of heterosulfated polysaccharidic nature, containing a large amount of carbohydrates in addition to proteins, fats, nucleic acids, and inorganic substituents (Rossi and De Philippis 2016; Halaj et al. 2018). Thermotolerant microalgae species have developed specific extracellular polymeric substances that make the cell grow and flourish under extreme temperature conditions (Mezhoud et al. 2014; Varshney et al. 2015) but real strategies to their industrial production have scarcely been studied.

EPSs are currently used as emulsifier, thickener, flocculent, and gelling agents (Mishra et al. 2011; Han et al. 2014). Certain microalgal EPSs are reported to possess diverse biological activities including anti-inflammatory (Baе et al. 2006), immunomodulatory (Guzmán et al. 2003), antitumor (Raposo et al. 2014), antiviral (Huheihel et al. 2002), antifungal, and antibacterial (Abedin and Taha 2008) activities.

Antioxidant and reactive oxygen species (ROS) scavenging properties of EPSs have been also reported in several microalgal species (Bkhairia et al. 2016; Zhang et al. 2019a, b), although, rarer than those reported from filamentous fungi (Lin et al. 2012) and bacteria (Li et al. 2013). In general, the antioxidant activity of microalga EPSs is attributed to their polysaccharidic fraction or inorganic substituents as sulfated groups (Tannin-Spitz et al. 2005; Sun et al. 2014). Protein fractions in EPSs are also implied in the antioxidant activity of several microalgae/cyanobacteria species (Gao et al. 2015). The ROS-scavenging role of EPSs produced by *Microcystis aeruginosa* was attributed to the water-soluble protein MAAs (mycosporine-like amino acids) (Hu et al. 2015).

Reactive oxygen species (ROS) and oxygen-derived free radicals produced in all vital organs are very dangerous and contribute to pathological effects and many illnesses, including DNA damage, cellular degeneration, Alzheimer’s, cancer, liver injury, and heart diseases (Gioti et al. 2007; Valko et al. 2007). Currently, synthetic antioxidants such as propylgallate, butylated hydroxytoluene, and butylated hydroxyanisole are used in order to reduce damage (Gülçin 2006) but they are suspected to be responsible of liver damage and carcinogenesis (Orćić et al. 2011). Therefore, the search for natural resources as potential and safe antioxidants is becoming important (Li et al. 2007).

Given the above considerations, the purpose of this study was to increase the production of EPSs from the thermotolerant chlorophyte strain *Graesiella* sp., isolated from Tunisian geothermal waters and in the perspective of industrial valorization. In view to make results more useful for industrial extrapolation, the experimental cultures were grown at pre-pilot scale (100 L culture volume) and in outdoor conditions. A two-step strategy was employed to enhance EPS production. Furthermore, the obtained EPSs were characterized using chromatographic and spectrophotometric methods, and EPSs antioxidant activity (total antioxidant, α,α-diphenyl-β-picrylhydrazyl radical scavenging, iron-reducing power, hydroxyl radical scavenging, and hydrogen peroxide radical scavenging) was studied. Due to their high importance, the presence of antioxidant enzymes in the EPSs received particular attention.

**Materials and methods**

**Algal strain and culture medium**

A thermophilic green algal strain identified by phylogenetic analysis as *Graesiella* sp. (Mezhoud et al. 2014) isolated from microbial mats anchored to submerged stones, in a hot spring (Ain Echffa) located in the northern part of Tunisia (36° 49’ N, 10° 34’ E) at a water temperature of 60 °C was used. The *Graesiella* strain (axenic colonies) was cultivated in batch culture under sterile conditions in Bold’s Basal Medium (BBM) (Bischoff and Bold 1963) modified according to the Elser concept for freshwater microalgae, with C:N:P ratio equal to 166:20:1 (Tang and Dam 1999). The strain was grown preliminarily in laboratory under optimal growth conditions at 30 ± 1 °C and 120 μmol photons m−2 s−1 as suggested by Mezhoud et al. (2014). The increase in the culture volume up to 20 L was carried out gradually by weekly subcultures in Erlenmeyer flasks.

**Experimental cultures**

To enhance the EPS production, *Graesiella* sp. was subjected to temperature stress conditions for 2 days, after the alga was grown in outdoor conditions; and step II: the stress step, where the concentrated biomass provided from step I, was subjected to temperature stress conditions for 2 days, after total medium renewal.

**Growth step—step I**

In this initial step, growth was achieved outdoor under August environmental conditions at the facilities of the Spanish Bank of Algae (BEA), Gran Canaria (Canary Islands). Cultures were grown in enclosed cylindrical photobioreactors consisting on glass columns (20 cm × 110 cm = diameter × height) and 108 L volume each and exposed for 9 days to natural light and temperature conditions. The pre-cultures (see the “Chlorophyta strain and culture medium” section) in the stationary phase were used to inoculate (Bt of
0.08 $g_{dw} \cdot L^{-1}$) 100 L culture volumes. The temperature in the water culture was between 30 and 35 °C, during the whole experimental period. Aeration was supplied 24 h on the day by an air compressor at a flow rate of 1.5 L min$^{-1}$.

**Stress step—step II**

In this step, the algal biomass harvested at the end of the exponential growth phase—step I by centrifugation (3985×$g$, 10 min at 4 °C) was washed with fresh BBM medium, centrifuged again, and re-suspended in 5 L flat glass flasks, at biomass density of 2 $g_{dw} \cdot L^{-1}$. EPSs induction was ensured by submitting the concentrated biomass to thermal stress for 2 days. Stressed cultures were placed in thermostat chamber (BOECO Orbital Shaker Incubator ES-20/80) at 50 ± 1 °C and 120 μmol photons m$^{-2}$ s$^{-1}$.

**Determination of biomass growth and EPS production**

The algal cell growth was monitored by the daily ($t$) changes in the biomass density. The biomass density ($g_{dw} \cdot L^{-1}$) was determined gravimetrically by measuring daily dry weight. Samples (1 L), in triplicate, were filtered over prewashed 0.2 μm microfiber filters (GF/F filter, Whatman, UK) and dried overnight at 80 °C. The filters were cooled to room temperature in a desiccator prior to weighing.

The EPSs were extracted from the *Graesiella* cultures by stirring the 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 biomass was washed with deionized water and centrifuged. The total recovered supernatant (culture medium and soluble EPS) was concentrated (10 times) by tangential ultrafiltration (Vivaflow 50) with Millipore membranes with a cut-off of 8 kDa. The elimination of low molecular weight substances and inorganics was ensured by a cycle of consecutive concentrations and dilutions with ultrapure water until constant conductivity (0.017 mS$^{-1}$). The recovered filtrate rich in EPS was freeze-dried and weighed for gravimetric EPS content determination.

The biomass and EPS productivities ($P_t; g_{dw} \cdot L^{-1} \cdot day^{-1}$) at any culture time ($t$) were calculated from Eq. 1 with $C_0$ and $C_t$ as the dry mass density ($g_{dw} \cdot L^{-1}$) at start and time $t$ (day), respectively.

$$P_t = \frac{C_t - C_0}{t} \quad (1)$$

**Biochemical characterization**

The carbohydrate content in the EPSs was determined by the phenolsulfuric acid method according to Dubois et al. (1956). In this method, the sugars in the EPSs are hydrolyzed by concentrated sulfuric acid during the phenol-sulfuric assay and form monomers, namely glucose, fructose, and galactose. The carbohydrate concentration was obtained using a calibration curve of glucose (99.5%, Sigma-Aldrich, G8270) and the absorption was recorded at 490 nm. Protein content was determined according to Lowry et al. (1951) using bovine serum albumin (98%, Sigma-Aldrich, B2064) as standard. Lipid amount was determined using the method of Folch et al. (1957). The elemental analysis (C, N, H, S, and P) of samples was conducted using a Flash Elemental Analyzer 1112. The results were expressed in % relative to the EPS dry weight ($d_w$).

**Fourier transformed infrared spectroscopy analysis**

Fourier transformed infrared (FTIR) spectra of the EPSs were recorded 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 by the Agilent software.

**Proton nuclear magnetic resonance spectroscopy analysis**

Proton nuclear magnetic resonance ($^1$HNMR) spectroscopy of the EPSs was achieved on Bruker ASX400-WB spectrometer equipped with a double resonance (1H/X) Broad Band Inverse z-gradient probe head. Samples were exchanged in 25 μL deuterated water ($D_2O$) with intermediate freeze-drying. Chemical shifts were expressed in ppm downfield from the signal of the methyl group of internal acetone ($\delta^1H \quad 2.225$ ppm at 300 K). The samples were analyzed in 5 mm susceptibility matched tubes (Shigemi, Japan). Integration of spectra and data analyses was performed with MESTRENOVA (version 2016).

**X-ray diffractometry analysis**

X-ray diffraction analysis of the crude EPSs was performed with an X-ray diffractometer CICECO Empyrean (JDX 3532; Japan). The diffraction angle range of observation was 5 to 60°, with a scan step of 0.02.

**High performance liquid chromatography analysis**

The high performance liquid chromatography (HPLC) apparatus used for the experiments was an Agilent Technologies HP 1200 series system, equipped with two preparative pumps (G1361A), a degasser, an injection valve with a 20 μL and 5 mL loops, an autosampler (DLA G2258A), a diode array multiple wavelength detector SL (G 1315D), and an Alltech conductivity detector model 650. A volume injection of 1 mL of water-soluble EPSs filtered through 2 μm syringe filter was
analyzed by reverse-phase HPLC. The analytical column used was an Agilent PL Aquagel OH 50 column (300 mm × 10 mm × 8 μm). The identification of compounds was based on the retention time, the UV visible spectra, and the conductivity. All samples were injected via a DLA G2258A autosampler provided with a 5 mL syringe. The EPS compounds were separated in isocratic conditions, using deionized water as mobile phase and detected at 246 nm. The flow rate was 1 mL min⁻¹ during the separation time (17 min).

**Electrophoresis profile**

Enzymatic hydrolysis of the EPSs (20 mg<sub>dw</sub> mL⁻¹) was performed using 0.05% Protamex (Novozymes A/S, Denmark, produced by Bacillus protease, containing both enzymes Neutrase and Alcalase). The mixture (enzyme + EPS solutions) was stirred continuously with a propeller in a water bath at 50 °C for 180 min. The pH was adjusted and kept at 8.0 by adding 1 M NaOH during the reaction (Córdova-Murueta et al. 2003). After that, the mixture was heated at 100 °C for 5 min and cooled to room temperature in order to stop the proteolysis. The obtained solution was centrifuged for 30 min at 5000×g and at 4 °C to obtain EPS protein hydrolysate.

Subunit components of the obtained EPS protein hydrolysate were analyzed using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Electrophoresis analysis was carried out in a vertical chamber using 12% polyacrylamide gel with sodium dodecyl sulfate (Laemmli 1970). Samples (20 μL) of the EPS hydrolysate were boiled for 15 min at 100 °C in 5 μL of sample buffer containing 0.5 mM Tris-HCl, pH 6.8, 25% glycerol, 1% bromophenol blue, 10% SDS, and 3% β-mercaptoethanol (added immediately before use). The molecular protein weight marker used was electrophoresis marker mol wt 10–200 kDa obtained from Sigma-Aldrich (mPAGE Unstained Protein Standard, SDS7B2). The SDS-PAGE was carried out with a constant current of 1–2 mA per track for 2–3 h. After electrophoresis, the gel was stained with Coomassie Blue overnight and the discoloration of the gel was made up with solution containing 40% ultrapure water, 40% methanol, and 10% acetic acid. Images of the gel were analyzed by the Software Gel Analyzer.

**Antioxidant enzymes assays**

The EPSs were used for the determination of enzymatic antioxidant superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX). A spectrophotometric quantification was used for the above-mentioned assays using a Beckman coulter DU 640B spectrophotometer. All the determinations were made in triplicate at ambient temperature. Enzymatic activities were expressed as unit per mg of protein. One unit is defined as the quantity of enzymes that catalyze the conversion of 1 μmol of substrate per min. Standard curves for all enzymes analysis were obtained at 4 °C using enzymatic assays kits (Merck Sigma-Aldrich).

CAT activity was determined following the decomposition of hydrogen peroxide (H₂O₂) spectrophotometrically at 240 nm, in a reaction mixture containing 50 mM H₂O₂ in 50 mM potassium phosphate buffer (pH 7) (Aebi 1984). APX activity was determined according to Simonetti et al. (2010), following the decrease in absorbance at 290 nm, of a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbate, 0.1 mM EDTA, and 0.1 mM H₂O₂. SOD activity was performed by using an EnzyChrom Superoxide Dismutase Assay Kit (ESOD-100). SOD activity was determined by calculating the difference between the absorbance at 0 min and 1 h, at wavelength of 440 nm. The obtained results were compared to the different concentrations of SOD in the ESOD-100 kit.

**Antioxidant activity**

**Total antioxidant capacity**

Total antioxidant capacity (TAC) was studied by the reduction of the green phosphomolybdenum complex according to Prieto et al. (1999). TAC was evaluated by the reduction of Mo (VI) to Mo (V) and measured by the absorbance at 695 nm. Briefly, 100 μL of EPS water solution (concentrations ranged from 20 to 2000 μg<sub>dw</sub> mL⁻¹) was added to 1 mL of a reagent mixture and incubated for 1 h at 95 °C. The reagent mixture solution consisting of 0.588 mL sulfuric acid, 0.049 g ammonium molybdate, and 0.036 g sodium phosphate made up to 10 mL with distilled water. The absorbance at 695 nm was used to evaluate the variation of the total antioxidant capacity. L-Ascorbic acid solution was prepared in the same conditions and was used as a standard (positive control).

**α,α-Diphenyl-β-picrylhydrazyl radical scavenging activity**

The α,α-diphenyl-β-picrylhydrazyl (DPPH) radical scavenging activity was evaluated according to the method described by Shimada et al. (1992) with slight modifications. The EPS solutions (concentrations ranged from 20 to 2000 μg<sub>dw</sub> mL⁻¹) were mixed with 500 μL of a methanolic solution containing DPPH⁻ (0.02%). The mixture was then incubated for 1 h in the dark, at room temperature. A control solution containing DPPH⁻ without EPSs was also prepared. The absorbance was measured at 517 nm and normalized to the DPPH⁻ control solution. The same procedure was made with L-ascorbic acid as a standard. The scavenging activity of DPPH⁻ (%) was determined using the Eq. 2 where A<sub>sample</sub> is the absorbance of the sample solution and A<sub>control</sub> is the absorbance of the sample control solution. The IC<sub>50</sub> defined as the
concentration of sample that scavenged 50% of DPPH• radical, was determined.

\[
\% = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100
\]  

Iron-reducing power The method developed by Adjimani and Asare (2015) was used for testing the iron-reducing power. The EPS solutions (concentrations ranged from 20 to 2000 μgdw mL⁻¹) or distilled water (control) were mixed separately with 0.5 mL of sodium phosphate buffer (0.02 M, pH 7) and 2.5 mL of potassium ferricyanide (1%, w/v). The mixture was then incubated for 30 min in a 50 °C water bath. After cooled, the resulting solution was mixed with 1.5 mL of 10% trichloroacetic acid and centrifuged (800×g for 10 min). The supernatant (2 mL) was then mixed with 0.5 mL of ferric chloride (0.1%, w/v). After allowing the reaction to proceed for 10 min, the absorbance at 700 nm was measured. The scavenging activity (%) was calculated using the Eq. 2. The same procedure was made with L-ascorbic acid as standard. The IC₅₀ was also determined.

Hydroxyl radical scavenging activity The hydroxyl radical (‘OH) scavenging activity was evaluated according to Chandran et al. (2013) where the ‘OH oxidize the Fe³⁺ into Fe²⁺, which react with 1,10-phenanthroline to form a red compound (1,10-phenanthroline-Fe²⁺) with a maximum absorbance at 536 nm. The concentration of ‘OH was determined by the degree of decolorization of the reaction solution. The EPS solutions (concentrations ranged from 20 to 2000 μgdw mL⁻¹) were mixed separately with 1,10-phenanthroline solution (1.0 mL, 1.865 mmol L⁻¹) and centrifuged and the supernatant absorbance measured. The ‘OH scavenging activity (%) was calculated using Eq. 2. The same procedure was made with L-ascorbic acid as standard. The IC₅₀ was also determined.

Hydrogen peroxide scavenging activity The hydrogen peroxide (H₂O₂) scavenging ability was determined following the procedure of Ruch et al. (1989). The EPS solutions (concentrations ranged from 20 to 2000 μgdw mL⁻¹) were dissolved in a solution of H₂O₂ (43 mM) mixed with phosphate buffer (0.1 M, pH 7.4). The absorbance value of the reaction mixtures (A₂₃₀ sample) was recorded at 230 nm. Distilled water was used as a control solution (A₂₃₀ control). The H₂O₂ inhibition (%) was calculated using Eq. 2. The same procedure was made with L-ascorbic acid as standard. The IC₅₀ was also determined.

Statistical analysis

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

Results and discussion

Biomass and EPS production

In this work, Graesiella sp. isolated from the Tunisian hot spring water was grown under controlled conditions in a large-scale outdoor photobioreactor of 100 L culture volume. Graesiella sp. biomass and EPS kinetics were evaluated by following the increase in biomass density and EPS concentration, of the batch cultures grown over 9 days under natural environmental conditions (Fig. 1). The biomass kinetics (Fig. 1a) showed a typical sigmoidal shape and the highest biomass density obtained was of 1.94 ± 0.25 gdw L⁻¹. The highest biomass productivity was registered at day 2 of culture and reached 0.50 ± 0.02 gdw L⁻¹ day⁻¹. These values are greatly higher than that obtained in optimized Graesiella laboratory culture conditions observed by Zili et al. (2017): 0.40 ± 0.01 gdw L⁻¹ of maximal biomass density and 0.17 ± 0.03 gdw L⁻¹ d⁻¹ as maximal biomass productivity.

Relatively to the EPSs, the production kinetics (Fig. 1b) showed a different shape marked by a long lag phase (4 days). The highest productivity of EPSs (0.38 ± 0.02 gdw L⁻¹ d⁻¹) was recorded later, day 5 of the culture period, with a maximal concentration of 1.46 ± 0.07 gdw L⁻¹ obtained at the end of the experimental culture period. These results are greatly higher than that obtained for Graesiella laboratory culture (0.29 gdw L⁻¹; Mezhoud et al. 2014). Comparing the daily EPS production kinetics to the biomass growth rate time course, it was clear that production of EPSs occurred when biomass growth was reduced. This confirms the role of EPSs in the defense response metabolism as shown in different types of microalgae stresses including thermal ones (Lupi et al. 1991). In most microalgae and cyanobacteria species, EPSs are produced during the stationary growth phase (Delattre et al. 2016). In some cases, EPS production occurred during all growth phases but increases during the stationary one (Fernandes et al. 1989; Lama et al. 1996). However, under specific starvation conditions, some strains such as Nostoc can produce more EPSs in the exponential growth phase (Mehta and Vaidya 1978; Gantar et al. 1995).

In the case where the maximum productivity of biomass and EPSs is in the opposite production phases, the use of the two-stage cultivation strategy is the solution to jointly and continuously ensure a maximized production of the algal biomass and the EPSs. This strategy has been commonly used in the production of several intracellular secondary metabolites.
such as lipids and carotenoids (Hosseini and Shariati 2006; Zili et al. 2017) but rarely for EPSs.

Thus, Graesiella was subjected to a stress condition (step II) to enhance the EPS production. In this step, the biomass recovered at the end of the exponentially growth phase (day 2 of cultivation) was concentrated (2 g dw L\(^{-1}\)) and subjected to higher temperature (50 °C) in order to inhibit the growth, and thus, all the energy was oriented to the EPS synthesis. The EPS production greatly increased in 2 days, reaching 1.30 g dw L\(^{-1}\). This value was slightly lower than that obtained at the end of step I of cultivation but 20-fold more than that recorded at the day 2 of maximum biomass productivity (0.04 g dw L\(^{-1}\)) representing an efficient way to ensure both high biomass yields and high EPS production.

The finding results emphasize that Graesiella sp. is an efficient EPS producer. The EPS production commonly found in Chlorophyta species ranges from 0.38 (Halaj et al. 2018) to 0.94 g dw L\(^{-1}\) (Mishra et al. 2011). Higher EPS rates to that recorded in this work have been reported for some algal strains such as Gyrodinium impudicum (1.347 g dw L\(^{-1}\), Yim et al. 2004), Porphyridium marinum (2.5 g dw L\(^{-1}\), Soanen et al. 2016), and Dictyosphaerium chlorelloides (2.1 g dw L\(^{-1}\), Kumar et al. 2017).

**EPS biochemical characterization**

The EPSs were analyzed for carbohydrate, protein, lipid, and mineral contents (Table 1). High content of carbohydrates (80.31%) with a relatively high amount of proteins (14.10%) and the presence of lipids were detected. These results reveal the heteropolymeric nature of the extracted EPSs from Graesiella sp. The elementary analysis supports this observation with high levels of nitrogen (12.3%), sulfur (0.6%), and phosphorous (0.5%). The total mineral components were less than 2%, which shows that the extracted EPSs were mostly constituted of organic components, demonstrating that ultrafiltration system used was adequate for EPS purification.

The heteropolymeric nature detected in the Graesiella sp. EPSs has been found in most microalgae and Chlorophyta. Compositional analysis of EPSs includes frequently, in
addition to carbohydrates (48 to 80%), several organic substituents such as protein (1 to 42%), lipids (2 to 8.7%), nucleic acids (0.01 to 10.9%), and sulfated groups (up to 10%) (Conrad et al. 2003; Zhu et al. 2012; Xiao and Zheng 2016). The concentrations of these components differ significantly with strain/species and culture conditions.

**FTIR, ¹H NMR, and X-ray analyses**

The FTIR and ¹H NMR spectra confirm the heteropolymeric nature of the *Graesiella* sp. EPSs. The IR spectrum (Fig. 2) displayed a broad band between 3500 and 3000 cm⁻¹ attributed mainly to the stretching vibration (ν) of O-H or N-H and a band centered to 2900 cm⁻¹ attributed to the νC-H (CH₃ and CH₂ sp³) groups, typical from the hydroxyl and alkyl functionality of carbohydrates, respectively.

The ¹H NMR spectrum of the EPSs (Fig. 3) confirms this observation by the signals found in the region 3.5–4.1 ppm, assigned to cyclic proton of many sugar residues and typical for polysaccharides (Kaplan Can et al. 2019).

The prominent absorption observed at 1640 cm⁻¹ in the FTIR, attributed to the bending vibration (δ) of N-H and the νC-N observed at 1240 cm⁻¹, indicates the existence of amino acids from peptide/proteins in the EPSs isolated from the *Graesiella* sp. These observations were confirmed by the ¹HNMR spectrum from the peak signal at 1.3 ppm, assigned to the N-H groups of primary amides and by the alkyl-halide peak observed at 3.1 ppm (Mishra et al. 2011). The peak at 1240 and 1080 cm⁻¹ could be attributed to the νS-O groups and νC-O-S. These data confirm the presence of sulfate groups in the EPSs, as reported for other Cyanobacteria and Chlorophyta exopolymers (Zou et al. 2008; Raposo et al. 2014). The peak recorded at 1020 cm⁻¹, attributed to the νC-O, is characteristic of uronic acids and o-acetyl ester linkage bond in carbohydrates (Bramhachari and Dubey 2006).

The X-ray diffraction (XRD) patterns of EPSs produced by *Graesiella* sp. (Fig. 4) exhibit numerous intense diffraction peaks ranged from 10 to 55° with interplanar spacing (d-spacings) ranged from 1 to 5 Å. The multitude of peaks with relatively sharp thin characteristics indicates high crystalline-like nature of *Graesiella* EPSs which justifies high interaction between the different components.

**Protein and antioxidant enzymes analysis**

The EPS HPLC chromatogram (Fig. 5) recorded at 246 nm showed one prominent peak at 9 min retention time and two
shoulders recorded near 10.5 and 12.5 min indicating that EPSs were constituted by at least two major groups of compounds. The compounds eluted at 9 min showed a great absorption at 200 and 220 nm, and a smaller absorption at 300 nm. On the other hand, the compounds eluted at 10.5 and 12.5 min have an absorbance at 200 nm, without the presence of the absorbance at 300 nm. The absorbance at 300 nm could be attributed to the presence of the hydrolysoluble protein mycosprin amino acids (MAA’s) linked to polysaccharides, confirmed by the yellowish color of EPS aqueous solution. This explains the high conductivity signals observed at 9 min which are probably correlated with the presence of charged amino acids.

The EPS protein content was evaluated at 14.10% of dw (Table 1) which is within the range of proteins reported in some Chlorophyta species, varying from 13 to 50% (Chiou et al. 2010; Goo et al. 2013). The molecular weight of the EPSs proteins determined by SDS-PAGE analysis showed that EPSs are constituted of ten bands gradually shifting from (25 to 195 kDa, Fig. 6), mainly with low molecular weight proteins as shown in some cyanobacteria species (Kawaguchi and Decho 2000).

The bands detected at 35, 69, and 74 kDa could be assigned respectively to the SOD enzyme (Keele et al. 1971), the catalase enzyme (Fu et al. 2014), and the peroxidase enzyme (Nicolas et al. 2003).

The enzymatic investigations of the Graesiella sp. EPSs confirm this hypothesis. Indeed, analysis showed the presence of high activity superoxide dismutase 119.70 ± 5.6 (U SOD) and catalase 13.8 ± 0.14 (U CAT) and peroxidase ascorbate 0.42 ± 0.14 (U APX).

Some extracellular enzymes such as protease (Kellam and Walker 1987), phenol oxidase (Otto and Schlosser 2014), and laccase like enzyme (Otto et al. 2015) have been identified in some Chlorophyta EPSs. Other exoenzymes were also found in several microalgae strains (Liu et al. 2016). However, as far we know, the presence of antioxidant enzymes such as SOD, CAT, and APX is not signaled as constituents of microalgae/cyanobacteria or bacterial EPSs. In fact, enzymatic antioxidants are underlined as principal endogenously defense mechanisms adapted by algae to scavenge ROS excess (Liu et al. 2012). SOD is known as the first line of defense against...
oxygen-derived free radicals (Fridovich 1995), APX as the key enzyme catalyzing the conversion of H$_2$O$_2$ into H$_2$O (Correa-Aragunde et al. 2013), and CAT as the key enzyme present in peroxisomes and mitochondria involved in removal of toxic peroxide (Góth et al. 2004). Maintaining the activity of enzymes in the extracellular matrix suggests a protective microenvironment afforded by EPSs (Hoffman and Decho 1999). Thus, enzymes may be attached to the EPS components through ionic and hydrophobic interactions (Gessesse et al. 2003) and/or embedded in the EPS extracellular matrix. Wingender et al. (1999) suggested that the aspect of the matrix architecture is the main responsible that maintain the activity of exoenzymes. In this regard, the high crystalline nature of Graesiella EPSs constitutes a reinforcing grid that enhances the retention of the enzymes in an active state even at high temperatures (Mazeau and Rinaudo 2004). Such findings suppose a potent antioxidant capacity of Graesiella sp. EPSs particularly via directly scavenging H$_2$O$_2$ and 'OH free radicals.

**Antioxidant activity**

The antioxidant activities have been attributed to various reactions and mechanisms; three types of stages are encountered in every free-radical reaction: initiation, propagation, and termination (Santos-Sánchez et al. 2019). The antioxidant activity of EPSs in vitro was studied by: (i) total antioxidant capacity (TAC) and α,α-diphenyl-β-picrylhydrazyl free radical scavenging activity (DPPH$^-$); (ii) iron-reducing power; and (iii) hydroxyl radical scavenging activity (•OH) and hydrogen peroxide scavenging activity (H$_2$O$_2$), to evaluate the effects on the initiation, propagation, and termination stages, respectively. TAC and the iron-reducing power of EPSs were expressed as L-ascorbic acid equivalent (equivalents per μg EPSsdw) by converting absorbance to equivalent activity of L-ascorbic acid based on standard curves. The scavenging ability of EPSs was compared to L-ascorbic by calculating the half inhibitory concentration (IC$_{50}$; Table 2).

TAC was evaluated by the reduction of Mo(VI) to Mo(V) and measured by the absorbance at 695 nm. Both EPSs and L-ascorbic TAC were dose dependent (Fig. 7).

The highest TAC value obtained at a concentration of 2000 μg$_{sdw}$ mL$^{-1}$ was slightly lower than that obtained by L-ascorbic acid at the same concentration. In terms of L-ascorbic...
acid equivalent, EPSs showed a TAC value of 1.4 μgdw. Our result highlighted a strong TAC compared to the *Chlamydomonas reinhardtii* EPSs obtained in laboratory scale under optimal growth condition where the TAC value was 5.6 μgdw (Bafana 2013).

The EPS antioxidant activity was also determined in relation to the DPPH· (Table 2). The present findings showed that *Græsiella* EPSs had a perceptible DPPH· activity. The inhibitory activity of EPSs varied from 10.61% at the concentration of 20 μgdw EPS mL⁻¹ and reached near 99% at the concentration of 2000 μgdw EPS mL⁻¹. The IC₅₀ of EPSs on DPPH· was 40 μgdw mL⁻¹ which was strongly lower than that of the standard L-ascorbic, evaluated at 100 μgdw mL⁻¹. In the case of other Chlorophyta species EPSs (*Chlorella zofingiensis* and *Chlorella vulgaris*, respectively), the IC₅₀ values were strongly higher reaching 1570 and 1860 μgdw of EPS mL⁻¹ (Zhang et al. 2019a).

The iron-reducing power was studied and compared to L-ascorbic acid (Fig. 8). It was clear that the increase of the EPS concentration induces the increase of the absorbance which means the increase of the reducing power. The iron-reducing power of EPSs attends its maximum potential to a concentration of 100 μgdw mL⁻¹. However, at whatever concentration tested, L-ascorbic acid showed higher (p < 0.05) reducing power, which suggests weaker ability of the EPSs as an electron donator. In terms of L-ascorbic acid equivalent,

### Table 2  Radical scavenging ability (%) of EPSs at different concentrations (μgdw mL⁻¹) and the half inhibitory concentration (IC₅₀: μgdw mL⁻¹) for EPSs and L-ascorbic acid

| Concentration (μgdw mL⁻¹) | DPPH· | ‘OH | H₂O₂ |
|----------------------------|-------|-----|------|
| 20                         | 20.01 ± 0.03 | 18.10 ± 0.01 | 50.09 ± 0.01 |
| 40                         | 67.00 ± 0.01  | 70.01 ± 0.08 | 79.41 ± 0.02 |
| 50                         | 70.04 ± 0.02  | 74.20 ± 0.01 | 86.11 ± 0.03 |
| 100                        | 82.01 ± 0.07  | 73.14 ± 0.03 | 86.31 ± 0.06 |
| 200                        | 83.12 ± 0.03  | 84.21 ± 0.02 | 98.10 ± 0.04 |
| 500                        | 91.08 ± 0.06  | 87.14 ± 0.04 | 98.20 ± 0.03 |
| 1000                       | 94.51 ± 0.02  | 93.21 ± 0.06 | 99.19 ± 0.01 |
| 2000                       | 99.27 ± 0.04  | 98.12 ± 0.01 | 99.22 ± 0.03 |
| EPSs (IC₅₀)                | 40.21 ± 2.21a | 40.17 ± 1.18a| 20.11 ± 1.21a|
| L-Ascorbic (IC₅₀)          | 100.12 ± 2.12b| 80.22 ± 3.12b| 40.21 ± 1.29b|

DPPH·: α, α-diphenyl-β-picrylhydrazyl free radical scavenging activity; ‘OH: hydroxyl radical scavenging activity; H₂O₂: hydrogen peroxide scavenging activity. Values (means ± SD of three replicates) not sharing a common letter are significantly different (p < 0.05)
**Graesiella** EPS iron-reducing power value was approximated to 3.1 μg<sub>dw</sub>, which shows lower activity than that recorded in the case of *Chlamydomonas reinhardtii* (2.3 μg<sub>dw</sub>; Bafana 2013).

Reactive oxygen species (ROS) including hydroxyl radical and hydrogen peroxide can react with biomolecules in living cell and caused severe damage, as mutagenesis, carcinogenesis, and cytotoxicity (Phaniendra et al. 2015). Then, EPS scavenging ability on ROS was estimated by the H<sub>2</sub>O<sub>2</sub> and ‘OH assays. The results show that EPSs have strong H<sub>2</sub>O<sub>2</sub> and ‘OH scavenging ability in a dose-dependent manner. When the concentration of the EPSs was from 20 to 2000 μg<sub>dw</sub> mL<sup>−1</sup>, the H<sub>2</sub>O<sub>2</sub> scavenging activity ranged from 50 to 98% and the ‘OH scavenging activity ranged from 20 to 73%. Compared to L-ascorbic, the EPSs exhibits a H<sub>2</sub>O<sub>2</sub> and ‘OH inhibitory IC<sub>50</sub> values (40.17 ± 1.18 and 20.11 ± 1.21, respectively) two time lower. *Graesiella* EPSs exhibited obvious scavenging activities on hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) than Isochrysis galbana EPSs (IC<sub>50</sub> 0.2 mg<sub>dw</sub> mL<sup>−1</sup>; Sun et al. 2014) and comparable to the EPSs isolated from *Scenedesmus* sp. (IC<sub>50</sub> 0.38 mg<sub>dw</sub> mL<sup>−1</sup>; Zhang et al. 2019b).

The in vitro results of antioxidant activity analysis indicated that the EPSs from *Graesiella* sp. possessed powerful scavenging capacity. This ability could be explained by the identified bioactive exoenzymes as SOD, CAT, and APX. Furthermore, reducing power analysis indicated that the EPSs performed the antioxidant activity by multiple mechanisms. The results show that the *Graesiella* sp. EPS has great potential to be developed as natural antioxidant or functional food additive.

**Conclusion**

The work goal was to propose an operation strategy to enhance the production of extracellular polymeric substances (EPSs) from *Graesiella* sp. in view of their industrial valorization. The two-step culture process developed and based on the interactive effects of the total medium renewal and heat stress could be easily adopted by the industry. Under these conditions, *Graesiella* sp. produce large amounts of EPSs rich in polysaccharides with potent antioxidant capacity against ROS free radicals mainly H<sub>2</sub>O<sub>2</sub> and ‘OH ones. This ability could be explained by the identified bioactive exoenzymes as SOD, CAT, and APX. The developed EPS production method has a great impact on the economic viability of microalgal-based industrial exploitation. *Graesiella* sp. is a promising candidate for large-scale production of EPSs, which are potentially new and safe therapeutic agents that can be incorporated in the food and pharmaceutical industry for the management of oxidative damage-derived disorders.

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