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Comparison on characterization and antioxidant activity of exopolysaccharides from two *Porphyridium* strains

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Abstract

The exopolysaccharides (EPS) excreted by the unicellular red alga, *Porphyridium*, have potential applications in medicine, cosmetics, and health care products. In the present study, two different *Porphyridium* stains, i.e., *Porphyridium cruentum* CCALA 415 and *Porphyridium purpureum* FACHB 806, were selected and their EPS productivity, chemical characteristics, and antioxidant activities were compared to determine the potential *Porphyridium* strain with high productivity and antioxidant activity for large-scale EPS production. The EPS productivity of *P. cruentum* CCALA 415 (EPS-C) and *P. purpureum* FACHB 806 (EPS-P) were 20.81 mg·L^{-1}·day^{-1} and 63.24 mg·L^{-1}·day^{-1}, respectively. The sulfate contents of EPS-C and EPS-P were 20.58% and 21.63%, respectively. The monosaccharide of these two EPS consisted of xylose, glucose, and galactose. EPS-C had a higher galactose content (40.16%), whereas EPS-P had a higher xylose content (36.62%), but the glucose content was less for both EPS. Compared with the scavenging superoxide anions and ABTS free radicals, the scavenging hydroxyl free radicals contributed to the antioxidant activity of *Porphyridium* EPS. The scavenging activities of EPS-C and EPS-P were 74.1% and 59.5% (1 mg·mL^{-1}), respectively. In conclusion, the EPS of *P. purpureum* FACHB 806 exhibited higher potential for application than those of *P. cruentum* CCALA 415 due to its high EPS productivity and antioxidant activity.

Keywords *Porphyridium cruentum* · *Porphyridium purpureum* · Rhodophyta · Exopolysaccharides · Chemical composition · Antioxidant activity

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Introduction

Microalgal exopolysaccharides (EPS) are the metabolites excreted as extracellular slime in the surroundings of the cell during growth and play a vital role in the microalgal life cycle (Xiao and Zheng 2016). For instance, EPS secreted from Cyanobacteria, Chlorophyta, Bacillariophyceae, and Rhodophyta are widely acknowledged for various applications as a potential feedstock for nutritional supplements, cosmetics, and pharmaceuticals due to their excellent antivirus, anti-inflammatory, anti-oxidation, immunomodulation, and anti-tumor activities (Rechter et al. 2006; Raposo et al. 2013; Bratchkova and Kroumov 2020; Tiwari et al. 2021).

The unicellular red alga, *Porphyridium*, excretes a large amount of sulfated polysaccharide consisting of galactan heteropolymers mutually joined by O-glycosidic bond with an average molecular weight between 2000 and 7000 kDa (Arad and Levy-Ontman 2010). Besides galactose, the monosaccharide of *Porphyridium* EPS also consists of xylose, glucose, and trace amounts of rhamnose,

arabinose, and mannose (Geresh et al. 1992). The aldobiouronic acid O-(α-d-glucopyranosyluronic acid)-L-galactopyranose disaccharide is a highly representative polysaccharide structure with a backbone unit of a linear and larger block containing (1 → 2)- or (1 → 4)-linked D-xylopyranosyl, (1 → 3)-linked D-glucopyranosyl, (1 → 3)-linked L-galactopyranosyl, and (1 → 3)-linked D-glucopyranosyl or glucopyranosyluronic acid residues (Geresh et al. 2009). Although the basic monosaccharide composition of this red microalgal EPS has been analyzed, very few studies have reported the structural differences between different species of *Porphyridium* EPS.

So far, nine *Porphyridium* species have been reported, i.e., *Porphyridium aerugineum*, *Porphyridium cruentum*, *Porphyridium griseum*, *Porphyridium marinum*, *Porphyridium purpureum*, *Porphyridium schinzii*, *Porphyridium sordidum*, *Porphyridium violaceum*, and *Porphyridium wittrockii* (Guiry 2021). Most of the studies on EPS focus on *P. aerugineum*, *P. cruentum*, and *P. purpureum*. For instance, the monosaccharide proportion of freshwater *P. aerugineum* and marine *P. cruentum* differed from each other; a higher xylose and galactose proportion was found in *P. cruentum* than in *P. aerugineum* (Percival and Foyle 1979). The structure and composition of EPS from different species are diverse leading to disparate biological activity. Accumulating studies have reported the differences in EPS antioxidant activity between different *Porphyridium* species. *Porphyridium cruentum* EPS showed weak antioxidant activity on lipid peroxidation inhibition of liver homogenate and erythrocytes hemolysis of mice, but the low molecular weight fragment activity after degradation significantly increased (Sun et al. 2009). The EPS of *Porphyridium* sp. UTEX 637 and *P. aerugineum* exhibited similar antioxidant activity on autoxidation/ferrous oxidation inhibition of linoleic acid and 3T3 cell oxidative damage. However, both were significantly higher than carrageenan produced by macroalgae (Tannin-Spitz et al. 2005). Therefore, it is necessary to screen better strains through comparative studies for higher EPS activity and more significant antioxidant effects.

Most of the research on *Porphyridium* EPS is currently focused on *P. cruentum*, limiting the exploration of other species and corresponding research on the differences of EPS produced by different *Porphyridium* species. In this study, two *Porphyridium* species, *P. cruentum* CCALA 415 and *P. purpureum* FACHB 806, were selected, and the differences in productivity, chemical properties, structural characteristics, and antioxidant activities of their EPS were compared. Based on the results, a potential *Porphyridium* strain with high EPS productivity and antioxidant activity was selected for large-scale production of *Porphyridium* EPS.

Materials and methods

Microalgae and culture conditions

Porphyridium cruentum CCALA 415 and *P. purpureum* FACHB 806 were obtained from the Culture Collection of Autotrophic Organisms in the Czech Republic and the Freshwater Algae Culture Collection in China, respectively.

These two *Porphyridium* species were inoculated in 6.0 cm × 60 cm glass column photobioreactors containing 1200 mL of modified ASW medium, which contained 462.0 mM NaCl, 26.8 mM $MgSO_4 \cdot 7H_2O$, 27.5 mM $MgCl_2 \cdot 7H_2O$, 10.2 mM $CaCl_2 \cdot 2H_2O$, 17.6 mM $NaNO_3$, 0.69 mM $K_2HPO_4 \cdot 3H_2O$, 0.48 mM $NaHCO_3$, 11.7 μM $EDTANa_2 \cdot 2H_2O$, 11.7 μM $FeCl_3 \cdot 6H_2O$, 0.91 μM $MnCl_2 \cdot 4H_2O$, 0.08 μM $ZnSO_4 \cdot 7H_2O$, 0.02 μM $Na_2MoO_4 \cdot 2H_2O$, 0.04 μM Co $(NO_3)_2 \cdot 6H_2O$, and 0.04 μM $CuSO_4 \cdot 5H_2O$ (Li et al. 2019). These strains were bubbled with CO_2-enriched compressed air (1% CO_2, v:v), filtered through a 0.2-μm sterile disposable filter are provided with 200 μmol photons·m^{-2} s^{-1} one-side illumination by T8 fluorescent lamp (Philips, China) at 25 ± 1 °C. The biomass concentrations of the initial inoculation for both strains were 0.20 ± 0.01 g·L^{-1}. After 12 days of cultivation, the biomass concentrations of *P. cruentum* CCALA 415 and *P. cruentum* FACHB 806 were 6.99 g·L^{-1} and 4.80 g·L^{-1}, respectively.

Preparation of EPS, determination of EPS concentration, and EPS productivity

EPS was prepared and purified according to the method reported by Chen et al. (2010). Briefly, the supernatant was obtained by centrifugation at 8000 rpm for 10 min, then concentrated by a RE-2000A rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory, China) at 60 °C, and finally dialyzed to remove small molecules. Later, 95% ethanol was added to the supernatant (95% ethanol to supernatant, 4:1, v:v). After centrifugation, EPS of *P. cruentum* CCALA 415 (EPS-C) and *P. purpureum* FACHB 806 (EPS-P) were obtained.

The obtained EPS was weighed to determine the EPS productivity using the following formula.

$$\text{EPS productivity} = (E_{t1} - E_{t0})/(t_1 - t_0) \quad (1)$$

where E_{t1} and E_{t0} are the EPS concentrations at day t_1 and day t_0, respectively.

Construction of phylogenetic tree

The DNA extraction of strains was based on the method described by (Chen et al. 2014). The primer for 18S rDNA

gene sequence was A (5′-ACCTGGTTGATCCTGCCAGT-3′) and B (5′-TGATCCTTCTGCAGGTTCACCTAC-3′). The PCR reaction using the KOD-Plus-Neo DNA polymerase (Toyobo, Japan) was performed according to the operation parameters reported by Li et al. (2016). PCR was performed under the following conditions: pre-denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 5 °C for 30 s, and extension at 68 °C for 7 min. PCR products were recovered and purified by DNA gel extraction kit (Tiangen, China). The 18S rRNA gene sequences were sequenced by BGI Tech Solutions Co., Ltd., in China. ClustalX 1.8 and MEGA 4.0 software were used to construct the phylogenetic tree.

Determination of EPS composition

Total carbohydrate and protein contents were determined by the phenol sulfuric acid method (Dubois et al. 1956) and Lowry method (Lowry et al. 1951), respectively. The meta-hydroxyphenyl method was employed to determine the uronic acid content (Blumenkrantz and Asboe-Hansen 1973). Sulfate content was calculated as reported by Reim (1991). The EPS was hydrolyzed by adding 2 mL of 1 M hydrochloric acid for 6 h at 100 °C. After filtering with a 0.45–μm microporous membrane, the distilled water volume was adjusted to 5 mL, and ion chromatography (IC) (DIONEX, USA) was used to determine the sulfate content.

Briefly, 2 mL of mixed acid (nitric acid:perchloric acid = 4:1, v:v) was added to the EPS and digested it at 160 °C for 4 h. After cooling, the distilled water volume was adjusted to 5 mL. The inductively coupled plasma mass spectrometer (ICP-MS) (PerkinElmer, USA) was employed to determine the metal ion content in EPS (Allen 1984).

The derivatization method of EPS and reference samples was according to the method reported by Luo et al. (2010). The monosaccharide components were determined by GC-2014 gas chromatograph spectrometer (Shimadzu, Japan) equipped with an SH-Rtx-5 capillary column (30 m × 0.25 mm × 0.25 μm, Shimadzu). Nitrogen was used as the carrier gas. The column was temperature-programmed from 120 °C (with a hold of 3 min) to 210 °C at 3 °C·min^{-1} (with a hold of 4 min). The temperature of the injection port and detector were 250 °C and 280 °C, respectively. The injection volume was 1.0 μL, and the split ratio was 30:1.

Fourier transform infrared spectroscopy (FT-IR) analysis

IR affinity-1 Fourier transform infrared spectrometer (Shimadzu, Japan) was employed to measure the infrared spectrum of EPS with the scanning interval of 400–4000 cm^{-1}.

Antioxidant activity assays

EPS was dissolved in distilled water to prepare a polysaccharide solution with a concentration of 0.2 to 5.0 mg·L^{-1}. Ascorbic acid was regarded as a positive control. The antioxidant activity evaluation methods are listed as follows.

2,2-Diphenyl-1-picrylhydrazyl radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity was determined according to the methods reported by Chen et al. (2018). Samples of different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg·L^{-1}) were added to an equal volume of 0.1 μM DPPH ethanol solution. The absorbance was measured at 517 nm using an Epoch 2 Microplate Spectrophotometer (Bio-Tek Instruments Inc., USA) after reaction in the dark for 30 min at room temperature. The DPPH radical-scavenging activity was calculated using the following equation:

$$\text{Scavenging activity}(\%) = [1 - (A_1 - A_2)/A_0] \times 100 \quad (2)$$

where A_0 is the absorbance of the control group (distilled water instead of the sample solution); A_1 is the absorbance of the experimental group (EPS solution); and A_2 is the absorbance of the blank group (ethanol instead of DPPH).

2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical-scavenging ability

2,2′-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity was carried out, as described by Li et al. (2012). Equal volume of 7.4 mM ABTS diammonium salt and 2.6 mM potassium persulfate was mixed and left in the dark for 12 h. The mixture was diluted with phosphate buffer at pH 7.4 so that its absorbance at 734 nm was 0.70 ± 0.02. The samples (0.2 mL) of different concentrations (1.0, 2.0, 3.0, 4.0, 5.0 mg·L^{-1}) were added to 0.8 mL of ABTS free radical working solution, shaken in the dark at 37 °C for 15 min, and the absorbance was measured at 734 nm using an Epoch 2 Microplate Spectrophotometer (Bio-Tek Instruments Inc., USA). The ABTS radical-scavenging ability was calculated using the following equation:

$$\text{Scavenging activity}(\%) = [(A_0 - A_1)/A_0] \times 100 \quad (3)$$

where A_0 is the absorbance of the control group (distilled water instead of the EPS solution) and A_1 is the absorbance of the experimental group (EPS solution).

Superoxide anion radical-scavenging activity

The superoxide anion scavenging activity of the sample (1.0, 2.0, 3.0, 4.0, and 5.0 mg·L^{-1}) was determined according to the instructions mentioned in the superoxide anion free radical test kit (Nanjing Jiancheng Bioengineering Research Institute, China). The mechanism of the kit is based on simulating the reaction system of xanthine and xanthine oxygenase in the body. The reaction system generates superoxide anion free radicals. After adding electron transport material and dye, the reaction system appears purple-red, and its absorbance can be measured by a spectrophotometer. When the sample contains a superoxide anion inhibitor, the absorbance of the measuring tube during colorimetry is lower than that of the control tube.

$$\text{Scavenging activity}(\%) = [1 - (A_1/A_0)] \times 100 \quad (4)$$

where A_0 is the absorbance of the control group (distilled water instead of the sample solution), and A_1 is the absorbance of the experimental group (EPS solution).

Hydroxyl radical-scavenging activity

Hydroxyl radical (OH)-scavenging activity was measured, as reported by Li et al. (2008). The samples of different concentrations (0.2, 0.4, 0.6, 0.8, and 1.0 mg·L^{-1}), 1,10-phenanthroline (0.75 mM), and $FeSO_4$ (0.75 mM) were dissolved in phosphate buffer (0.15 M, pH 7.4), and 0.01% of H_2O_2 was added in the end. The mixture was shaken at 37 °C for 30 min, and the absorbance was measured at 536 nm using an Epoch 2 Microplate Spectrophotometer (Bio-Tek Instruments Inc., USA). The hydroxyl radical-scavenging activity was calculated using the following equation:

$$\text{Scavenging activity}(\%) = [(A_1 - A_0)/(A_2 - A_0) \times 100] \quad (5)$$

where A_0 is the absorbance of the negative control group (distilled water instead of the sample solution), A_1 is the absorbance of the experimental group (EPS solution), and A_2 is the absorbance of the normal group (distilled water instead of hydrogen peroxide).

Statistical analysis

Data are shown as mean and standard deviations of two independent biological replicates and three technical replicates. SPSS 18.0 was used for data analysis (ANOVA) and IC_{50} (half maximum inhibitory concentration) was used to describe the effective concentration of the EPS when the scavenging activity of free radicals in the system reaches 50% which was calculated using GraphPad Prism 7. The $p < 0.05$ was considered statistically significant.

Results

Phylogenetic analysis and comparison of EPS productivity

Total base pairs of 18S rDNA gene of *P. cruentum* CCALA 415 and *P. purpureum* FACHB 806 were obtained by sequencing. BLAST analysis showed that *P. purpureum* FACHB 806 had a close relationship with *P. purpureum* UTEX 637 (Fig. 1). However, *P. cruentum* CCALA 415 had a larger single cell (> 5 μm), and the inner star-shaped chromophore was darker and larger in size than *P. purpureum* FACHB 806.

Significant differences between the EPS productivity and biomass of the two strains were observed (Fig. 2). The biomass productivity of *P. cruentum* CCALA 415 was 565.83 mg·L^{-1}·day^{-1}, which was higher than that of *P. purpureum* FACHB 806 (383.33 mg·L^{-1}·day^{-1}). However, the EPS productivity of the two strains showed the opposite result. The productivity of EPS-P was 63.24 mg·L^{-1}·day^{-1}, which was three times than EPS-C (20.81 mg·L^{-1}·day^{-1}).

Characterization and comparison of chemical composition

The carbohydrate, protein, uronic acid, and SO_4^{2-} contents of EPS-C and EPS-P were determined (Table 1). The carbohydrate and protein contents of EPS-P were 46.69% and 9.61% of dry weight (DW), respectively, which were significantly higher than those of EPS-C ($p < 0.05$). The SO_4^{2-} contents were 20.58% for EPS-C and 21.63% for EPS-P ($p > 0.05$). EPS-C exhibited the similar uronic acid content (5.65% DW) with EPS-P (4.25% DW) ($p > 0.05$).

Both EPS-C and EPS-P contained several types of transition-metal ions, including Cu, Cr, Fe, Mn, and Ni (Table 1). The transition-metal proportion in EPS-P (0.68) was higher than that in EPS-C (0.37). Of them, the Fe and Cr contents of EPS-P were 4.38% DW and 2.48% DW, which were 2.86% DW and 1.65% DW higher than EPS-C.

Comparison of monosaccharide composition and FT-IR

The monosaccharide composition of EPS-C and EPS-P were determined by gas chromatograph spectrometer. The retention time of xylose, glucose, and galactose

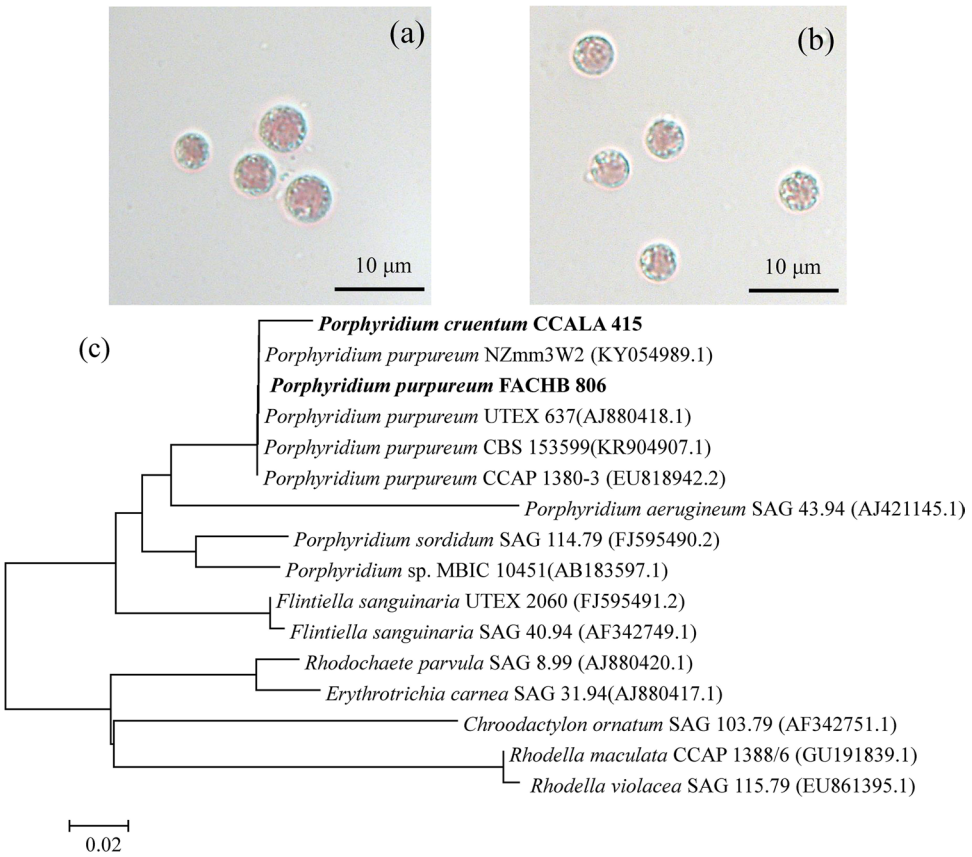

Fig. 1 Cell morphology of *Porphyridium cruentum* CCALA 415 (**a**) and *Porphyridium purpureum* FACHB 806 (**b**), and the phylogenetic tree based on 18S rDNA gene sequence (**c**)

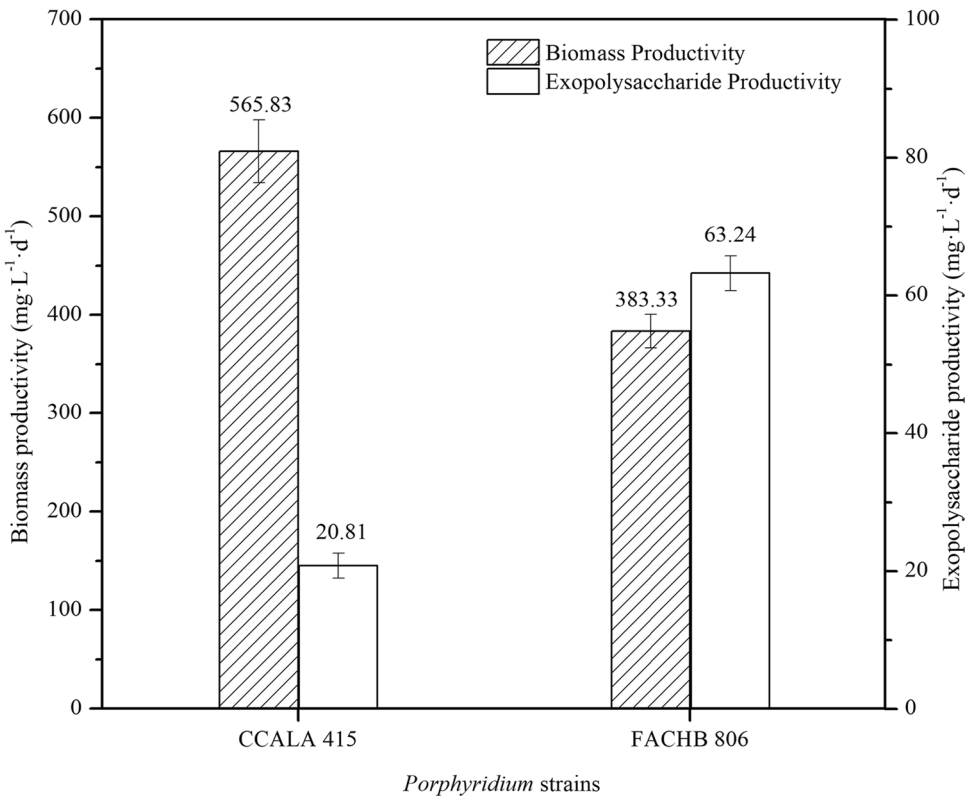

Fig. 2 The productivity of biomass and exopolysaccharides of *Porphyridium cruentum* CCALA 415 and *Porphyridium purpureum* FACHB 806

Table 1 Chemical composition of exopolysaccharides

Chemical composition (%)		Sample	
		EPS-C	EPS-P
Total carbohydrate		42.81 ± 0.72[a1]	46.69 ± 0.40[b1]
Protein		6.20 ± 0.02[a2]	9.61 ± 0.50[b2]
Uronic acid		5.94 ± 0.41[a3]	4.22 ± 0.05[b3]
Sulfate		20.58 ± 0.61	21.63 ± 1.14
Metal ions	Ca	4.25 ± 0.53[a4]	3.10 ± 0.18[b4]
	Cu	0.10 ± 0.05	0.30 ± 0.07
	Cr	0.83 ± 0.28[a5]	2.48 ± 0.34[b5]
	Fe	1.52 ± 0.52[a6]	4.38 ± 0.93[b6]
	Mg	0.31 ± 0.05	0.24 ± 0.00
	Mn	0.06 ± 0.05	0.17 ± 0.04
	Ni	0.25 ± 0.21	0.75 ± 0.32
	$\frac{\text{Transition-metal ions}}{\text{Total metal ions}}$	0.37	0.68

Different letters denoted significant differences among the values of the total carbohydrate, protein, uronic acid, sulfate, and metal ions of EPS-C and EPS-P (a1–a6: EPS-C; b1–b6: EPS-P). The metal ions include calcium (Ca), copper (Cu), cobalt (Cr), iron (Fe), magnesium (Mg), manganese (Mn), and nickel (Ni). Among them, the transition metal ion was Cu, Cr, Fe, Mn, and Ni. The values shown are the averages of two biological replicates and three technical replicates ± standard deviation

were 20.13 min, 27.87 min, and 28.63 min, respectively (Fig. 3). The EPS-C and EPS-P had the same monosaccharide composition (xylose, galactose, and glucose) but had different monosaccharide percentages (Table 2). The galactose percentage of EPS-C (40.16%) was significantly higher than that of xylose (30.63%) and glucose (29.22%) ($p < 0.05$). There was no significant difference between the xylose (36.62%) and galactose contents (36.11%) of EPS-P ($p > 0.05$), but both were significantly higher than glucose (27.27%) ($p < 0.05$).

FT-IR is a powerful tool for assessing the structural and functional organic groups of polysaccharides. The infrared absorption spectra of the two EPS within the range of 400–4000 cm^{-1} are depicted in Fig. 4. The broad absorption peaks at 3375.4 cm^{-1} (EPS-C) and 3363.8 cm^{-1} (EPS-P) were the stretching vibrations of hydroxyl (-OH), respectively. The weak absorption peaks at 2949.2 cm^{-1} (EPS-C) and 2922.2 cm^{-1} (EPS-P) were the stretching vibrations of C-H. The absorption peaks might have appeared at 1639.5 cm^{-1} (EPS-C) and 1633.7 cm^{-1} (EPS-P) due to the crystalline water (Wang et al. 2012). The bands appearing at 1415.8 cm^{-1} (EPS-C), 1419.6 cm^{-1} (EPS-P), and 1234.4 cm^{-1} (EPS-C), 1240.2 cm^{-1} (EPS-P) corresponded to the stretching vibration of C-O bond in C–OH and the asymmetrical stretching vibration of S = O bond in sulfate group (Wang et al. 2013). The absorption peaks at 1029.9 cm^{-1} (EPS-C) and 1035.7 cm^{-1} (EPS-P) might have appeared due to the C-O stretching vibration in C–O–C (Kacurakova et al. 2000). The absorption bands at 864.3 cm^{-1} (EPS-C) and 856.4 cm^{-1} (EPS-P) might have appeared due to the vibration of the β-configuration pyran ring (Choi et al. 2021). The infrared spectroscopy results showed typical absorption peaks for both the EPS and pyranose ring with a glycosidic bond type of β-configuration.

Evaluation and comparison of antioxidant activity

DPPH scavenging activity

DPPH scavenging activities of EPS-C and EPS-P with a concentration ranging from 0 to 1 $mg \cdot mL^{-1}$ are shown in Fig. 5a. EPS-C and EPS-P showed no DPPH scavenging activity.

ABTS scavenging activity

ABTS scavenging activities of EPS-C and EPS-P (0–5 $mg \cdot mL^{-1}$) are shown in Fig. 5b. The ABTS scavenging activity by EPS-C and EPS-P increased in a dose-dependent manner, with IC_{50} of 8.92 $mg \cdot mL^{-1}$ and 6.59 $mg \cdot mL^{-1}$, respectively, which was significantly lower than ascorbic acid ($IC_{50} = 0.003$ $mg \cdot mL^{-1}$) (Table 3). When the concentration was less than 4 $mg \cdot mL^{-1}$, there was no significant difference in the scavenging activity of EPS-C and EPS-P ($p > 0.05$). However, the upward trend of the scavenging activity of EPS-P was more obvious. The scavenging activity of EPS-C was only 35.97% at 5 $mg \cdot mL^{-1}$ concentration, while the scavenging activity of EPS-P reached 47.02%, which was significantly higher than that of EPS-C ($p < 0.05$).

Superoxide anion radical-scavenging ability

The superoxide anion scavenging activity of EPS at a concentration ranging from 0 to 5 mg mL^{-1} is shown in Fig. 5c. The superoxide anion scavenging activity of EPS-C and EPS-P also increased in a dose-dependent manner, and the effects of EPS-C were significantly higher than those of EPS-P ($p < 0.05$). The differences between the two EPS reached the maximum at 3 mg mL^{-1}, with scavenging activities of 19.38% and 7.54%, respectively. However, with the increased concentration, the activity of EPS-C tended to be stable, while the activity of EPS-P continued to rise. In this study, the EPS activity achieved the maximum at 5 $mg \cdot mL^{-1}$ concentration, and the scavenging activity of EPS-C and EPS-P were 22.29% and 17.03%, respectively. The IC_{50} of EPS-C and EPS-P were 14.51 $mg \cdot mL^{-1}$ and 29.73 $mg \cdot mL^{-1}$, respectively, and the IC_{50} of ascorbic acid was 0.066 $mg \cdot mL^{-1}$ (Table 3). The superoxide anion scavenging activity of the two EPS was significantly lower than the positive control group ($p < 0.05$).

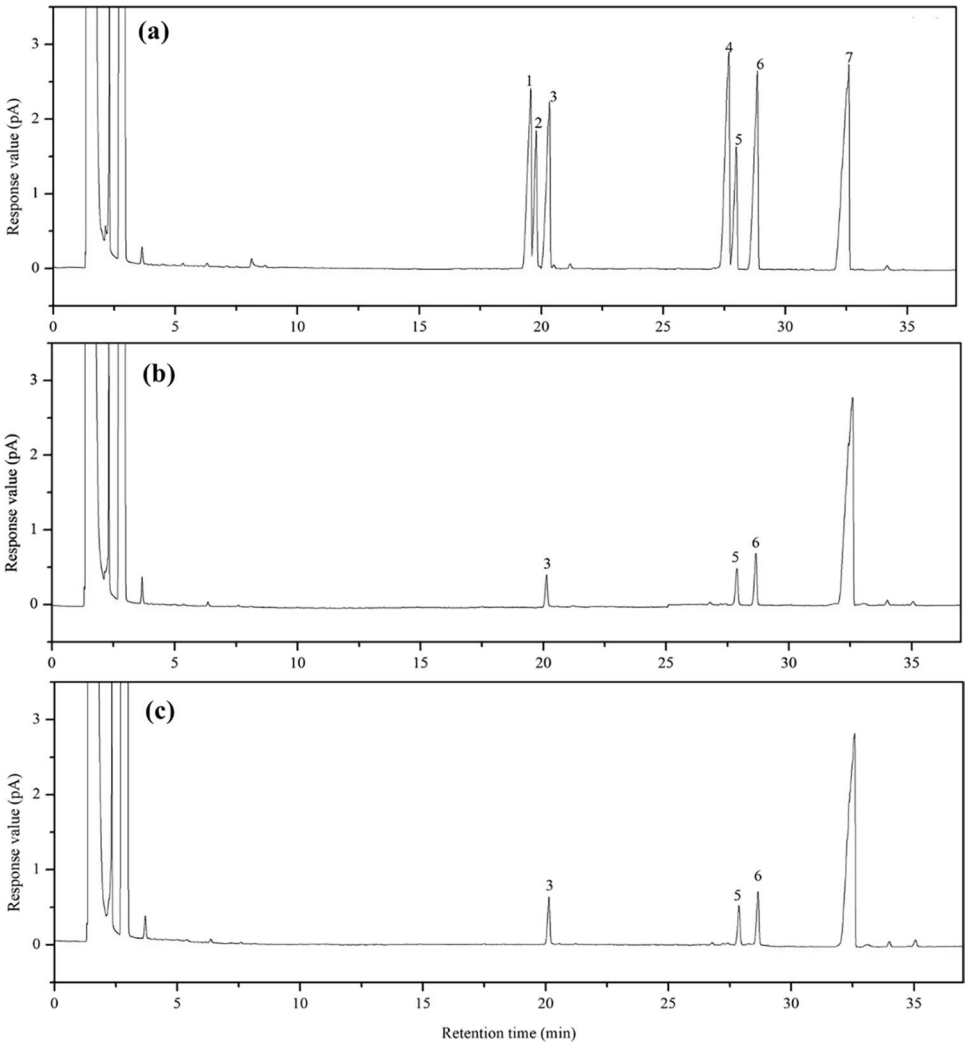

Fig. 3 Gas chromatography profiles of mixed standard monosaccharides (**a**), EPS-C from *Porphyridium cruentum* CCALA 415 (**b**), and EPS-P from *Porphyridium purpureum* FACHB 806 (**c**). The peaks in chromatography profile (**a**) from left to right order are as follows: (1) arabinose; (2) fucose; (3) xylose; (4) mannose; (5) glucose; (6) galactose; (7) inositol

Hydroxyl radical-scavenging ability

The hydroxyl radical scavenging ability of EPS ranging from 0 to 1 mg·mL^{-1} concentration is depicted in Fig. 5d. For the scavenging activity of hydroxyl radicals, high EPS concentrations were not used in the study. When the EPS concentration was 1.0 mg L^{-1}, the scavenging activity of EPS-C and EPS-P both exceeded 50%. The IC_{50} calculated by GraphPad Prism 7 software is accurate, only when the scavenging activities of all test points were distributed around 50%. Therefore, EPS concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 mg L^{-1} were chosen to evaluate the scavenging activity of hydroxyl free radicals. When the concentration was less than 0.2 mg·mL^{-1}, no significant differences in the scavenging activities of EPS-C, EPS-P, and ascorbic acid were observed. The scavenging activity of ascorbic acid in the concentration range of 0.4–1.0 mg·mL^{-1} was significantly higher than those of the two groups ($p < 0.05$), and the activity was ascorbic acid > EPS-C > EPS-P. However, the difference between EPS-C and EPS-P was not significant ($p < 0.05$). The maximum scavenging activities of EPS-C and EPS-P were 74.05% and 59.52%, respectively, corresponding to IC_{50} of 0.59 mg·mL^{-1} and 0.71 mg·mL^{-1}. The EPS-C activity was slightly higher up to 44% of ascorbic acid concentration. Compared with the other three free radicals mentioned above, EPS-C and EPS-P had significant hydroxyl radical-scavenging activity.

Table 2 Monosaccharide composition analysis

Sample	Sugar composition (%)		
	Xylose	Glucose	Galactose
EPS-C	30.63 ± 1.95^{b1}	29.22 ± 0.11^{b1}	40.16 ± 2.07^{a1}
EPS-P	36.62 ± 0.82^{a2}	27.27 ± 1.92^{b2}	36.11 ± 1.10^{a2}

Different letters denoted significant differences among the values of the xylose, glucose, and galactose of EPS-C and EPS-P (a1–b1: EPS-C; a2–b2: EPS-P). The values shown are the averages of two biological replicates and three technical replicates ± standard deviation

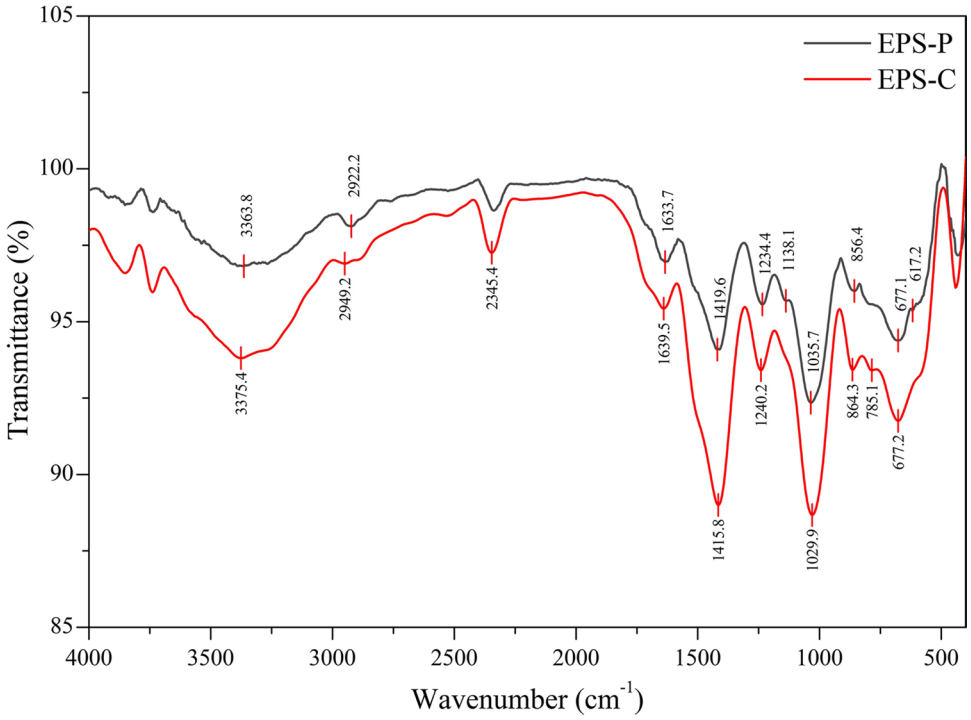

Fig. 4 FT-IR spectra of *Porphyridium cruentum* CCALA 415 (**a**) and *Porphyridium purpureum* FACHB 806 (**b**)

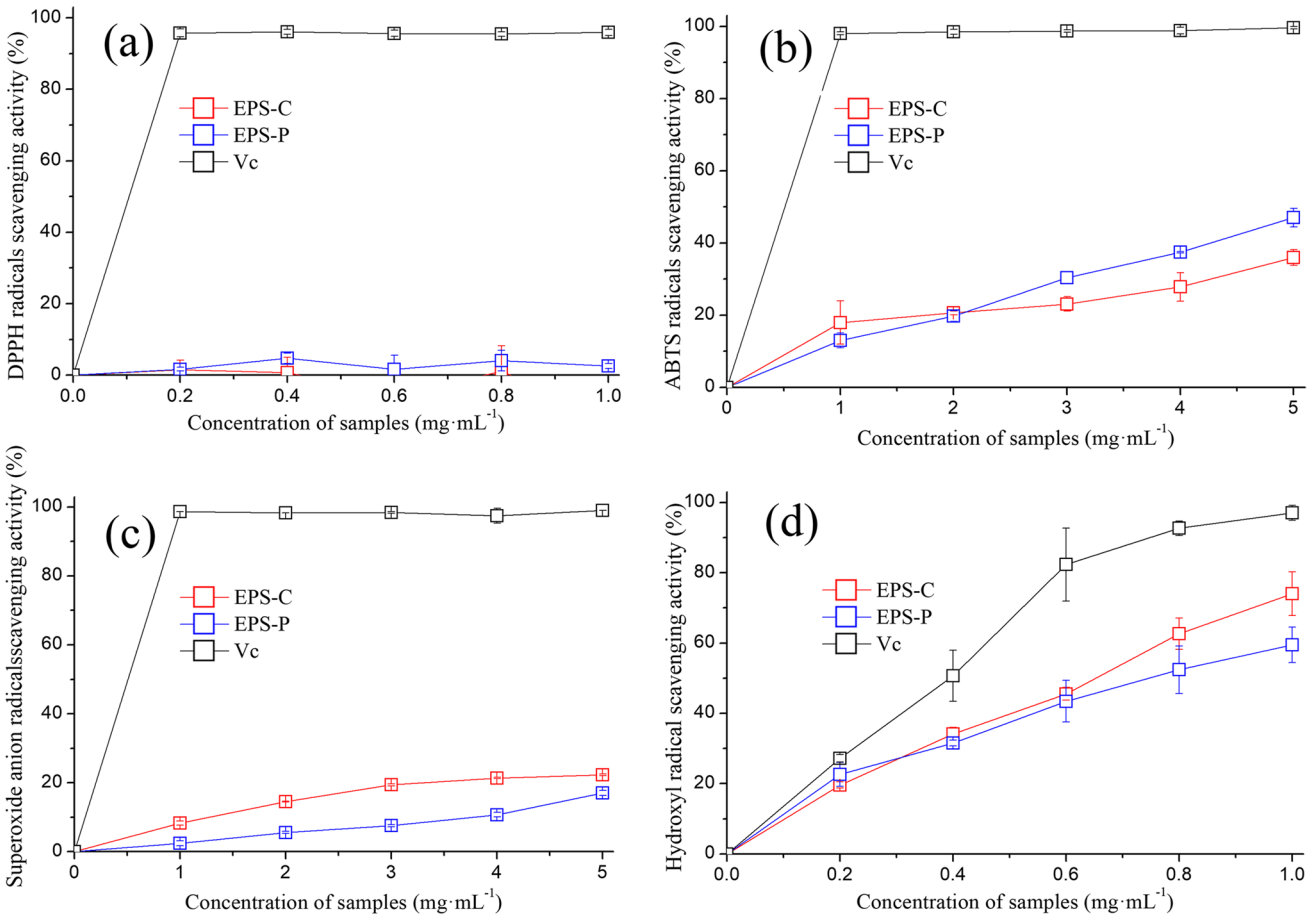

Fig. 5 Radical-scavenging activity of *Porphyridium cruentum* CCALA 415 and *Porphyridium purpureum* FACHB 806

Table 3 IC_{50} of exopolysaccharides

IC_{50} (mg·mL^{-1})	Sample		
	EPS-C	EPS-P	Ascorbic acid
DPPH	–	–	0.005 ± 0.001
ABTS	8.92 ± 0.28^{a1}	6.59 ± 0.26^{b1}	0.003 ± 0.001^{c1}
O_2^-	14.50 ± 0.37^{b2}	29.73 ± 0.34^{a2}	0.066 ± 0.001^{c2}
OH·	0.59 ± 0.03^{a3}	0.71 ± 0.15^{a3}	0.260 ± 0.041^{b3}

Different letters denoted significant differences among the values of IC_{50} of different free radical-scavenging activity of EPS-C and EPS-P (a1–c1: ABTS; a2–c2: O_2^-; a3–b3: OH). The values shown are the averages of two biological replicates and three technical replicates ± standard deviation

Discussion

The two *Porphyridium* strains selected for this study were *P. cruentum* and *P. purpureum*. Frutarom Industries Ltd. (Israel) has successfully extracted the EPS and converted it into cosmetics with large-scale cultivation. However, no other species of *Porphyridium* EPS have been reported for commercial application. *Porphyridium purpureum* was selected as its genome determination was completed. Previous studies have proved that the EPS secreted by *Porphyridium* have excellent antiviral, anti-inflammatory, antioxidant, immunomodulatory, and anti-tumor biological activities (Huleihel et al. 2001; Matsui et al. 2003; Tannin-Spitz et al. 2005; Sun et al. 2009; Raposo et al. 2013). It has potential application prospects in medicine, health care products, cosmetics, and other industries, and has gradually become a research hotspot. In our study, although *Porphyridium* EPS has excellent biological activities, compared with the 30–50-g·L^{-1} production of xanthan gum, *Porphyridium* EPS does not yet have a large-scale production capacity. Our laboratory has currently been conducting some basic research, such as optimizing culture conditions and designing novel photobioreactors to increase EPS productivity, so as to realize the large-scale production of *Porphyridium* EPS.

Although these two strains shared a similar genetic relationship and little difference in morphology and classification, the EPS production of *P. purpureum* is three times higher than that of *P. cruentum*. Therefore, *P. purpureum* might be a strong substitute for the commercial production of *Porphyridium* EPS in the future.

Previous studies have revealed some proteins (covalently linked to polysaccharides of *Porphyridium* EPS) linked to carbohydrate via serine-xylose and threonine-xylose linkages (Heaney-Kieras et al. 1977). Besides, the uronic acid residues of *Porphyridium* EPS are mainly linked to glucose and galactose, containing 3-O-(α-D-glucopyranosyluronic acid)-L-galactose, 3-O-(2-O-methy1-α-D-glucopyranosyluronic acid)-D-galactose and -D-glucose, indicating a positive correlation of galactose and glucose content with uronic acid (Heaney-Kieras and Chapman 1976). In this study, the EPS structure was not further analyzed, but the chemical composition results confirmed similar conclusions. EPS-C had a higher uronic acid content, and correspondingly, galactose content is also higher than EPS-P. The EPS-P protein content was higher than EPS-C, and its monosaccharide composition was xylose-dominant.

The ratio of the sulfated polysaccharides in EPS has attracted much attention to study the relationship between the EPS properties and activities. It is generally believed that polysaccharides with high-sulfated content have stronger biological activity. The low molecular weight polysaccharides treated by hermetical microwave on *P. cruentum* EPS have a higher sulfate group content and antioxidant activity than the undegraded polysaccharides (Sun et al. 2009). The study about the antioxidant activity of red and brown seaweed polysaccharides had demonstrated that the sulfate content was positively correlated with the antioxidant activity (de Souza et al. 2007). However, our results were not consistent with the previous studies. In this study, the sulfate contents of these two *Porphyridium* strains were not significantly different, but the antioxidant activities were significantly different. These results indicated that the sulfate content is not the only factor affecting the EPS antioxidant activity. The protein and uronic acid contents and the monosaccharide composition may have a non-negligible effect on the EPS biological activity.

The superoxide anion free radical-scavenging results showed that the EPS-C activity was significantly higher than that of EPS-P ($p < 0.05$). Chen et al. (2004) proved that the higher the uronic acid content of tea polysaccharide conjugates, the stronger reactive oxygen species scavenging activities. However, the relationship between sulfate content and antioxidant activity was not involved. The sulfate content of EPS-P was higher than that of EPS-C, while the content of uronic acid showed the opposite result. It is speculated that the content of uronic acid in the crude polysaccharide of *Porphyridium* may have a greater impact on the scavenging performance of peroxide anions than sulfate.

Of the reactive oxygen radicals, hydroxyl radicals have a strong oxidation ability, leading to unnecessary carbohydrate peroxidation, amino acids, proteins, nucleic acids, and other substances (Li et al. 2012). The peroxidation reaction will further lead to oxidative damage and destruction, resulting in cell necrosis or mutation and diseases, such as aging and cancer (Li et al. 2008). In this study, the scavenging activity of the two EPS types against hydroxyl radicals was measured. The results suggested that EPS-C (IC_{50} = 0.59 mg·mL^{-1}) and EPS-P (IC_{50} = 0.76 mg·mL^{-1}) had no significant difference in the hydroxyl radical-scavenging activity. The excellent hydroxyl radical scavenging activity of *Porphyridium* EPS is related to the high sulfation

level of polysaccharides (Heaney-Kieras et al. 1977). The difference between the two EPS types could be explained by the difference in uronic acid contents and transition metal ion contents. Studies have exploited that the transition-metal ions could promote the superoxidation reaction to generate more hydroxyl radicals, and uronic acid groups with carboxyl groups could reduce hydroxyl radical formation by chelating metal ions (Macdonald et al. 2003; Shen et al. 2018). EPS-P has a higher transition-metal ion proportion, resulting in an increase in the free radicals during the reaction, and reduces its scavenging activity. In contrast, the higher uronic acid content of EPS-C allows it to chelate more metal ions and improve its scavenging activity. Therefore, it was inferred that uronic acid and transition-metal ions play a non-negligible role in the active oxygen radical-scavenging process of *Porphyridium* EPS.

Although the DPPH and ABTS assay use non-physiological free radicals, they can reflect whether a substance can scavenge free radicals directly (Floegel et al. 2011). It has been reported that DPPH can form stable DPPH-H molecules through hydrogen atoms (H·) provided by antioxidants, and the removal of ABTS is caused by the electron (e) transfer reaction (Bondet et al. 1997; Prior and Cao 1999). The antioxidant experiments on five different extracts of *Riczoma cimicifugae* confirmed that natural antioxidants directly scavenge free radicals by providing hydrogen atoms (H·) and electrons (*e*-) (Wang et al. 2012). The scavenging activities of DPPH at 1.0, 2.0, 3.0, 4.0, and 5.0 $mg{\cdot}L^{-1}$ concentrations of EPS were not measured in the present study. DPPH usually needs to be dissolved in organic reagents, such as ethanol, which caused partial precipitation of EPS and made the absorbance reading wrong. When using higher concentrations of EPS, this situation will be more serious. Thus, the concentrations of EPS used in DPPH assays did not exceed 1.0 $mg{\cdot}L^{-1}$. This result was consistent with the results of Sun et al. (2009). Interestingly, ABTS showed different scavenging activities than hydroxyl radicals and superoxide anions, and the IC_{50} of EPS-P (6.59 $mg{\cdot}mL^{-1}$) was lower than EPS-C (8.92 $mg{\cdot}mL^{-1}$), indicating higher ABTS radical-scavenging activity of EPS-P. The results of the ABTS free radical-scavenging assay were different from other antioxidant assays. The scavenging activity of EPS-P was higher than that of EPS-C, but the emergence of this result is completely understandable. Because the active groups in natural products can usually be combined with other substances, the interaction of the substances in the system leads to an impact on the antioxidant capacity. The study of Zhang et al. (2003) showed that the antioxidant activity of sulfated polysaccharide fractions from *Porphyra haitanesis* could be influenced by protein contents in addition to sulfate content. The higher the protein content, the stronger the antioxidant activity. Therefore, when using different analytical methods to explain the antioxidant capacity, it is necessary to consider the influence of different chemical components on the antioxidant activity of the natural macromolecular mixture of EPS.

Based on the results of two different strains of *Porphyridium* on four different free radical-scavenging experiments, EPS-C has stronger in vitro antioxidant activity. The antioxidant activity of EPS is closely related to the structure, the type and number of substituent groups, and the composition of monosaccharides. From the chemical composition of EPS-C and EPS-P determined in this study, the reason for the difference in antioxidant activity of the two EPS cannot be drawn. In the future, we will plan to explore the reasons for the difference in antioxidant activity from the structure of EPS.

Conclusion

The present study provides a comprehensive analysis of the differences between two *Porphyridium* strains, i.e., *P. cruentum* CCALA 415 and *P. purpureum* FACHB 806. Although the genetic relationship and cell morphology of the two *Porphyridium* strains are similar, there are still differences in the productivity, structure, composition, and activity of their EPS. EPS-C has higher content of galactose and uronic acid, and its antioxidant activity is mainly reflected in its scavenging capacity of superoxide anion and hydroxyl radical; EPS-P has higher content of xylose, protein, sulfate, and transition metal ions, and its scavenging activity on ABTS is higher than that of EPS-C. Notably, the productivity of EPS-P reached three times than that of EPS-C, which makes *P. purpureum* a promising strain for large-scale production of EPS in the future. Besides, the uronic acid content exhibited a better effect on the active oxygen radical-scavenging activity, while the sulfate group content exhibited a greater effect on the ABTS and DPPH free radical-scavenging activity.

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Author contribution Wei-Nan Wang designed the study, performed most of the experiments, analyzed the results, and drafted the manuscript. Yi Li carried out the cultivation of *P. cruentum* CCALA 415 and *P. purpureum* FACHB 806 and measured EPS concentrations. Ying Zhang performed the statistical analysis and helped to draft the manuscript. Wenzhou Xiang assisted in the determination of monosaccharide components. Tao Li and Ai-Fen Li designed and coordinated the study, and edited the manuscript.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

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