Journal of Applied Phycology (2019) 31:3831–3839
https://doi.org/10.1007/s10811-019-01864-z

Effects of bromide-enriched natural seawater culture medium on protein and monoterpenes output of *Ochtodes secundiramea* (Rhodophyta, Gigartinales)

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Received: 26 February 2019 / Revised and accepted: 8 July 2019 / Published online: 25 July 2019
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Abstract

Benthic marine algae, especially Rhodophyta, are within a well-known marine group able to produce secondary metabolites with bioactive properties. Among them are halogenated terpenes, synthesized by vanadium-dependent haloperoxidase (V-BPO). The red alga *Ochtodes secundiramea* (Montagne) M. Howe produces high amounts of halogenated monoterpenes (HMT) which have the potential to control phytopathogenic fungi. Culture closed a system is a tool used to manipulate conditions to increase the synthesis of HMT and other metabolites. The apical segments of *O. secundiramea* were cultivated in a bioreactor, either in seawater enriched with half strength von Stosch's solution (VSES/2) without (control) and with the addition of 250 mg L^{-1} bromine (treatment). In response to these conditions, specific growth rate, HMT profile, soluble protein, phycobiliproteins, soluble carbohydrate, and chlorophyll as well as the V-BPO activity, uptake, and accumulated bromine within the biomass were evaluated. The treatment did not promote significant alterations in the growth but increased the V-BPO activity and bromine uptake and incorporation (2.5 fold). No changes in the HMT profiles of the extracts from both cultures conditions were observed by gas chromatography–mass spectrometry analyses, whereas the values of total soluble protein and phycobiliproteins were 2-fold higher in samples from the bromine-enriched medium when compared to samples from control. These results support the evidences of protective effects promoted by V-BPO activity. Furthermore, carbohydrate and chlorophyll contents were not affected by experimental conditions. These results provided new low-cost strategies that can be applied to increase of certain biomass components, which here are algal proteins, especially in Rhodophyta-based biotechnological industries for food and agriculture.

Keywords Bioreactor · Halogenated monoterpenes · Antifungal · Protein content · Bromoperoxidase · Rhodophyta

Introduction

Benthic marine macroalgae are the main primary producers in the coastal and marine ecosystem. Extreme abiotic conditions that are dynamic in this environment (e.g., daily tide variations, hydrodynamic regime, rain, seasonal oceanic currents, and light and ultraviolet radiation intensities) lead to alterations in nutrient availability, pH, salinity, temperature, drought, and light regime. Along with these features, a complex biotic network (e.g., herbivory, competition, epiphytes, and parasitism) also influences the relationship of benthic marine macroalgae with that environment (Lobban and Harrison 2004).

This intricate network compels macroalgae to face multiple stress-inducing factors, as oxidants (Lesser 2006) against which the organism produces a wide array of enzymes and metabolites (Bischof and Rautenberg 2012). In Rhodophyta, macroalgae may have defense strategies that take advantage of the high

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10811-019-01864-z) contains supplementary material, which is available to authorized users.

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availability of halogenated anions (Cl, Br, I) in the marine environment, enhancing the haloperoxidase activities in order to eliminate free radicals through the synthesis of halogenated compounds (Fenical 1975; Butler and Walker 1993). Terpenes, alkaloids and steroids (Güven et al. 2010), saponins (Jeeva et al. 2012), and lignans and phenolics (Machu et al. 2015) are substrates for haloperoxidase enzymes (Wever and Horst 2013).

Volatile halogenated metabolites (particularly terpenoids and essential oils) are within the group of known responses to environmental stress (Dring 2006). Among terpenoids, halogenated monoterpenes (HTMs) worth special attention because of their ecological functions, such as anti-herbivory, anti-biofouling, and anti-environmental stress (Paul et al. 1987). Regarding some biotechnological applications, it has been demonstrated that these compounds exhibit antitumor and antifungal activities, which highlight their potential for cancer therapeutics and control of pathogens of tropical fruits, respectively (Fuller et al. 1992; Butler and Carter-Franklin 2004; Machado et al. 2014a, 2014b).

The aforementioned defense mechanisms and potential applications demonstrate that the use of macroalga as a renewable resource might be an interesting strategy to evaluate the commercial value of halogenated compounds. Among macroalgae, *Ochtodes secundiramea* is one of the most promising source of essential terpene oils with significant biological activities (Machado et al. 2014c; Paula Pérez-López et al. 2016).

Maliakal et al. (2001) verified that the chemical profile of halogenated terpenoids produced by this alga was qualitatively and quantitatively influenced via nitrogen and phosphorous availabilities. Same results were observed after adding the halogens bromine and vanadate to the medium (Polzin et al. 2003)—known to have positive effects on bromoperoxidase activity (Rorrer et al. 2001). Together, these studies contributed to the identification of *O. secundiramea* strategies that allow the prediction and optimization of target halogenated terpenoid production (Rorrer and Cheney 2004; Ahmed and Taha 2011; Pérez-López et al. 2016). Other bioactive compounds like proteins can be improved by this enzymatic protection of free-radical attack (Ohsawa et al. 2001; França et al. 2007).

Synthesis of halogenated monoterpenes was directly related and can be manipulated by the availability of bromide and vanadate confirmed in the culture of *O. secundiramea* under bromide-free and enriched pulse condition (Polzin et al. 2003). These increases of bio-halogenation process pattern were verified in other Rhodophyta species, a well-known halogenated terpenes producer in enriched bromide responses (Paradas et al. 2010).

In this paper, unialgal apical segments of vegetative *Ochtodes secundiramea* (Montagne) M. Howe (Gigartinales, Rhodophyta) were cultured under naturally available (control at 50 mg L^{-1} of Br^-) and augmented (treatment at 250 mg L^{-1}, followed by weekly additions of 100 mg L^{-1} of Br^-) concentrations of bromine in natural seawater. Physiological responses were evaluated via the specific growth rate and protein, carbohydrate, and pigment contents. Profile of HTMs, bromine uptake, and accumulation were compared to bromoperoxidase activity. Lastly, among the potential applications of *O. secundiramea* extracts, their antifungal potentials were tested against relevant agriculture phytopathogens *Cladosporium sphaerospermum* Penzig (SPC 491) and *Colletotrichum lagenarium* Ellis & Halst (IB Col. 19 - Cucumber anthracnose).

Material and methods

Collection and laboratory cultivation conditions

Female gametophytes of the red alga *Ochtodes secundiramea* were collected from Ponta da Baleia beach, Espirito Santo State, Brazil (20°15′54″ S and 40°11′20″ W). After collection, the specimens were transported to the laboratory where they were isolated and cultivated. The voucher specimen was registered at the herbarium of Instituto de Botânica, São Paulo, Brazil, with the accession number SP401725.

Apical segments (2 cm) from female gametophytes were cultured in flasks containing the medium (80 mL), in which the composition was sterilized with seawater (salinity 32 ± 2 psu) enriched with half strength of von Stosch's solution (VSES/2 medium), as described by Oliveira et al. (1995) and modified by reducing 50% vitamin concentrations (Yokoya 2000). Medium renewal and procedure of seaweed decontamination (following Fernandes et al. 2011) were carried out weekly. Cultures were incubated at 23 ± 2 °C, under irradiance of 30 ± 10 μmol photons m^{-2} s^{-1}, provided by cool-white fluorescent lamps, under a 14:10-h light:dark cycle, without aeration. Irradiance was measured with a quantum photometer (LI-250; Li-Cor, Lincoln, NE, USA) equipped with underwater spherical quantum sensor (LI-192 SA; Li-Cor). After isolation, unialgal cultures were maintained with VSES/2 medium, which was renewed weekly.

Bioreactor culture conditions and analytical procedures

Experiments were performed in a bioreactor system (model TE-BIT-E3; Tecnal—Brazil) composed by six reaction vessels (volume 2.5 L) with stainless steel lids coupled with a thermometer (Tecnal), pH (Mettler Toledo—USA), oxygen (Mettler Toledo), and CO_2 (Mettler Toledo) sensors. A control system (Tecbio-soft; Tecnal) monitored the temperature and pH parameters as well as changes in dissolved O_2 and CO_2 concentrations in the culture medium. Constant aeration was

provided by the module Bio-Tec A (Tecnal), throughout the experimental period, at an airflow rate of 0.5 L min^{-1}.

The cultivation of *O. secundiramea* followed the same culture parameters described previously, except irradiance (enhanced to 90 ± 10 μmol photons m^{-2} s^{-1}). Culture medium consisted of sterile seawater enriched with VSES/2 solution, without (control, i.e., regular supplement) and with (treatment, i.e., augmented supplement) KBr addition (140 mg of fresh biomass L^{-1}). Experiments were carried out simultaneously with three replicates ($n = 3$). The VSES/2 media were renewed weekly over 21 days.

Specifically, control medium consisted of sterilized seawater with 50 ± 5 mg L^{-1} Br^- (median concentration of 52 mg L^{-1}). The bromine content in seawater measured before sterilization was 58 mg L^{-1} being in agreement with the one found by Saenger (1972). Regarding the treatment group, bromine concentration was 250 ± 10 mg L^{-1} at time zero. The weekly addition of a bromine reposition solution (100 mg L^{-1} KBr) to the culture medium provided high levels of bromine over all the experiment period. This protocol did not alter the salinity of seawater.

Determination of daily growth rate

The daily growth rate (DGR) values were evaluated weekly from the ratio of the fresh mass variation (Δm: $ln\,[m_{final} / m_{initial}]$) and the time in days ($\Delta t$:$t_{final} - t_{initial}$): $DGR = \Delta(m / t)$.

Determination of bromine concentration in culture media and macroalgae biomass

Changes in concentrations of dissolved bromine from both control and treatment culture media ($n = 3$) were assessed weekly by spectrophotometric assays (Saenger 1972). Briefly, after the reaction of 20 mL culture medium with 5.0 mL H_2SO_4 (16 N), 5.0 mL of chloroform and 1.5 mL chloramine T (15 g L^{-1}) (Sigma-Aldrich, EUA), the bromine concentration was determined spectrophotometrically in a 1-cm path length cuvette at 400 nm against a bromine standard curve (concentration range, 0 to 600 mg L^{-1} and linearity coefficient of 0.981). Additionally, control and treatment experiments were performed without algae for over 21 days (Mendes et al. 2013). This approach was important to ensure that the variations observed in bromine concentrations were exclusive because of *O. secundiramea* metabolism. There were no observed statistical differences between bromine concentration initial and after 21-day control ($F = 2.3128$; $p = 0.1524$) and ($F = 2.0996$; $p = 0.1784$) treatment (Supplementary Fig. 1). Also, data collected through the bioreactor sensors obtained during the experiments are presented in Supplementary Fig. 2.

In order to evaluate the concentration of bromine in *O. secundiramea*, the fresh biomasses (100 mg each; $n = 3$) were rinsed twice with distilled water and dried in an oven at 65 °C over 24 h. After, 10 mL of 10% Na_2CO_3 aqueous solution was added to each dried and powdered biomass to prevent bromine volatilization. Then, the samples were incinerated in a muffle furnace (440 °C for 8 h). The ashes were suspended in 5 mL of distilled water and filtered through Whatman No. 541 filter paper. The samples were washed with deionized water continuously up to a final volume of 20 mL.

Bromoperoxidase activity

The principle of the bromoperoxidase (BPO) activity assay is the brominating of monochlorodimedone (MCD) with Br^- and H_2O_2 (Butler and Walker 1993), being the quantification accomplished according to Rorrer et al. (2001). Briefly, fresh biomasses (100 mg, $n = 3$) collected at the end of the experiment (21 days) were immediately ground in $N_{(l)}$. Afterward, samples were suspended in 50 mM KH_2PO_4 buffer (pH 6.0) containing 25 mM H_2O_2—used to avoid the inactivation promoted by phosphate (Soedjak et al. 1991). The BPO activity was determined by spectrophotometry in a 1-cm cuvette following the absorbance decay at 290 nm (UV-1650PC, Shimadzu), in comparison with controls containing 0.10 mL of a 1.25 U mL^{-1} of purified bromoperoxidase commercially available (Cat. B2170, Lot No. 105H38993, Sigma-Aldrich, USA) (Rorrer et al. 2001).

Pigments analyses

Fresh biomasses (80 mg, $n = 3$) were ground in $N_{(l)}$, then each sample suspended with 1 mL of 50-mM phosphate buffer (pH 5.5) and centrifuged at 12,000×*g* for 10 min (4 °C). The supernatants were used for the quantification of phycobiliproteins (allophycocyanin, phycocyanin, and phycoerythrin) through the formulas described by Kursar et al. (1983). Afterwards, each pellet was re-suspended in 1 mL of 90% acetone and the solutions centrifuged at 12,000×*g* for 15 min (4 °C). Supernatants containing chlorophyll *a* were transferred to vials, sealed and kept in dark until the spectrophotometric analyses. Determination of chlorophyll concentration was carried out according to Jeffrey and Humphrey (1975).

Total soluble carbohydrates analyses

Each $N_{(l)}$ ground fresh sample (300 mg, $n = 3$) was suspended in 1 mL of 70% ethanol. Samples were incubated for 15 min at 50 °C and centrifuged at 3000×*g* for 15 min. The supernatants were collected and concentrated with a rotary evaporator at 60 °C. The total carbohydrate concentrations were determined by spectrophotometry in a 1-cm pathlength cuvette at 490 nm after the addition of phenol and sulfuric acid (5%), according to Dubois method (Dubois et al. 1956). The concentrations of total soluble carbohydrates were obtained in comparison to a

standard curve (range 0 to 600 mg L^{-1} and linearity coefficient of 0.998) of glucose (Sigma-Aldrich, USA).

Total soluble proteins assay

Each $N_{(l)}$ ground fresh sample (80 mg, $n = 3$) was suspended in extraction buffer (0.2 M phosphate buffer, pH 7.5, 8.5 mM EDTA; 1 mM DTT) at a ratio of 0.1 g per liter. The solutions were centrifuged at 12,000×*g* for 15 min (4 °C). Total soluble protein content was determined by spectrophotometry in a 1-cm path length cuvette at 595 nm, after addition of Coomassie Blue solution (Bio-Rad) following the Bradford (1976). The concentrations of total soluble proteins were obtained in comparison to a standard curve (range 0 to 600 mg L^{-1} and linearity coefficient of 0.991) of bovine serum albumin (BSA, Bio-Rad, USA).

Extraction and analysis of compounds and antifungal activity

Crude extraction preparation and chemical and antifungal analyses were based upon our previous study (Machado et al. 2014a). Samples ($n = 3$) were extracted by maceration of seaweed with 10 mL of DCM/MeOH (2:1, *v*/*v*) per gram. After 1 week of incubation, the extracts were filtered through filter paper no. 5 (Whatman, UK) and concentrated under nitrogen flow. The dried extracts were analyzed by gas chromatography–mass spectrometer (GC–MS) QP2010 Plus (Shimadzu—Japan), using a 30 m × 0.25 mm × 0.1 μm HP-5MS capillary column. Samples were injected in split mode at 220 °C. Transfer line was set to 240 °C. Helium (99.999%) was used as carrier gas at a constant flow rate of 1 mL min^{-1}. A linear temperature program was employed at a rate of 3 °C min^{-1}; ranging from 60 to 260 °C and then held at 260 °C for 40 min. Detection was performed in full scan mode over mass-to-charge ratios ranging from 50 to 650 m/z. Electron impact ionization (collision energy = 70 eV) was employed and the ion source temperature maintained at 240 °C. Compounds were identified by their GC retention times and mass spectra (NIST 08 library). Compounds that were not listed in the library's database were identified and supported by data from the literature. Peaks were integrated to obtain their respective areas.

Bioautography antifungal assays were performed with spores of two model fungi: *Cladosporium sphaerospermum* Penzig (SPC 491) and *Colletotrichum lagenarium* Ellis & Halst (IB Col. 19—Cucumber anthracnose). These fungi were cultured for 12 days until sporulation. *Cladosporium sphaerospermum* was cultured in potato dextrose agar (Difco—USA) while *C. lagenarium* in oatmeal agar (Difco—USA). Spore suspensions of these fungi were collected with a solution containing glucose and salts (Homans and Fuchs 1970; Rahalison et al. 1994). Both solutions at 10^8 spores mL^{-1} were used for qualitative and quantitative antifungal tests.

Qualitative antifungal activity was determined by bioautography assay. Briefly, 10 μL of a solution containing 100 μg of crude extract was applied to Al-backed, silica gel GF 254 TLC plates (Merck—Germany) and developed with DCM/MeOH (99:1, *v*/*v*). Limit of detection of antifungal activity was assessed using the same test by evaluating 10 μL solutions containing 1, 5, 10, 25, and 50 μg of crude extract. Then, the solvent was completely removed, and TLC plates, sprayed with fungal spore suspensions, were incubated for 72 h at 28 °C. After incubation, clear inhibition zones appeared against the dark backgrounds of the TLC plates. The retention factor (Rf), defined as the ratio of the distance from the origin to the location of the solvent front over the distance from the origin to the center of each spot, was recorded. Cinnamic acid (1.0 μg) was used as a standard.

Statistical analyses

A completely randomized design was used to analyze the extraction yield. Each treatment was tested in triplicates. Data are presented as means and their standard deviations, with error bars representing the standard deviation. Firstly, the normal distribution was checked by Shapiro-Wilk test. Statistical analyses were performed using Assistat Software Version 7.7 (Universidade Federal de Campina Grande—Brazil) (Silva and Azevedo 2016). One-way analysis of variance (ANOVA) was performed when statistically significant differences were detected by Tukey's test. Differences were considered significant at a minimum level of $p < 0.05$.

Results

Bromine enrichment in *O. secundiramea* culture did not promote statistically significant changes in growth rate ($p > 0.05$), growth curve, nor biomass weight during the 21-day experiment (Fig. 1). The treated and control biomasses weights were 2.378 g and 2.382 g, respectively.

Bromine uptakes by *O. secundiramea* from culture media were, respectively, 1.84 mg $L^{-1}day^{-1}$ and 14.47 mg $L^{-1}day^{-1}$ for control and treatment (Fig. 2).

The results of bromine and enzymatic activity were presented in Table 1. As expected, the bromine amount in treated alga (106.35 mg of bromine per gram of fresh algae) was greater than in control ones (45.87 mg of bromine per gram of fresh algae). The amount of bromine found in treated alga was more than twice as large compared to the value seen in non-treated one ($p < 0.05$). The same was observed in bromoperoxidase activity (BPO) ($p < 0.05$).

The treatment did not affect both macroalgae growth and total sugar content ($p > 0.05$). However, both total soluble protein (Fig. 3I) and pigments (Fig. 3II) increased significantly. At the end of the experiment, total protein values were

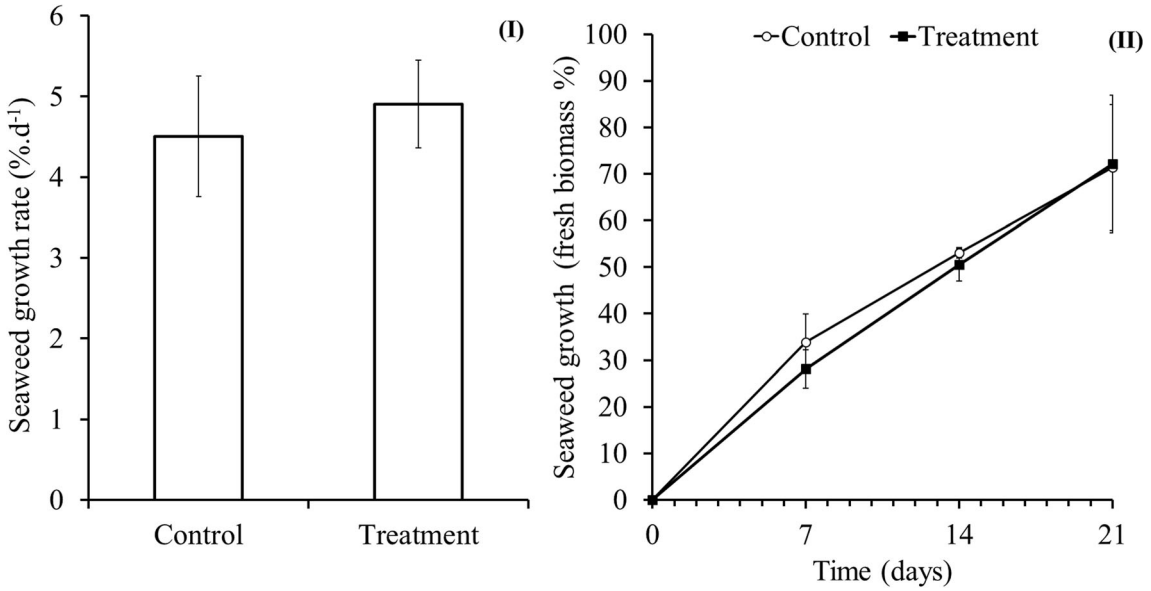

Fig. 1 (I) Growth rate (% day^{-1}) and (II) growth curve measured by fresh weight (%) of *O. secundiramea* after 21 days of growth in bioreactors in response to control conditions (50 mg L^{-1}) and treatment (250 mg L^{-1} of bromine with weekly addition of 100 mg L^{-1}). Values correspond to mean ± standard deviation ($n = 3$)

5.913 mg g^{-1} for treated algae while 3.146 mg g^{-1} for untreated ones, i.e., almost doubled.

There was no significant difference ($p > 0.05$) between the extraction yields (%) of control (0.87 ± 0.06) and treated (0.93 ± 0.09) algae. GC–MS chromatographic profiles of both extracts were qualitatively similar: 10 peaks were observed in control samples and 9 in treated ones. The major compounds, which were determined by semi-quantitative analyses, were peak 9 (57.52%) in the control and peak 8 (59.88%) in the treated extract.

The main differences in the composition of these *O. secundiramea* extracts were the presence of mono- and

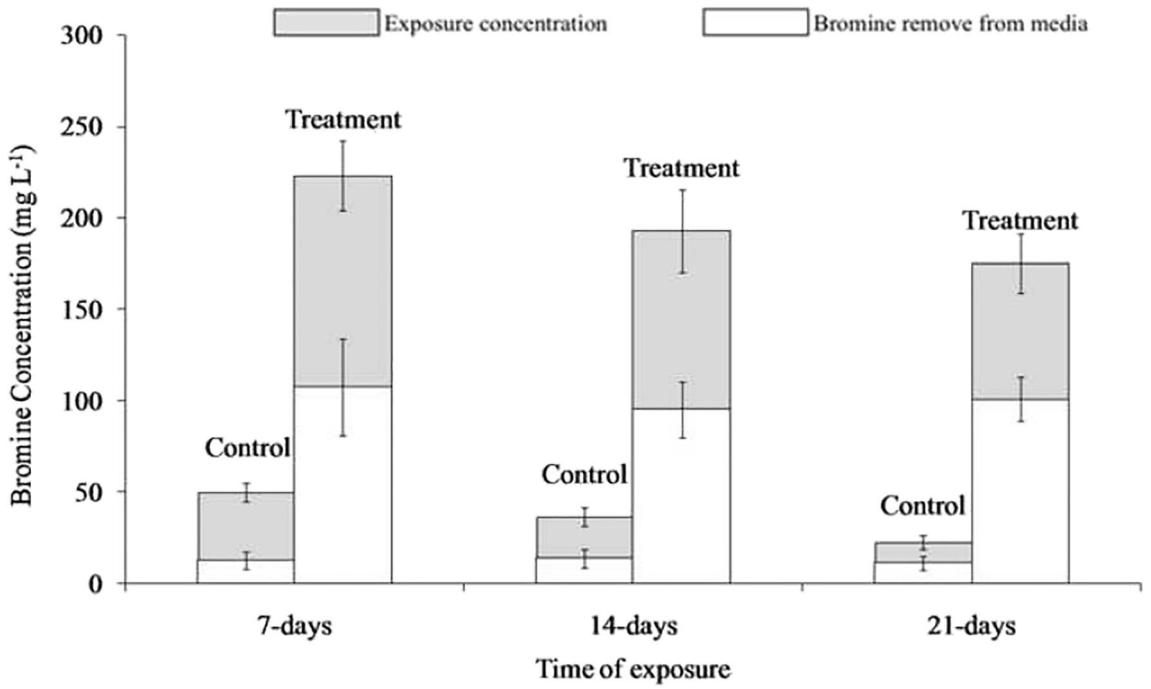

Fig. 2 Bromine exposure concentration (mg L^{-1}) represented by shadows bars measured in culture media through 21 days of cultivation in bioreactor in response to control (50 mg L^{-1}) and treatment (250 mg L^{-1} and weekly addition of 100 mg L^{-1} of bromine) groups. (II) Macroalga bromine incorporation at the end of experiment (day 21) in control and treatment groups. Values correspond to mean ± standard deviation ($n = 3$)

Table 1 Final content of bromine incorporated in biomass, total bromine added in each experiment, bromine daily uptake rate, and specific activity of BPO in enzyme units (U mg^{-1}) by *O. secundiramea* cultivated in bioreactors. Results followed by different letters differ from Tukey test, considering 5% of significance

Treatments	Control group	Treated group
Bromine concentration in *O. secundiramea* after 21 days of exposure	45.87 mg g^{-1} a	106.35 mg g^{-1} b
Total amount of bromine used over 21 days of exposure	0 mg	450 mg
Bromine uptake	1.84 ± 0.16 mg day^{-1} a	14.47 ± 0.71 mg day^{-1} b
Specific activity of BPO in enzyme units	0.34 ± 0.04 U mg^{-1} a	0.63 ± 0.07 U mg^{-1} b

*Mean ± SD ($n = 3$)

di-HMTs ($C_{10}H_{15}OBr$, 229 *m/z*, RT 31.342 min and $C_{10}H_{12}OBr_2$, 311 *m/z*, RT = 35.167 min) in control samples, and the prevalence of di-HMTs in treated ones. Their composition was identified based on comparison of fragmentation patterns with those from the available literature data (Paul et al. 1980; Gerwick 1984; Coll and Wright 1987; Maliakal et al. 2001; Polzin et al. 2003; Barahona and Rorrer 2003; Machado et al. 2014a, 2014b). Table 2 shows these compounds and provides additional information about them [retention time, relative concentrations (area %), molecular formula]. Total ion chromatogram (TIC) and mass fragments of identified compounds were available in Supplementary Figs. 3 and 4. Cholesterol (386 *m/z*, RT 76.734; similarity superior to 95% according to NIST database library) was also detected in both extracts.

The same fungal inhibitory effect was observed in both control and treated extracts: in both cases, the inhibition zones had the same size and retention factors (Rf 0.85). The activity detection limits were 5 μg and 10 μg against the fungi *Cladosporium sphaerospermum* and *Colletotrichum lagenarium* (Supplementary Fig. 5), respectively.

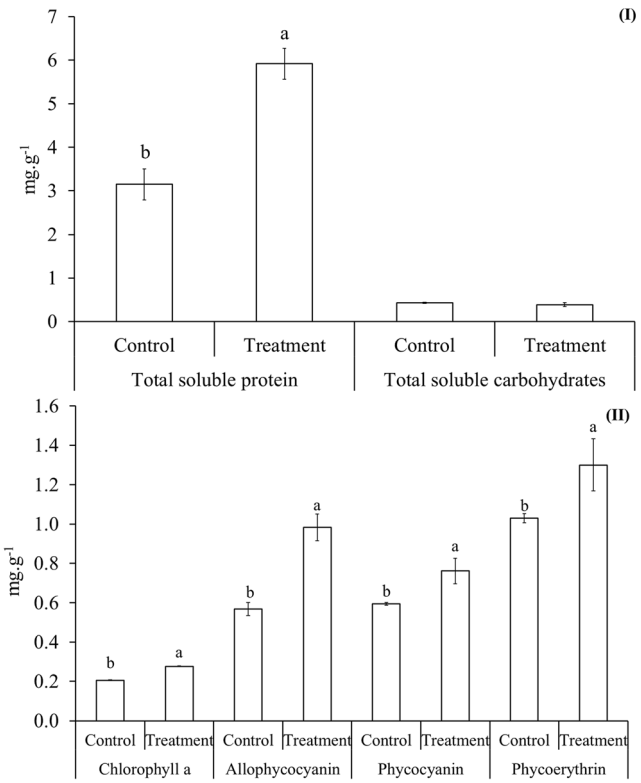

Fig. 3 Pigments, total soluble proteins, and carbohydrates (mg g^{-1}) concentration in *O. secundiramea* after 21 days in the control (50 mg L^{-1}) and the treatment (250 mg L^{-1} and weekly additions of 100 mgL^{-1} of bromine) groups. The values correspond to the mean ± standard deviation ($n = 3$). Treatments followed by different letters differ by Tukey test, considering 5% of significance

Discussion

Ochtodes secundiramea is a known producer of essential oils, particularly HTMs (Pérez-López et al. 2016). HTMs are secondary metabolites synthesized against environmental stress and some from red macroalgae have been described to have antifungal activities (Machado et al. 2014c), antifouling (Sudatti et al. 2006; Paradas et al. 2010), and therapeutic (Blunt et al. 2007). These metabolites are stored in vesicles that are transported to the cell wall region under environmental stress, such as the bacterial surface colonization in the red macroalgae *Laurencia obtusa* (Hudson) J.V. Lamouroux (Paradas et al. 2010) and *Asparagopsis armata* Harvey (Paul et al. 2006). In both macroalgae, the vesicular transport mechanisms seem to be bromine-dependent. For example, the bromine's absence in the culture medium causes vesicles' loss in *A. armata* related to the absence of HTMs syntheses (Paul et al. 2006), whereas different concentrations of this halogen affected the mean frequency of vesicle traffic in *L. obtusa* (Paradas et al. 2010).

Despite the pivotal role of bromine in the aforementioned cellular mechanism and the structure-related alterations against environmental stress, the presence and absence of this halogen into the seawater appear to not influence growth rate (Paul et al. 2006). That was in accordance with the results from the present study, where growth rate values for control (4.8% day^{-1}) and treatment (5.0% day^{-1}), were similar to 4.4% day^{-1} observed by Polzin et al. (2003) and Polzin and Rorrer (2018) in free-bromine culture of *O. secundiramea.*

Table 2 Retention time (RT), peak area percentage in the chromatogram, molecular ion, corresponding molecule, and reference for each peak observed in the GC–MS analysis chromatogram of *O. secundiramea* extracts from bioreactor cultures under control conditions (cultured 50 mg L^{-1} of bromine) and treatment (cultured at 250 mg L^{-1} and weekly additions of 100 mg L^{-1} of bromine)

Bioreactor seaweed culture conditions	Peak	RT	Area (%)	Molecular ion	Molecular formulae	Reference
Control (culture at 50 mg L^{-1} of bromine)	1	31.342	4.1	229	$C_{10}H_{15}OBr$	Paul et al. (1980)
	2	35.167	5.81	294	$C_{10}H_{14}Br_2$	Barahona and Rorrer (2003)
	3	40.310	5.13	249	$C_{10}H_{14}BrCl$	Maliakal et al. (2001)
	4	41.738	3.7	311	$C_{10}H_{12}OBr_2$	Paul et al. (1980)
	5	42.166	4.4	284	$C_{10}H_{13}BrCl_2$	Polzin et al. (2003)
	6	43.596	4.81	294	$C_{10}H_{14}Br_2$	Barahona and Rorrer (2003)
	7	44.330	4.56	325	$C_{10}H_{13}Br_2Cl$	Polzin et al. (2003)
	8	45.002	5.17	410	$C_{10}H_{14}Br_3Cl$	Gerwick (1984)
	9	47.507	57.52	329	$C_{10}H_{15}Br_2Cl$	Coll and Wright (1987)
	10	76.734	4.8	386	$C_{27}H_{46}O$	Patterson (1971)
Treatment (culture at 250 mg L^{-1} and weekly additions of 100 mg L^{-1} bromine)	1	35.164	3.36	294	$C_{10}H_{14}Br_2$	Barahona and Rorrer (2003)
	2	40.307	4.18	249	$C_{10}H_{14}BrCl$	Maliakal et al. (2001)
	3	41.738	6.16	311	$C_{10}H_{12}OBr_2$	Paul et al. (1980)
	4	42.162	4.97	284	$C_{10}H_{13}BrCl_2$	Polzin et al. (2003)
	5	43.593	4.58	294	$C_{10}H_{14}Br_2$	Barahona and Rorrer (2003)
	6	44.331	3.87	325	$C_{10}H_{13}Br_2Cl$	Polzin et al. (2003)
	7	45.005	4.95	410	$C_{10}H_{14}Br_3Cl$	Gerwick (1984)
	8	47.507	59.88	329	$C_{10}H_{15}Br_2Cl$	Coll and Wright (1987)
	9	76.734	3.75	386	$C_{27}H_{46}O$	Patterson (1971)

The macroalgae present different sensitivities in response to bromide addition, from inhibition to stimulation growth in red algae species (Fries 1966; Iwasaki 1967). Synthesis of halogenated monoterpenes is strongly related to bromide and vanadate availability, while concentration more than 0.1 mM can promote saturation of that processes (Polzin et al. 2003). Absence alterations of HMT's GC–MS profile of *O. secundiramea* in our results were explained by natural bromine available, and enriched treatment conditions were superior to saturation point.

However, the overexposure to bromine had a positive effect on halogenated compound production as previously observed in the red macroalgae *Laurencia obtusa* (Paradas et al. 2010) and *Asparagopsis armata* (Paul et al. 2006). Here, the highest bromine uptake rate was observed, and incorporation associated a common response pattern of Rhodophyta to this challenge.

Otherwise, tested bromine availability increases in the specific activity of bromoperoxidase and that affected positively total soluble proteins and phycobiliproteins (allophycocyanin, phycocyanin, and phycoerythrin), resulting from the protective effect of this enzyme on damage caused by oxidative stress (Ohsawa et al. 2001). This presents an extensive application considering the relationship of biostimulant potential applied to agriculture which is related to these molecules (Machado et al. 2018).

The results found here for bromoperoxidase activity and enzyme concentration fit with those reported by Rorrer et al. (2001) which were obtained from biomass harvested at exponential growth phase as well. The increased specific concentration of BPO enzyme and its activity in *O. secundiramea* bromine-enriched cultures were a response to high Br^- concentrations. This enzyme activity is an important step for halogenation of organic compounds, and this process is one of the main mechanisms to alleviate oxidative stress in seaweeds (Fenical 1975; Rorrer et al. 2001; Dring 2006).

Enrichment of seawater with KBr promoted an increase in the amount of BPO enzyme concentration, its activity, uptake, and incorporation of Br^- by seaweed. However, no changes were detected in the HMT profile. This result is related to the non-production of terpenes, possibly caused by low carbon availability as well as nitrogen balance (C/N ratio) (Mata et al. 2012). Additionally, lack of stimuli to myrcene synthase, responsible for the synthesis of monoterpenes, could be an important factor as well (Wise et al. 2002; Polzin and Rorrer 2018). Another possible fate to bromine is its combination of different molecules such as fatty acids or phenols because BPO lacks specificity (Carvalho and Roque 2000; Butler and Carter-Franklin 2004).

The knowledge regarding the mechanisms and activity of myrcene synthase (Wise et al. 2002), increasing synthesis of volatile compounds in CO_2 enriched media (Mata et al. 2012),

and higher rate of halogenated compounds in red seaweeds exposed to H_2O_2 (Mata et al. 2011) in addition to the findings of the present work (demonstration of increased BPO activity as well as higher bromine uptake in *O. secundiramea* supplemented with KBr) pave the way to develop platforms to grow seaweed with biotechnology applications seeking to induce and obtain high yield of bioactive compounds.

Therefore, KBr enrichment in bioreactor cultures of Rhodophyta did not affect growth rate but promoted an increase in protein and pigment contents by 2-fold. Thus, the combination of these factors is a very interesting biotechnological strategy to increase the nutritional attributes of seaweed, considering the low cost of bromine salt and the high added value of metabolites.

Funding information The financial support of the present study was provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). ES was funded by FAPESP2010/14893-9. NSY was provided grants from CNPq (Proc. 308879/2013-9) and CAPES (AUXPE-CIMAR 1991/2014).

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