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Improvement in carbohydrate and phlorotannin extraction from *Macrocystis pyrifera* using carbohydrate active enzyme from marine *Alternaria* sp. as pretreatment

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Abstract The commercial importance of brown seaweed has been increasing over the past decade, especially due to industries interested in the extraction of phycocolloids and, more recently, of polyphenol compounds such as phlorotannins. The objective of this work was to optimize the extraction conditions of carbohydrates and phlorotannins from *Macrocystis pyrifera*, evaluated enzymatic pretreatment and different parameters of extraction using design of experiment. The optimal conditions upon extraction of the carbohydrates and phlorotannins were determined by means of a pretreatment protocol taking advantage on a carbohydrate active enzyme, followed by an alkaline hydrolysis with 0.5 N NaOH at 100 °C, 180 min, and S/L ratio of 1/20. In order to extract the carbohydrates, the best conditions found for the pretreatment procedure were 37 °C, pH 7.0 for 24 h, and a S/L ratio of 1/10, giving an extraction yield (EY) of 89.67 ± 12.3 wt.%. In turn, for the extraction of phlorotannins, the best conditions identified in terms of the pretreatment were 25 °C, pH 7.0 for 36 h, and a S/L ratio of 1/20, thus giving a yield (EY) of 2.14 ± 0.25 wt.%. Statistical analysis of both processes revealed a maximum EY of 91.24 wt.% for carbohydrates and 3.31 wt.% EY for phlorotannins.

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Introduction

In recent years, the quest for more environmentally benign and economical processes to produce biofuels and biomolecules has been accelerating. One alternative is to adopt the biorefinery philosophy in the production of biofuels and bioproducts, thus maximizing the use of biomass and increasing the profitability of the processing plant as well to decrease of the amount of biomass discarded as waste (Hafting et al. 2015). Recently, algae have received a significant interest as a potent biomass for biorefinery processes due to their fast growth rate relative to terrestrial plants and not needing extra nutrients, fresh water, or fertilizer for growth.

The brown seaweed *Macrocystis pyrifera* is the most widely distributed kelp species in the cold temperature waters of the Northern and Southern hemispheres, forming ecologically diverse and productive kelp forests. In Chile, *M. pyrifera* is distributed along the coast from Iquique to Cape Horn. The commercial importance has increased over the past decade, especially due to the interest to extract phycocolloids such as alginate (Buschmann et al. 2008).

The cell wall of seaweeds is constituted of complex polysaccharides, proteins, and polyphenol compounds which together form a physical barrier in the algae and limit the extraction process of different compounds (Wijesinghe and Jeon, 2012). The polysaccharides found in brown seaweeds mainly consist of alginate (up to 40%), laminarin (up to 35%), and fucoidan (up to 15%) (Hahn et al. 2012; Jung et al. 2013). Consequently, in order to improve the extraction yield of bioactive compounds, the cell wall must be disrupted by means of a pretreatment. The open literature describes that enzymatic

hydrolysis is a less aggressive alternative (in reaction temperature and time) compared to acid and alkaline hydrolysis. For an efficient enzymatic pretreatment, an optimal mixture of enzymes is necessary for the degradation of the cell wall and for obtaining the maximum recovery of the desired compounds (Wijesinghe and Jeon, 2012).

Phenolic compounds known as phlorotannins are produced as secondary metabolites in many brown algae (Phaeophyceae) and, importantly, are not found in terrestrial plants or other types of algae. These compounds have been extensively studied for their potential health benefits, and reportedly they have shown promising effects against radical-mediated oxidative stress, photon-induced cell damage, cancer, allergy, diabetes, inflammation, and viral as well as microbial infections (Le Lann et al. 2016; Zenthoefer et al. 2017). In *M. pyrifera*, different extraction conditions of phlorotannins and their partial identification have been evaluated (Kindleysides et al. 2012; Leyton et al. 2016).

The aim of this work was to determine the best extraction conditions of carbohydrates and phlorotannins contained in *M. pyrifera* for which different conditions and extraction variables, such as an enzymatic pretreatment, pH of the aqueous solvents, extraction time, temperature, and solid-to-liquid ratio upon extraction were evaluated.

Materials and methods

Characterization of algal material

Macrocystis pyrifera, donated by Dr. A. Buschmann (Universidad de Los Lagos), was collected by scuba diving 30 km Southwest of Puerto Montt, Chile. The sample was harvested in May 2013, dried at room temperature, and ground to an average size of <0.5 mm. Chemical analysis of the *M. pyrifera* sample was carried out in order to determine the initial composition of the algae in terms of proteins, lipids, carbohydrate, and ash elements. The analyses were carried out at the Institute of Agroindustry, University of Frontera, Chile. The moisture content, as well as protein, lipid, ash, and fiber contents were quantified following the official methods of the Association of Official Analytical Chemistry (AOAC): Methods 930.04, 978.04, 991.36, 930.05, and 962.09, respectively (AOAC, 2000). The carbohydrate content was calculated from the difference between the initial mass and the sum of values reported for proteins, ash, lipids, and fibers (Merril and Watt 1973).

Influence of the pH of the aqueous solvent in carbohydrate and phlorotannin extraction

In our previous study, we found that washing the algae with hexane followed by extraction with water improved the extraction of phlorotannins (Leyton et al. 2016). Considering these parameters, the influence of pH on the extraction of carbohydrates and phlorotannins was evaluated. For this, 0.2 g of dry algae (washed with hexane) was dispersed in 2 mL of water at different pH (1, 3, 4, 6.5, 10, 12, and 4–10, adjusted with H_2SO_4 or NaOH for pH 1.3 and 12, and H_3PO_4 or Na_2CO_3 for pH 4, 6.5, and 10) (Pham et al. 2013; Briceño-Dominguez et al. 2014; Gómez-Ordóñez et al. 2014). All of the above-mentioned procedures were carried out on a shaker operated at 300 rpm and 120 °C for 1 h. The mixture was separated by filtration using glass fiber filters; supernatant was stored at −20 °C and for further analysis for the quantification of total concentration of phlorotannins and carbohydrates.

Optimization conditions of carbohydrate and phlorotannin extraction using the Box-Behnken design

In order to optimize carbohydrate and phlorotannin extraction, different extraction variables, namely the extraction time (60, 120, and 180 min), extraction temperature (80, 100, and 120 °C), and the solid-liquid (S/L) ratio in terms of the algae (1:10, 1:15, and 1:20) as well as the best pH of the extractant (described in the "Influence of the pH of the aqueous solvent in carbohydrate and phlorotannin extraction" section) were evaluated. All these variables were simultaneously evaluated using a Box-Behnken design (Tahmouzi and Ghodsi, 2014) according to Table 1. At the end of the incubation time of each every experimental design run, the samples were centrifuged at 3600×*g* for 10 min at 4 °C, and the supernatant was stored for further analyses. The best extraction conditions were determined in terms of the extraction yield (EY) of phlorotannins and carbohydrates.

The predicted response values were matched into an empirical second-order polynomial equation using the expression below:

$$\begin{aligned}\text{Response} = {} & \beta_0 + \beta_1{}^{*} A + \beta_2{}^{*} B + \beta_3{}^{*} C + \beta_{11}{}^{*} A^2 \\ & + \beta_{12}{}^{*} A^{*} B + \beta_{13}{}^{*} A^{*} C + \beta_{22}{}^{*} B^2 - \\ & + \beta_{23}{}^{*} B^{*} C + \beta_{33}{}^{*} C^2 \end{aligned} \quad (1)$$

where *response* represents the dependent variables of the yields obtained from the carbohydrate and phlorotannin extraction; *A*, *B*, and *C* represent the independent variables: time, temperature, and S/L ratio, respectively. B_0 is a constant coefficient in the model; β_1, β_2, and β_3 are the coefficients of the linear equation; β_{11}, β_{22}, and β_{33} are the quadratic coefficients; and β_{12}, β_{13}, and β_{23} represent the interactive terms. The second-order polynomial coefficients were calculated and analyzed using the Statgraphics software (Montgomery, 2001).

Table 1 Box-Behnken experimental design with independent variables and their levels used for optimizing the extraction conditions of carbohydrates and phlorotannins from *Macrocystis pyrifera*. Both experimental and predicted values are given. The ± shows the standard deviation where $n = 3$

Runs	Time (min)	Temperature (°C)	S/L ratio	CEY experimental (%)	CEY predicted (%)	PEY experimental (%)	PEY predicted (%)
1	60	100	1/20	42.53 ± 2.96	49.61	1.36 ± 0.05	1.43
2	180	100	1/20	77.57 ± 8.47	72.34	1.61 ± 0.13	1.57
3	180	120	1/15	62.1 ± 3.24	71.29	1.66 ± 0.22	1.63
4	60	120	1/15	59.95 ± 3.44	56.81	2.66 ± 0.92	2.50
5	120	120	1/20	65.57 ± 2.12	61.63	2.13 ± 0.09	2.23
6	60	80	1/15	43.27 ± 2.66	34.10	0.86 ± 0.01	0.90
7	180	80	1/15	51.06 ± 5.3	54.21	1.22 ± 0.05	1.39
8	120	100	1/15	56.39 ± 5.58	61.21	2.35 ± 0.85	1.65
9	120	80	1/20	44.18 ± 5.35	46.27	1.51 ± 0.26	1.41
10	120	100	1/15	57.16 ± 2.37	61.21	1.36 ± 0.05	1.65
11	120	100	1/15	54.91 ± 1.58	61.21	1.39 ± 0.39	1.65
12	120	80	1/10	28.71 ± 3.16	32.65	1.51 ± 0.18	1.43
13	60	100	1/10	40.72 ± 1.27	45.96	1.83 ± 0.3	1.88
14	180	100	1/10	64.88 ± 3.54	57.81	1.43 ± 0.41	1.36
15	120	120	1/10	59.16 ± 8.45	57.08	2.34 ± 0.10	2.45
				R^2	88.97	R^2	96.08

S/L solid/liquid ratio of algae/extractant, *CEY* carbohydrate extraction yield, *PEY* phlorotannin extraction yield, R^2 correlation coefficient

Effect of the enzymatic pretreatment in the carbohydrate and phlorotannin extraction using the Taguchi experimental design

The effect of an enzymatic pretreatment before optimized conditions of carbohydrate and phlorotannins extraction was evaluated. The enzymatic pretreatment was carried out using a set of extracellular carbohydrate active enzymes (alginate lyase, fucoidanase, and 1,3-β-D-glucanase) produced by the marine fungus *Alternaria* sp. (accession N°: KU163454 of database NCBI). The strain was precultured in 250-mL Erlenmeyer flasks with 100 mL fermentation broth: 70 vol.% artificial seawater (ASW, 27.5 g L^{-1} NaCl; 5.38 g L^{-1} $MgCl_2 \cdot 6H_2O$; 6.78 g L^{-1} $MgSO_4 \cdot 7H_2O$; 0.72 g L^{-1} KCl; 0.2 g L^{-1} $NaHCO_3$; and 1.6 g L^{-1} $CaCl_2$) (Shene et al. 2013), 4 g L^{-1} yeast extract, and 1 wt.% dry *M. pyrifera*, by 3 days at 25 °C under continuous shaking at 200 rpm. Afterwards, 5 mL of precultured was introduced in fresh fermentation broth as inoculum (5 vol.% of total volume) and incubated at 25 °C for 3 days. Finally, the culture was centrifuged at 8500×*g* at 4 °C for 5 min and the phase liquid (enzymatic extract) was collected for use. The activity of the enzyme extract was 60.34 ± 4.97 U mg^{-1}, where one unit of enzyme activity, U, is defined as the amount of enzyme that catalyzed the liberation of 1 μmol glucose equivalent reducing end in a time interval of 1 mg per minute, under the assay conditions (An et al. 2008; Ghose 1987).

The parameters selected for the enzymatic pretreatment were the incubation time (12, 24, and 36 h), temperature (25, 37, and 50 °C), pH (4.5, 5.5, and 7.0), and S/L ratio of the algae-enzyme extract (1:10, 1:20, and 1:30). All these parameters were simultaneously evaluated using the Taguchi experimental design detailed in Table 2 (Taguchi, 1990). Upon completion of the incubation time of each and every experimental design run, the mixture was centrifuged at 3000×*g*, for 5 min and at 4 °C, and the pellet was dried and stored for further extraction processes under the optimized extraction conditions defined in the "Optimization conditions of carbohydrate and phlorotannin extraction using the Box-Behnken design" section. The optimized conditions of pretreatment of the algae were determined based on the liquid

Table 2 Influence of parameters in the pretreatment of *Macrocystis pyrifera* using the Taguchi experimental design on carbohydrate and phlorotannin extraction yields. The ± shows the standard deviation, where $n = 3$

Runs	Time (h)	Temperature (°C)	pH buffer	S/L ratio	Carbohydrate EY (%)	Phlorotannin EY (%)
1	12	25	4.5	1/10	71.11 ± 3.45	1.14 ± 0.10
2	12	37	5.5	1/20	53.74 ± 9.63	1.44 ± 0.16
3	12	50	7	1/30	71.45 ± 2.91	1.45 ± 0.08
4	24	25	5.5	1/30	66.33 ± 5.25	1.75 ± 0.02
5	24	37	7	1/10	89.67 ± 12.27	1.58 ± 0.03
6	24	50	4.5	1/20	65.57 ± 8.83	1.39 ± 0.12
7	36	25	7	1/20	69.02 ± 1.33	2.14 ± 0.25
8	36	37	4.5	1/30	59.56 ± 4.05	1.78 ± 0.09
9	36	50	5.5	1/10	70.64 ± 6.39	1.56 ± 0.09

S/L solid/liquid ratio of algae/enzyme extract, *EY* extraction yield

phase obtained after the extraction process and evaluated in terms of the extraction yield of phlorotannins and carbohydrates.

Determination of the concentration of phlorotannins (TPC)

The amount of total phlorotannins compounds in the extracts was determined according to the Folin–Ciocalteu assay (Singleton and Rossi, 1965) adapted to 96-well plates. Standards containing phloroglucinol with concentrations varying from 20 to 100 mg L^{-1} were prepared to measure the amount of phlorotannins in the extracts. Samples and standard (20 μL) were introduced separately into the 96-well plates, each containing 100 μL of Folin–Ciocalteu's reagent diluted with water (10 times) and 80 μL of sodium carbonate (7.5% *w*/*v*). The plates were mixed and incubated at 45 °C for 15 min. The absorbance was measured at 765 nm using an UV–visible spectrophotometer. Finally, the phlorotannin concentration was determined from the calibration curve and expressed as gram of phloroglucinol per gram of dry seaweed, i.e., phlorotannin extraction yield (wt.%).

Determination of the total carbohydrate concentration

Total carbohydrate concentration was measured using the phenol-sulfuric acid method (Dubois et al. 1956). Consequently, 200 μL of the sample was added to a 200 μL phenol solution (5 *w*/*v* %) and supplemented with 1 mL of concentrated sulfuric acid. The mixture was equilibrated for 20 min at room temperature. As the next step, the absorbance was measured at 476 nm against a distilled water blank sample. A calibration curve of glucose, at different concentrations (0.02–0.1 g L^{-1}) was prepared and the results were expressed as gram of glucose per gram of total carbohydrate present in the algae, i.e., the carbohydrate extraction yield (wt.%).

Determination of the radical scavenging activity, total antioxidant activity (TAA)

In order to determine the integrity of phlorotannins extracted, the free radical scavenging activity was evaluated using the modified method of Von Gadow et al. (1997). Briefly, 40 μL of 0.4 M 1,1-diphenyl-2-picryl-hydrazyl (DPPH) solution in ethyl alcohol was added to 50 μL of the sample solution, supplemented with 110 μL of ethanol. The plates were mixed and kept for 30 min in the absence of UV light to avoid any potential decomposition. The absorbance was measured at 520 nm against an ethyl alcohol blank sample. The calibration curves of Trolox (0–24 mg L^{-1}) were prepared and the results were expressed as mg of the equivalent Trolox per gram of dry seaweed (mg TE g^{-1} DS).

Determination of the carbohydrate species present

The carbohydrates in the extract were quantified following the acid methanolysis method complemented by sample silylation and gas chromatography (GC) analysis, as described in Pezoa-Conte et al. (2015). Additionally, the total glucan content was analyzed by acid hydrolysis followed by derivatization with silylation agents and analysis by GC (Pezoa-Conte et al. 2015).

Determination of the phlorotannin species present

The phlorotannins in the extract were identifying by high-precision liquid chromatography mass spectrometry (HPLC-ESI-MS/MS) analysis described in Leyton et al. (2016). Twenty microliter of sample was analyzed using a LC-ESI-MS/MS system which consisted of the HP1100 liquid chromatography (Algilent Technologies, USA) connected to the mass spectrophotometer (Esquire 4000 ESI-Ion Trap LC/MS (n) system, Bruker Daltonik, Germany). A Luna C18 150 × 4.6 mm, 5 μm, and 100 Å analytical column (Phenomenex, USA) was used in the analysis; at the exit of the column, a split divided the eluent for simultaneous UV and mass spectrometry detection. The mobile phase used was 1% *v*/*v* formic acid in water deionized (solvent A) and acetonitrile (solvent B), fed at a flow rate 1 mL min^{-1} according to the following elution gradient: 0–15 min, 5% B; 15–75 min, 5–100% B; 75–85 min, 100% B; and 85–90 min, 100–5% B (Sundberg et al., 2003). The detection wavelength was set to 280 nm. The mass spectral data were acquired in positive and negative modes; ionization was performed at 3000 V assisted by nitrogen as nebulizing gas at 45 psi, drying gas at 345 °C, and flow rate 10 L min^{-1}. All scans were performed in the range 20–2200 *m*/*z*. The trap parameters were set in ion charge control using the manufacturer's default parameters. The collision-induced dissociation (CID) was performed by collisions with the helium background gas present in the trap and automatically controlled trough SmartFrag option.

Statistical analysis

All the extracts were analyzed in triplicate. The measurements are presented as average ± standard deviation. The experimental data was processed with the higher-the-better analysis for determining the best conditions for the carbohydrate and phlorotannin extraction. The significance and the relative influence of each and every individual factor in the pretreatment and extraction process were determined using the variance analysis (ANOVA). The significance of the factors was determined at a 5% confidence level.

Results

Characterization of *M. pyrifera*

The characterization of the alga was carried out to determine the intrinsic extraction potential. The chemical analyses of *M. pyrifera* (Fig. 1a) indicated that ash, protein, lipid, and carbohydrate contents were 19.28, 14.49, 1.42, and 64.81 wt.%, respectively. The most abundant carbohydrate present in *M. pyrifera* was alginate, amounting to 62.54 wt.%, followed by mannitol and fucose in concentrations of 8.05 and 6.34 wt.%, respectively (Fig. 1b).

Influence of the pH of the aqueous solvent on the carbohydrate and phlorotannin extraction yields

Different pH of the aqueous extractants were evaluated in order to improve the extraction yield (EY) of carbohydrates and phlorotannins (see Fig. 2). It was found that the best pH was 12 (alkaline extract), resulting in an EY of 46.93 ± 8.78 wt.% for carbohydrates and 2.20 ± 0.40 wt.% for phlorotannins, respectively.

Influence of the extraction parameters on the carbohydrate and phlorotannin extraction yields

The evaluation of the extraction parameters was performed using the Box-Behnken design. The conditions under which the EY of carbohydrates increased are as follows: 100 °C for 180 min and an algae-to-alkaline extract ratio of 1:20, thus giving rise to an EY of 77.58 ± 8.48 wt.% (run 2, Table 1). The condition that allowed the EY of phlorotannins to be optimized was 120 °C for 60 min and an algae-alkaline extract ratio of 1:15, thus obtaining an EY of 2.66 ± 0.92 wt.% (run 4, Table 1). Under both of the aforementioned conditions, an increase of EY (65 and 21% compared to the initial conditions; 46.93 ± 8.78 wt.% for the carbohydrates and 2.20 ± 0.40 wt.% for the phlorotannins) was documented.

The antioxidant activity of phlorotannins obtained under conditions of runs 2 and 4 of Table 1 were 227.15 ± 3.14 and 138.61 ± 6.71 mg TE g^{-1} DS^{-1}, respectively (Fig. 3). These results demonstrated a lower phlorotannin activity in run 4 compared to run 2, an observation which could be due to the degradation of these compounds at higher temperatures used upon the extraction (120 °C). This is the reason why the best extraction conditions were finally selected considering both of the compounds: 100 °C for 180 min and an algae-alkaline extract ratio of 1:20 (run 2, Table 1).

Consequently, a second-order polynomial quadratic equation was fitted to the carbohydrate and phlorotannin extraction yields, as follows:

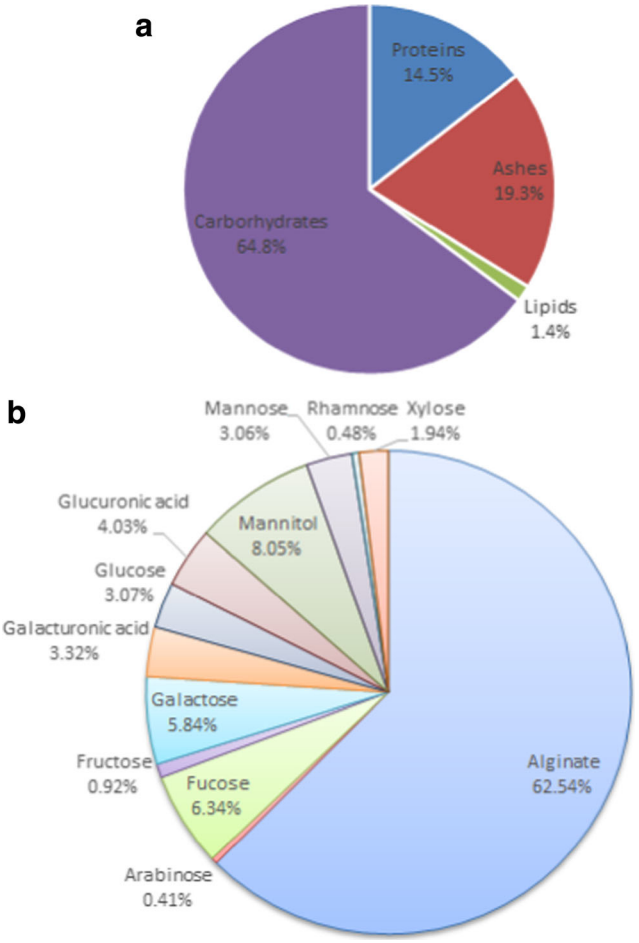

Fig. 1 Composition of *Macrocystis pyrifera* harvested in May 2013. **a** Chemical composition. **b** Type of carbohydrate

$$\begin{aligned}\text{EY-of-carbohydrates} = {} & -270.6 + 0.13\,{}^{*}A + 4.51\,{}^{*}B + 7.77\,{}^{*}C \\ & -0.00001\,{}^{*}A^2 - 0.001\,{}^{*}A{}^{*}B + 0.01\,{}^{*}A{}^{*}C \\ & -0.0\,{}^{*}B^2 - 0.02\,{}^{*}A{}^{*}C - 0.19\,{}^{*}C^2\end{aligned} \quad (2)$$

$$\begin{aligned}\text{EY-of-phlorotannins} = {} & 0.03 + 0.03\,{}^{*}\text{A} - 0.003\,{}^{*}\text{B} - 0.13\,{}^{*}\text{C} \\ & -0.0001\,{}^{*}A^2 - 0.0003\,{}^{*}A{}^{*}B + 0.001\,{}^{*}A{}^{*}C \\ & +0.0003\,{}^{*}B^2 - 0.001\,{}^{*}B{}^{*}C + 0.004\,{}^{*}C^2\end{aligned} \quad (3)$$

In case of both equations (Eqs. 2 and 3), it was possible to determine the predicted value for each experimental run (Table 1, columns 6 and 8). These predicted values had a correlation coefficient of 96% in case phlorotannins and 89% in case of carbohydrates, respectively. According to the statistical analysis (Table 3) the factors contributing to the significant effects ($p < 0.05$) on the EY were time and temperature in case of carbohydrates whereas in case of phlorotannins, the temperature and the interaction of the

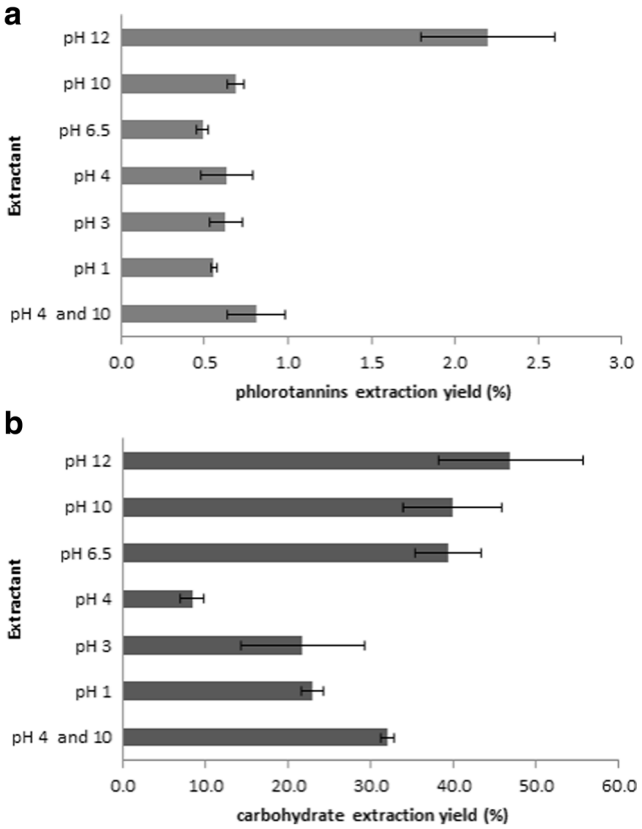

Fig. 2 Extraction yields of **a** phlorotannins and **b** carbohydrates from *Macrocystis pyrifera* using different pH of extraction. Mean ± standard deviation (*n*=3)

time-temperature variables were significant. The predicted optimum conditions for obtaining the maximum EY of carbohydrates and phlorotannins constituted an extraction time of 60 min, at 120 °C and a S/L ratio of 1:10, (Figs. 4 and 5, respectively). Under these conditions, the maximum EY predicted was 81.72 wt.% in case of the carbohydrates and 3.31 wt.% in case of the phlorotannins, respectively.

Influence of the pretreatment with a carbohydrate active enzyme on the carbohydrate and phlorotannin extraction yield

The pretreatment with carbohydrate active enzyme followed by an alkaline extraction (with 0.5 N NaOH at 100 °C, 180 min and using an algae-to-alkaline solvent ratio of 1:20) was evaluated. The best conditions observed upon the pretreatment that allowed to increase the extraction yield (EY) of carbohydrates can be found in run 5 in Table 2, whereupon the incubation pretreatment was carried out for 24 h at 37 °C, pH 7.0, and a S/L ratio of 1:10, accounting a 89.67 ± 12.27 wt.% EY of carbohydrates. The conditions that gave rise to increased EY of phlorotannins were 36 h of incubation at 25 °C, pH 7.0, and S/L ratio of 1:20, run 7 in Table 2, thus yielding an EY of 2.14 ± 0.25 wt.% for the phlorotannins.

Phlorotannins are between 3 and 10% DW (Connan et al. 2004) in brown seaweed, and carbohydrates are between 30 and 50% DW (Westermeier et al. 2012), phlorotannins being the compounds in a smaller proportion in the alga. Therefore, the best pretreatment conditions for the extraction of both of the compounds were selected considering the condition where the highest concentration of phlorotannins is obtained: 36 h of incubation at 25 °C, pH 7.0, and S/L ratio of 1:20 (run 7, Table 2).

The statistical analysis determined that the S/L ratio, pH, and the incubation time had significant influence ($p < 0.05$), both in terms of the EY of carbohydrates and phlorotannins, respectively (Table 4). The strongest influence was observed for the S/L ratio, followed by pH with 33.3 and 29.1%, respectively. The sum of these two factors accounted for 61.4% of the carbohydrates, in terms of the EY. On the other hand, the influence of

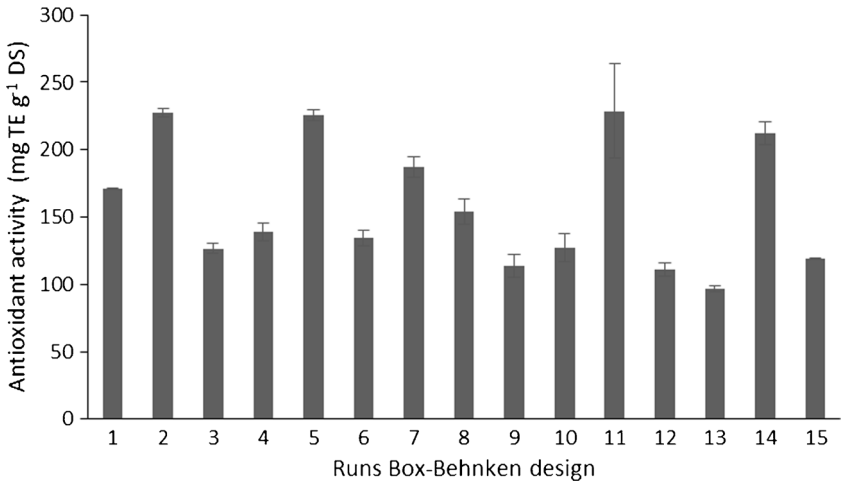

Fig. 3 Antioxidant activity of the liquid phase obtained from each experimental condition of the Box-Behnken design. See Table 2 for further details with respect to each run. Mean ± standard deviation (*n*=3)

Table 3 Analysis of variance (ANOVA) results for the extraction parameters of carbohydrates and phlorotannins from *Macrocystis pyrifera* with the alkaline extraction process

Extraction process						
Response	Factors	DOF	SS	Variance	*F* value	*p* value
Phlorotannin extraction yield (%)	A:time	1	0.076	0.076	3.57	0.1173
	B:temperature	1	1.684	1.684	79.14	*0.0003*
	C:S/L ratio	1	0.028	0.028	1.3	0.3062
	A^2	1	0.121	0.121	5.7	0.0626
	AB	1	0.462	0.462	21.73	*0.0055*
	AC	1	0.109	0.109	5.12	0.0731
	B^2	1	0.069	0.069	3.22	0.1326
	BC	1	0.011	0.011	0.52	0.5038
	C^2	1	0.031	0.031	1.45	0.2831
	Other/error	5	0.106	0.0212		
	Total	14	2.71			
Carbohydrate extraction yield (%)	A:time	1	597.715	597.715	7.8	*0.0383*
	B:temperature	1	791.622	791.622	10.33	*0.0236*
	C:S/L ratio	1	165.347	165.347	2.16	0.2018
	A^2	1	0.007	0.007	0	0.9929
	AB	1	792.423	792.423	0.1	0.7608
	AC	1	295.936	295.936	0.39	0.5616
	B^2	1	184.299	184.299	2.4	0.1817
	BC	1	205.662	205.662	0.27	0.6265
	C^2	1	828.698	828.698	1.08	0.3461
	Other/error	5	383.199	76.6398		
	Total	14	2247.99			

F-value significant of results, *p*-value probability of error

S/L solid/liquid ratio of algae/alkaline solvent, *DOF* degree of freedom, *SS* sums of squares

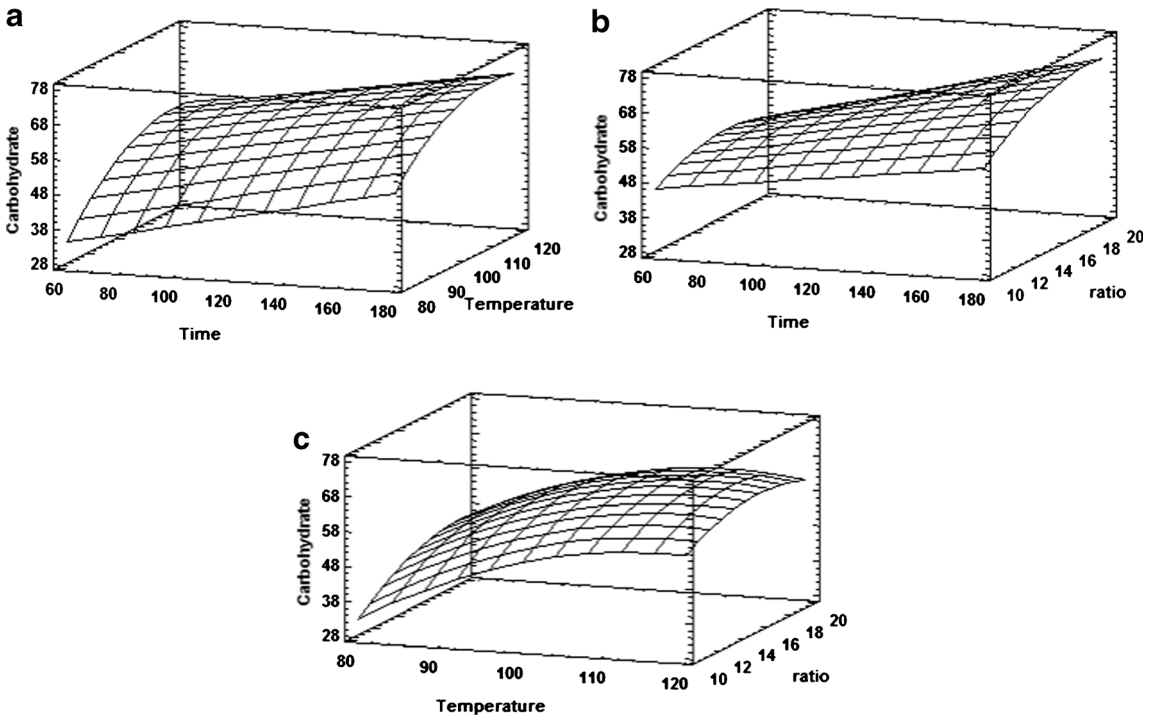

Fig. 4 Response surface (3-D) plots showing the effects of **a** extraction time and temperature, **b** extraction time and the alkaline solvent to raw material ratio, and **c** extraction temperature and alkaline solvent-raw material ratio on the extraction yield of carbohydrates

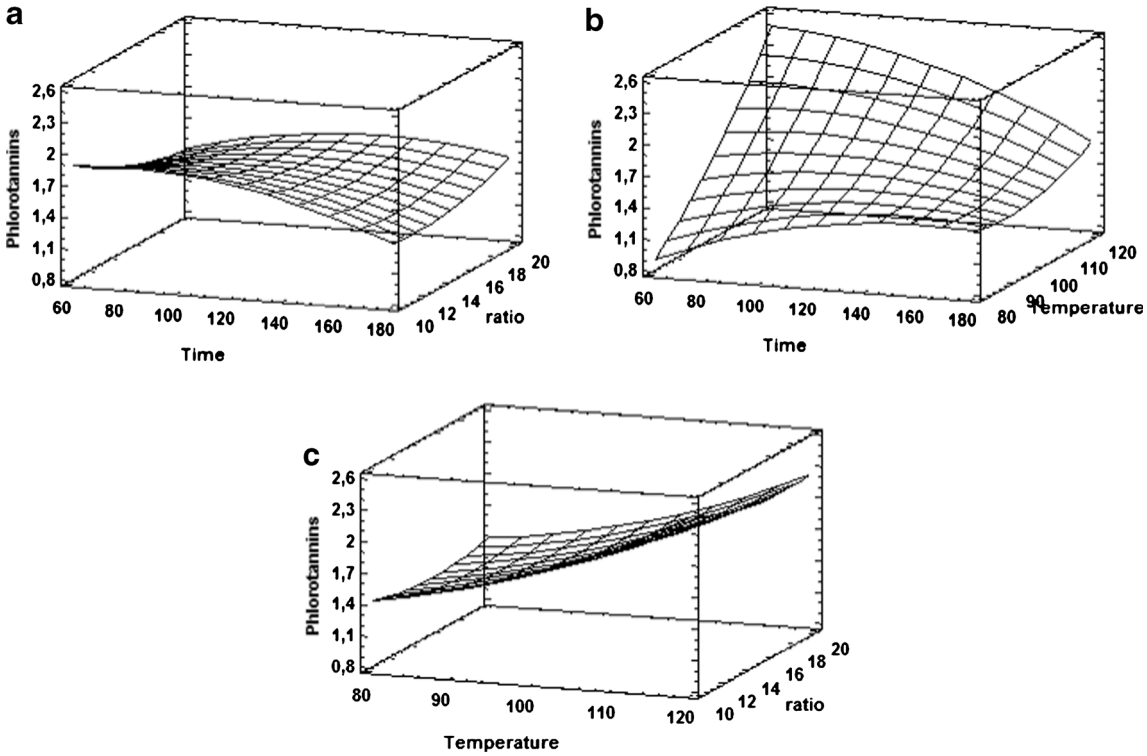

Fig. 5 Response surface (3-D) plots showing the effects of **a** extraction time and temperature, **b** extraction time and the alkaline solvent to raw material ratio, and **c** extraction temperature and the alkaline solvent to raw material ratio on the extraction yield of phlorotannins

time in the EY of phlorotannin was 46%. The statistical methodology applied predicts that a maximum carbohydrate EY of 91.24 wt.% can be reached under the optimal extraction conditions, 24 h incubation at 50 °C, pH 7.0, and a S/L ratio of 1:10. Additionally, the optimal conditions predicted for the phlorotannin EY were the in accordance to the best pretreatment conditions, as in run 7.

Characterization of the type of carbohydrate and phlorotannins present in the extract under the best conditions extraction

The identification of the type of carbohydrate present in the extract obtained under the combined process of hydrolysis enzymatic following of alkaline extraction was realized. The type of carbohydrate present was alginate with 2.72%, glucose

Table 4 Analysis of variance (ANOVA) results for the pretreatment of *Macrocystis pyrifera* with carbohydrolase enzymes

Enzymatic pretreatment							
Response	Factors	DOF	SS	Variance	*F* value	Pure sum	%
Phlorotannin extraction yield (%)	Time	2	1.07	0.54	32.17	1.04	46
	Temperature	2	0.21	0.1	6.19	0.17	7.6
	pH	2	0.37	0.19	11.16	0.34	15
	S/L ratio	2	0.31	0.15	9.3	0.28	12.2
	Other/error	18	0.3	0.02		0.43	19.2
	Total	26	2.26			2.26	100
Carbohydrate extraction yield (%)	Time	2	381.99	191	5.97	317.96	10.9
	Temperature	2	11.84	5.92	0.18	−52.19	0
	pH	2	911.32	455.66	14.23	847.28	29.1
	S/L ratio	2	1033.26	516.63	16.14	969.23	33.3
	Other/error	18	576.32	32.02		832.46	28.6
	Total	26	2914.74			2914.74	100

S/L solid/liquid ratio of algae/enzyme extract, *DOF* degree of freedom, *SS* sums of squares

9.81%, mannitol 86.96%, and fucose 0.52% with respect to total carbohydrate present in the extract.

The identification of phlorotannins in the extract by HPLC-ESI-MS/MS was realized. The phlorotannins identified were phloroeckol and a tetramer of phloroglucinol.

Discussion

The composition of carbohydrate suggests the use of specific carbohydrate active enzymes upon polysaccharide hydrolysis in major proportion in the algae, alginate, laminarin (related to mannitol), and fucoidan, as alginate lyase, 1,3-β-D-glucanase and fucoidanase, respectively. According to Deniaud-Bouët et al. (2014), the alginate–phenol linkages play an essential role in the brown algal cell wall structure, and it is expected that a high alginate concentration correlates with a high phlorotannin concentration in the brown alga.

The variability of the chemical composition of *Macrocystis* species in the Southern Chile was observed by Westermeier et al. (2012). The protein levels observed in the cultured *Macrocystis* varied in a range from 12% dry weight (DW) (January–February) and 15% DW (March–June). The values decreased in summer and increased mildly during early autumn and winter. The lipid and carbohydrate contents were in a range between 0.2 and 0.7% DW for lipids and between 36.2 and 49.9% DW for carbohydrates, respectively, reaching their respective maximum levels in winter and summer. The protein concentration was found to correlate with the environmental supply and internal reserves of nitrogen that are depleted during growth (Gorham and Lewey, 1984). In case of the carbohydrates, the correlation was in respect to photosynthetic activity, and surplus carbon fixed during the summer was translocated as a reserve for growth under more light-restricted periods (Chapman and Craigie, 1978).

In this regard, it is well documented that the extraction of alginate from brown algae is best performed using alkaline solvents with pH between 10 to 12 (Gomez et al. 2009; Hernández-Carmona et al. 2012). Therefore, since alginate is the most prominent carbohydrate present in *M. pyrifera* where phlorotannin are found in linkage with the carbohydrate. It is hypothesized that a high EY of carbohydrate is directly related with a high EY of phlorotannins. The alkaline extraction of polysaccharide from seaweeds has been reported. The EY obtained from *Salicornia brachiata* and *M. pyrifera* were 58.1 and 33 wt.%, respectively (Sanandiyaa and Siddhanta 2014; Gomez et al. 2009). On the other hand, the combination of an acid pretreatment, followed by an alkaline extraction from *Laminaria digitata* by Blanco-Pascual et al. (2014) resulted in an EY of 78.02 ± 16.81 and 0.34 ± 0.02 wt.% for carbohydrates and polyphenols, respectively. These results are lower than those obtained in this work, which could are relate to the lower temperature of extraction used, 75 °C in comparison with 100 °C used by us. The same effect of temperature and type of solvent was observed in polyphenol compound extraction from *Fucus vesiculosus* with ethanol (95 vol.%) with an EY of 0.10 wt.% (Peinado et al. 2014).

Thus, an EY increase of 33% for phlorotannins was obtained. Consequently, the enzymatic pretreatment improved the digestibility of the algae, increasing the amount of phlorotannins extracted upon the alkaline extraction. Most probably, the intensive cleavage of the polysaccharides contained in the cell wall of the tissue during the pretreatment resulted in an improved accessibility of the solvent to the phlorotannin fraction during the alkaline treatment.

A similar study, albeit with different algae, was carried out by Wu et al. (2014) and Borines et al. (2013), in which sequential acid and enzymatic hydrolysis resulted in an EY of 56.26% for sugars from *Gracilaria* sp. and 12 wt.% for carbohydrates from *Sargassum* ssp. Olivares-Molina and Fernández (2016) compared carbohydrate and phlorotannin extraction from brown seaweed by enzymatic, and conventional method of extraction (maceration) and obtained high yield extraction with enzymatic extraction, 37.7% and 10 μg PGE mg^{-1} seaweed for carbohydrate and phlorotannins respectively, with a better bioactivity as natural inhibitors of the angiotensin I-converting enzyme. These results emphasize the use of enzymes for the extraction of biocompounds in order to protect the bioactivity of the compounds.

In conclusion, in order to increase the extraction yields of carbohydrates and phlorotannins from *M. pyrifera*, a combined enzymatic pretreatment followed by an alkaline extraction process was evaluated. Combining both processes increased the extraction efficiency of phlorotannins from 1.6 to 2.1 wt.% and in the case of the carbohydrates from 46.9 to 69.0 wt.%. Future work includes isolation process of the phlorotannin fraction and the use of extracted carbohydrates and phlorotannins in biochemical and nutraceutical platforms.

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