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DESIGN OF AN EXTRACTION PROCESS OF PHLOROTANNINS AND CARBOHYDRATE FROM *Macrocystis pyrifera,* INTEGRATING THE USE OF MARINE ENZYMES IN THE STEP OF CARBOHYDRATE HYDROLISIS

TESIS PARA OPTAR AL GRADO DE DOCTORA EN CIENCIAS DE LA INGENIERÍA MENCIÓN INGENIERÍA QUÍMICA Y BIOTECNOLOGÍA

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La macroalga parda *Macrocystis pyrifera* es una especie de algas marinas que posee una amplia distribución en aguas templadas y frías tanto del hemisferio norte como sur, formando bosques productivos de alta diversidad biológica. En Chile *M. pyrifera* se distribuye a lo largo de la costa desde Iquique al Cabo de Hornos. La importancia comercial de esta especie se ha incrementado en la última década, especialmente en la extracción de alginato y su uso como alimento de abalones. El alga se compone principalmente de carbohidratos heterogéneos (> 50% peso seco del alga) como fucoidano, alginato y laminarina, los cuales pueden ser empleados como plataforma para la producción de variados compuestos. Además, posee compuestos polifenólicos únicos en su especie conocidos como florotaninos los cuales han sido ampliamente estudiados por sus beneficios potenciales para la salud como anti-oxidantes, anti-cáncer, anti-diabetes, anti-inflamación y anti-microbianas, entre otras.

En este trabajo se estudió el diseño de un proceso de extracción de florotaninos y carbohidratos desde *M. pyrifera*, usando enzimas marinas en la etapa de hidrolisis de los carbohidratos. Para lo cual se procedió en un primer paso a determinar condiciones que mejoran la extracción de florotaninos desde el alga, tales como temperatura de secado del alga, parámetros de extracción e identificación de los compuestos. En una segunda etapa se optimizó la producción de enzimas carbohidrasas, alginato liasa, fucoidanasa y 1,3-β-D-glucanasa, desde microorganismos marinos asociado a la macroalga, para ser empleadas posteriormente en el pre-tratamiento enzimático *de M. pyrifera.* En una tercera etapa se determinó condiciones de extracción simultánea de carbohidratos y florotaninos desde el alga incorporando la etapa previa de pre-tratamiento enzimático. Finalmente, se estudió la separación de florotaninos desde el extracto final usando resinas macroporosas. La evaluación de estas etapas permitió determinar que un pre-tratamiento enzimático del alga con carbohidrasas, producidas por el hongo marino *Alternaria sp,* a 25°C por 36 horas a un pH 7.0 y una razón alga/extracto enzimático de 1/20, seguido por extracción alcalina de fase solida con NaOH 0.5N a 100°C por 180 min en una razón sólido/líquido de 1/20. Obteniendo un extracto con concentración final de 452 mg carbohidrato/g alga y 2.14 mg florotaninos/g alga, lo cual representa un rendimiento de extracción específico de 89.6% para carbohidratos y 21.4% para florotaninos. Posteriormente, el uso de resina XAD-16N permitió la separación del 42% de los florotaninos desde el extracto. La fracción enriquecida en florotaninos fue liofilizada presentando una concentración de 14.2 mg florotaninos/g liofilizado.

Abstract

The brown macroalgae *Macrocystis pyrifera* is a kind of seaweed that has a wide distribution in temperate and cold waters of both the Northern and Southern hemispheres, forming productive forests of high biological diversity. In Chile *M. pyrifera* it is distributed along the coast from Iquique to Cape Horn. The commercial importance of this species has increased in the last decade, especially in alginate extraction and use as food abalones.

M. pyrifera (>50 % of the alga dry weight) as fucoidan, laminarin and alginate, which can be used as a platform for the production of many compounds is mainly composed of heterogeneous carbohydrate. In addition, the brown seaweeds have unique polyphenolic compounds in their species known as phlorotannins which have been widely studied for their potential health benefits such as prevention of oxidative stress-mediated radical, anti-cancer, anti-diabetes, anti-inflammation and anti-microbial, among others.

In this work was studied in this paper the design of an extraction process phlorotannins and carbohydrates from *Macrocystis pyrifera*, using marine enzymes in the hydrolysis step carbohydrates. For which we proceeded in a first step to determine the conditions that improve phlorotannins extraction from algae such as seaweed drying temperature, parameters extraction and identification of compounds. In a second stage production carbohydrate active enzymes, alginate lyase, fucoidanase and 1,3- β-D-glucanase was optimized from microorganisms associated with marine macroalgae, to be employed subsequently in the enzymatic pretreatment of *M. pyrifera*. In a third stage of simultaneous extraction conditions of carbohydrate and phlorotannins was determined from *M. pyrifera* incorporating the previous stage enzymatic pretreatment. Finally, the separation of phlorotannins was studied from the final extract using macroporous resins.

Evaluation of these stages allowed to determine that an enzymatic pretreatment with carbohydrate active enzymes, alginate, fucoidanasa and 1,3-β-D-glucanase produced by the marine fungus *Alternaria sp,* at 25°C for 36 hours pH 7.0 and algae/enzyme extract of 1/20, followed by alkaline extraction of the solid phase with 0.5 N NaOH at 100°C for 180 min in a solid/liquid ratio of 1/20 allowed obtain a final extract with a concentration 452 mg carbohydrate/g of alga and 2.14 mg phlorotannins/g of alga, which represents a specific extraction yield of 89.6% for carbohydrates and 21.4% for phlorotannins.

Subsequently, the use of macroporous resin XAD- 16N allowed separation of the 42% of phlorotannins from the extract. Finally, the enriched fraction with phlorotannins was lyophilized presenting a concentration of 14.2 mg phlorotannins/g lyophilized.

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Chapter 1

1. Introduction

In recent decades, the chemistry of natural products of marine origin has been the subject of intense research that has uncovered new substances with pharmacological and nutraceutical properties. A great source of biologically active compounds are marine organisms in which compounds with potential antibacterial uses, anticancer, anti-viral, anti-tumor, anti-inflammatory and anticoagulant [De Lara- Issasi et al., 1989] among others, have been found, pointing to algae as a major source of these compounds [Faulker 1984; Stein and Borden 1984; Hay 1996]

Macroalgae are multicellular organisms that are distributed globally, commercially around 20,000 species are grown to be used primarily as a source of food, with a production increase of 10% over the last 10 years [FAO, 2012]. Macroalgae are classified according to the composition of pigments in green alga (*Chorophyta*), brown algae (*Phaeophyta*) and red algae (*Rhodophyta*), being mainly cultivated for use as a source of food and secundary as source of polysaccharides (hydrocolloids) for industrial use, such as alginate, carrageenan and agar used in industry as thickeners, gelling and/or encapsulating matrix [Roesijadi et al., 2010; Reith et al., 2009]. Currently, there are globally 3.1 million metric tons of annual macroalgae, of which 72 % are produced by China growing genera such as *Laminaria*, *Undaria Porphyra*, *Eucheuma* and *Gracilaria* [Roesijadi et al., 2010].

Macroalgae also can be differentiated by their principal polysaccharide composition being alginate, starch and carrageenan characteristic of brown, green and red algae, respectively. Table 1.1 presents polysaccharides and monosaccharides detailed present in each one.

Table 1.1. Content of carbohydrates in macroalgae [Jang et al., 2012; Roesijadi et al., 2010; Turvey and Christison, 1967; Wegeberg and Felby, 2010].

Macroalgae		
Green algae	*Red algae*	*Brown algae*
Polysaccharide		
Ulvan	Carrageenan	Laminarin
Starch	Agar	Alginate
Cellulose		Fucoidan
		Cellulose
		Mannitol
Monosaccharide		
Glucose	Glucose	Glucose
Mannose	Galactose	Galactose
Ramnnose	Agarose	Fucose
Xylose		Xylose
Uronic acid		Uronic acid
Glucuronic acid		Mannuronic acid
		Guluronic acid
		Glucuronic acid

Seaweeds are also a source of many bioactive compounds, which are functional compounds that confer a health benefit associated with their consumption [Diplock et al., 1999] as carotenoids, polyunsaturated fatty acids, vitamins, sulfated polysaccharides and polyphenols. Recently, several studies have shown the variety of biological benefits associated with the polyphenols present in brown algae, including antioxidant, anticoagulant, anti-bacterial, anti-inflammatory and anti-cancer [Mayer and Hamann, 2005; Heo et al., 2008; Kong et al., 2009].

1.1. Carbohydrate in brown seaweed

Among the polysaccharides (carbohydrates) found in the brown seaweed are mostly alginate, fucoidan and laminarin, and a lesser proportion mannitol and derivatives thereof, which provide the alga flexibility and maintenance of ion balance. Alginate, alginic acid is a linear polysaccharide consisting of anionic binding β -D- mannuronic acid (M) and acid α -D- guluronic (G) can be found as polysaccharide units MM, GG and MG. Laminarin is a linear polysaccharide consisting of units (1,3) -β -D-glucans and mannitol, is being last a

polyol. Fucoidans are complex sulfated polysaccharides (heteropolysaccharide) composed mainly of L -fucose and sulfated small proportions of galactose, mannose, xylose, glucose, rhamnose and uronic acid. Importantly, fucoidans are not present in other divisions of algae or plants.

The concentration of carbohydrates and other compounds will vary depending on the water, season, temperature, nutrient availability, age, geographical location and stress conditions in the algae [Adams et al,. 2011; Gutierrez et al., 2006; Westermeier et al., 2012].

1.2 Phlorotannins

Phlorotannins are formed by the polymerization of monomers units of phloroglucinol (1,3,5-trihydroxybenzene) and biosynthesized by ethyl malonate pathway, also known as polyketide pathway. The hydrophilic compounds are phlorotannins with highly molecular size range between 126 Da and 650 kDa [Ragan and Glombitza, 1986]. Brown seaweed accumulate a variety of polyphenols based on phloroglucinol, as phlorotannins, low, intermediate and high molecular weight containing phenyl and phenoxy units. Based on the links, the phlorotannins can be classified into four subclasses as fuhaloles, floretoles (phlorotannins with an ether bond), fucoles (with a phenyl bond), fucofloroetoles (with an ether and phenyl bond), and eckoles (with a link dibenzo -1,4 - dioxin). Figure 1.1 shows the structure of different phlorotannins prsented in Table 1.2 and the phlorotannins extracted from various brown seaweeds detailed.

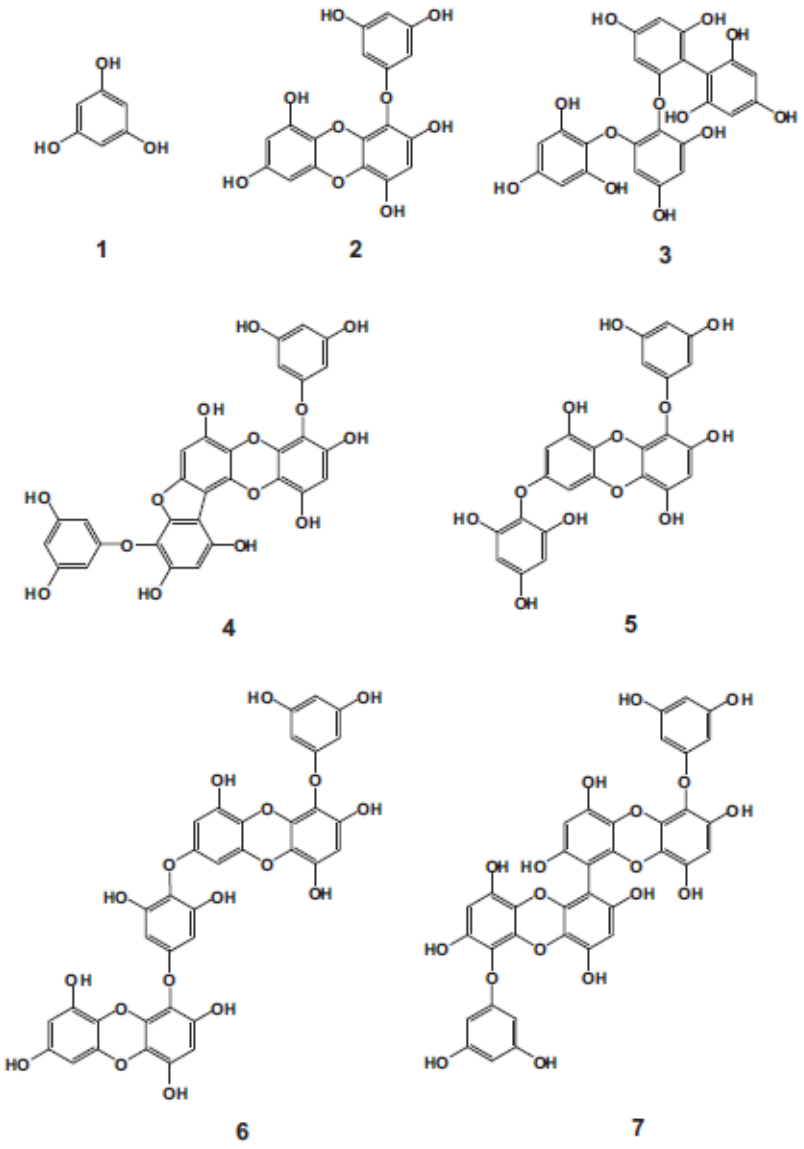

Figure 1.1. Phlorotannins structure derived from brown seaweed. (1) phloroglucinol, (2) eckol, (3) fucodiphloroetol- G, (4) Phlorofucoroeckol A, (5) 7- phloroeckol, (6) dieckol, and (7) 6,6- bieckol [Adapted from Li et al., 2011].

Table 1.2. Phlorotannins derived from brown seaweed.

Phlorotannins	Brown seaweed	Reference
Phloroglucinol	*Ecklonia cava*	Yoon, et al, 2008
Eckol	*E. cava, Eisenia bicyclis*	Yoon, et al, 2008; Nagayama, et al, 2003
Fucodiphloroetol G	*E.cava*	Yoon, et al, 2008
Phlorofucofuroeckol A	*E.cava, E.stolinifera, E.kurome*	Yoon, et al, 2008; Nagayama, et al, 2003; Yoon, 2008
Phlorofucofuroeckol B	*E.bicyclis*	Yoon, 2008
7-Phloroeckol	*E.cava, E.stolinifera*	Yoon, et al, 2008; Yoon, 2008
Dieckol	*E.cava*	Yoon, et al, 2008
6,6-Bieckol	*E.cava, Ishige okamurae*	Yoon, et al, 2008; Li, et al, 2009
Triphloroetol B	*E.stolinifera*	Nagayama, et al, 2003
2-Phloroeckol	*E.stolinifera*	Nagayama, et al, 2003
6,8-Bieckol	*E.bicyclis*	Yoon, 2008
8,8-Bieckol	*E.bicyclis*	Yoon, 2008

The phlorotannins are in the algae in soluble form, stored in physodes, and insoluble form forming part of the structure of the cell walls of algae, forming complexes with proteins and alginic acid [Singh and Sidana, 2013]. Concentration and molecular size of these compounds vary according to intrinsic factors (reproductive condition, age and size of the algae), as well as by extrinsic factors (environmental and ecological stimuli) [Singh and Sidana, 2013; Ortiz et al., 2006; Sanchez-Machado et al., 2004]. These compounds have been extensively studied due to their pharmacological and nutraceutical properties exhibiting antioxidative, antiangiogenic, antiallergic, anti-inflammatory, and antidiabetic effects [Singh and Sidana, 2013; Ortiz et al., 2006; Sanchez-Machado et al., 2004; Glombitza and Pauli, 2003; Breton et al., 2011; Kim and Himaya, 2011; Zubia et al., 2008; Zhao et al., 2008; Li et la., 2011].

Global nutraceutical market (association of nutrition and drug concept) is estimated at $ 151 billion of dollars in 2011. The increase is estimated at $ 207 billion of dollars with a projected annual growth rate of 6.5% between 2011 and 2016. According to the latest Transparency market Research, in terms of volume, the market for balanced antioxidant food is estimated to grow at an annual net rate of 3.6% from 2012 to 2018, so as to reach $ 216.8 million of dollars [Transparency market Research, 2015], which makes it attractive for

further research on new antioxidants source. Besides, in the market there is widespread use of antioxidants of synthetic origin being the most widely used in the industry Hidroxibutilanisol (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG), Octilgalato (OG) and tertiary butyl hydroquinone (TBHQ), used to preserve oils, fats or other compounds spoilt by oxidation. But the use of these compounds has been linked to increased tumor activity (particularly BHA and BHT) [Kahl and Kappus, 1993] resulting in strict regulation of its use in foods [Hettiarachchy et al., 1996] and search for sources of natural antioxidants, being the phlorotannins a good alternative.

1.3 Macrocystis pyrifera

Macrocystis pyrifera, known in Chile as "Huiro", is an alga belonging to the group of *Phaeophyta* (brown algae) family of Lessoniaceae, order Laminariales, Phaeophyceae classification. They are fast growing and large and can reach 30 m in length , serving as a substrate to a large number of shellfish , fish, sea urchins , etc. [Ortiz, 2011] . They have a high content of nitrogen-free extract up to 70 % approximately, being mainly soluble carbohydrates [Klasing, 1988]. In addition it synthesizes an important secondary metabolite phlorotannins, which can represent in this group 15% of the dry weight of alga [Steinberg, 1985, 1989; Ragan and Glombitza, 1986]. These algae are exploited in Chile, 7,600 tons by year [Sernapesac, 2014], their main use being a source of polysaccharides, particularly alginate and as a feed source for abalone cultivation.

1.4 Extraction process of bioactive compounds

In order to improve the extraction of bioactive compounds from brown seaweed a previous step of hydrolysis or pre-treatment of the algae is necessary to facility output of biomolecules from cellular wall. The hydrolysis can be carried through physical processes (drying, milling, irradiation or hydrothermolysis), chemical (treatment with acids, alkalis, solvents or other chemicals), biological (hydrolysis with microorganism or enzymes) and/or combined process (mixture of one or more pre- treatment). The average size of macroalgae varies between 0.3-30 meters long [FAO, 2004], then an initial physical size reduction treatment being necessary for increasing the surface area to facilitate the extraction of compounds of interest, hydrolysis physical, grinding being the most widely used. Among the

chemical process hydrolysis is the most commonly used with dilute sulfuric acid (0.1-3% v/v) in conjunction with high temperature (120-130°C for 15-120 min) [Meinita et al., 2012; Nguyen et al., 2009; Lee et al., 2011], but the presence of inhibitors, product of acid hydrolysis process and high temperature, as molecules of low molecular weight 5-hydroxy-methyl-furfural (HMF) and levulinic acid, have a negative impact on the efficiency for their subsequent use (fermentative process) [Klinke et al., 2004; Adams et al., 2009]. Therefore, it is necessary to employ a hydrolysis process which minimizes the presence of inhibitors. An alternative to this equipment is the use of specific carbohydrate active enzymes for each polysaccharide type present in brown algae such as: alginate lyase (EC4.2.2.-; family PL 7, 15 and 17) which acts on alginate via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end; 1,3-β-D-glucanase (laminarinase, EC3.2.1.39; family GH16) which acts on laminarin via hydrolysis of a glycosidic bond between two or more carbohydrates; and finally fucoidanase (EC3.2.1.44; not information of family type) which acts on fucoidan via hydrolysis of methyl L-fucoside and fucosidic linkages of fucoidan [Cazy, 2016].This enzyme should have the capacity of tolerate the presence of inhibiting compounds, such as metal ions and high salt concentrations in the algae themselves [Lee and Lee 2012b].

In this context, several studies have pointed out that marine microorganisms are a suitable source of carbohydrate active enzymes [Khambhaty et al. 2012; Trivedi et al. 2013; Chi et al. 2009; Alderkamp et al. 2007]

1.5 Previous work with Macrocystis pyrifera

The main works developed with *M. Pyrifera* consist of characterization and cultivation techniques as well as techniques for extracting alginate (profitably developed alginate extraction systems obtaining high yields and a controlled molecular weight for different applications) [Arvizu-Higuera et al., 1997; Hernandez-Carmona et al., 1991; Gómez et al., 2009; Goh et al., 2012]. Other less developed works are in the antioxidant capacity of extracts [Kindleysides et al., 2012], role of phlorotannins in the alga (protection against stress) [Swanson and Druehl, 2002], and biogas production [Vergara-Fernández et al., 2008; Bird and Ryther, 1985].

Further work done with the alga grown in Chile, have focused on the characterization (proximal composition, lipids, amino acids, pigments and concentration of antioxidants, such as sugar composition) [Ortiz, 2011; Molina, 2013; Westermeir et al., 2012; Buschmann et al., 2008], pretreatment by acid hydrolysis evaluating different process variables (particle size, time, hydrolysis temperature, and acid concentration) [Wilken, 2012] and conditions of polyphenols compounds extraction [Consuegra, 2014].

1.6 Objectives

1.6.1 Main objective

Design an extraction process of phlorotannins and carbohydrate from *Macrocystis pyrifera*, using marine enzymes in the carbohydrate hydrolysis step.

1.6.2 Specific objectives

- Study drying conditions of *Macrocystis pyrifera* that allow alga storage, minimizing damage of phlorotannins.
- Identify the type and amount of phlorotannins present in *Macrocystis pyrifera* , as well as those released in the various stages of the process
- Identify the best extraction conditions of phlorotannins post- drying of *Macrocystis pyrifera*, minimizing the loss of carbohydrates.
- Identify the type and amount of carbohydrates present in *Macrocystis pyrifera*, as well as those released at different stages of the process.
- Identify the best extraction conditions of carbohydrates post- drying of *Macrocystis pyrifera*, minimizing loss and /or damage of phlorotannins.
- Identify marine enzymes capable of hydrolyzing carbohydrates present in *Macrocystis pyrifera.*

1.7 Summary of methodology and principal results

This thesis is based on the following publications that are referred to in the text.

- *Chapter 2*: Leyton, A., Pezoa-Conte, R., Barriga, A., Buschmann, A.H., Mäki-Arvela, P., Mikkola, J-P., Lienqueo, M.E. (2016). Identification and efficient extraction method of phlorotannins from the brown seaweed *Macrocystis pyrifera* using an orthogonal experimental design. Algal Research. 16, 201-208.
- *Chapter 3*: Leyton, A., Pezoa-Conte, R., Mäki-Arvela, P., Mikkola, J-P., Salazar, O., Lienqueo, M.E. (2016). Carbohydrate active enzymes from macroalgae-associated microorganisms: optimization of production using experimental design. Marine Biotechnology. Submitted
- *Chapter 4*: Leyton, A., Pezoa-Conte, R., Barriga, A., Buschmann, A.H., Mäki-Arvela, P., Mikkola, J-P., Lienqueo, M.E. (2016). Improvement in carbohydrate and phlorotannin extraction from *Macrocystis pyrifera* using carbohydrate active enzyme from marine Alternaria sp as pretreatment. J Appl Phycol. Submitted
- *Chapter 5*: Leyton A.; Vergara-Salinas J.R; Pérez-Correa J.R; Lienqueo M.E. (2016). Purification of phlorotannins from *Macrocystis pyrifera* using macroporous resins. In review

In summary, the optimal conditions for phlorotannins and carbohydrate extraction were: drying temperature of the algae 40°C, particle size below 1.4 mm, washed with hexane, enzymatic pre-treatment with carbohydrate-active enzyme from marine *Alternaria sp*. HN (with an activity of 60.34 U/mg), at 37°C, pH 7.0 for 24 hours and an S/L ratio of 1:10 in the case of carbohydrates and, at 25°C, pH 7.0 for 36 hours and a S/L ratio of 20 in the case of the phlorotannins; following for both compounds an alkaline extraction with NaOH 0.5 N, at 100°C, 180 min and an algae-to-alkaline solvent ratio of 1:20. Combining both processes allowed for an increase in the extraction efficiency of the phlorotannins of 21.4% and, in the case of the carbohydrates, of 89.67%. Finally, a subsequent process of phlorotannins purification was carried out using macroporous resin XAD-16N; the operation conditions that improve the purification of phlorotannins were at 25°C for 9 hours of adsorption and desorption. Under these conditions, the adsorption capacity was of 183.19 mg PGE/g resin, with desorption ratio of 38.2% and a purification level of 42.0%. The phlorotannins types

present in *M. pyrifera* were phloroeckol and a tetrameric phloroglucinol. These phlorotannins have been reported in the literature to have an antidiabetic effect and prevention of Alzheimer's disease for phloroeckol, and free radical scavenging ability and antiallergic effect for tetrameric phloroglucinol.

Chapter 2

Identification and efficient extraction method of phlorotannins from the brown seaweed *Macrocystis pyrifera* using an orthogonal experimental design

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Published on Algal Research accepted 8 march 2016 (A. Leyton; R. Pezoa-Conte; A. Barriga; A.H. Buschmann; P. Mäki-Arvela; J-P Mikkola, M. E Lienqueo. See appendix A)

Abstract

The brown seaweed contains a type polyphenol compound characteristic of it is species, the phlorotannins, which are produced from the polymerization of phloroglucinol units. They have been extensively studied due to their pharmacological and nutraceutical properties, but there is still a need for an optimized extraction protocol. In this study, the brown seaweed *Macrocystis pyrifera* was employed to determine the best conditions for extraction of phlorotannins. A set of different variables were evaluated such as the use of pre-treatment, type of solvent, drying temperature, particle size, temperature and extraction time as well as the solid/liquid ratio upon extraction. The optimal conditions for the extraction of phlorotannins were: pre-treatment with hexane, extraction with water, drying temperature 40°C, particle size below 1.4 mm, at 55°C for 4 hours and a solid-to-liquid ratio of 1:15. Under these conditions, the concentration of phlorotannins achieved in the extract was 200.5±5.6 mg galic acid equivalent (GAE)/100 g dry seaweed (DS) and total antioxidant activity of the extract of 38.4±2.9 mg trolox equivalent (TE)/100 g DS. Further, it was possible to identify two phlorotannins through HPLC-ESI-MS analyses: phloroeckol and a tetrameric phloroglucinol. These phlorotannins have been reported in the literature to have an antidiabetic effect and prevention of Alzheimer's disease for phloroeckol, and free radical scavenging ability and antiallergic effect for tetrameric phloroglucinol. Therefore, the extract of phlorotannins has potential as medicinal foods or therapeutics for human health applications.

Keywords: Brown algae; extraction optimization; *Macrocystis pyrifera*; Phlorotannins identification; Taguchi Orthogonal array.

2.1 Introduction

In recent decades, the quest to identify new natural products with antioxidant potential has been increasing as several studies have recognized synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), as substrates with potential health hazards [Botterwerck et al., 2000; Jung et al., 2008]. Consequently, natural antioxidants of marine origin have been the subject of intense research, and new substances with pharmacological and nutraceutical properties have been identified [Stein, 1984]. Interestingly, seaweed is one of the main sources of these compounds [Faulkner, 1984; Hay, 1996].

The macroalgae that occupy the littoral zone can be classified as red (*Rhodophyta*), brown (*Phaeophyta*) or green (*Chlorophyta*) types, depending on pigment present and chemical composition [Dawczynski et la., 2007; Gupta and Abu-Ghannam, 2011]. The brown algae contain a substantial amount of soluble carbohydrates and they completely lack lignin, which is important when aiming for an extraction process. Also, they contain unique compounds known as phlorotannins [Arvizu-Higuera et al., 1996; Klasing, 1988]. These compounds are polyphenols produced by seaweed as secondary metabolites and biosynthesized via the acetate malonate pathway [Heo et la., 2005]. Further, they are derived from the polymerization of phloroglucinol units, which constitute up to 15% of the dry weight of the seaweeds [Arnold and Targett, 2002; Ragan and Glombitza, 1998]. Depending on the types of interlinkage present, phlorotannins can be classified into various subclasses, i.e., phlorotannins with phenyl linkages, ether linkages or ether and phenyl linkages that are characteristics for different types of phlorotannins [Targett and Arnold, 1998; Shibata et al., 2004].

The phlorotannins are present in the algae in soluble form, stored in physodes, and insoluble form forming part of the structure of the cell walls of algae, forming complexes with proteins and alginic acid [Singh and Sidana, 2013]. Concentration and molecular size of these compounds varies according to intrinsic factors (reproductive condition, age and size of the algae), as well as by extrinsic factors (environmental and ecological stimuli) [Singh and Sidana, 2013; Ortiz et al., 2006; Sanchez-Machado et al., 2004]. The molecular size of

phlorotannins reported vary between 126 Da (phloroglucinol) and 650 kDa (phlorofucoroeckol) [Glombitza and Pauli, 2003] and have been extensively studied due to their pharmacological and nutraceutical properties exhibiting antioxidative, antiangiogenic, antiallergic, anti-inflammatory, and antidiabetic effects [Singh and Sidana, 2013; Ortiz et al., 2006; Sanchez-Machado et al., 2004; Glombitza and Pauli, 2003; Breton et al., 2011; Kim and Himaya, 2011; Zubia et al., 2008; Zhao et al., 2008; Li et la., 2011].

The extraction of phlorotannins has been performed from different brown seaweed (*Himanthalia elongata, Stypocaulon scoparium, Ascophyllum nodosum, Ecklonia stolonifera, Fucus vesiculosus* between others). Also, the influence of temperature, drying time and nature of the extractant as well as the quality of antioxidant compounds, phlorotannins, have been studied [Ortiz et al., 2006; Sanchez-Machado et al., 2004; Glombitza and Pauli, 2003; Tello-Ireland et al., 2011; Li et al., 2006; Gupta et al., 2011; Koivikko et al., 2007]. However, there is only limited information on extraction of phlorotannins from *Macrocystis pyrifera* [Kindleysides et al., 2012; Ortiz, 2011]. This brown seaweed, *M. pyrifera* is abundantly present in the Pacific Ocean [Arvizu-Higuera et al., 1996; Ortiz, 2011; Buschmann et al., 2014] and is very abundant in the coastal areas of Chile. *M. pyrifera* is an alga with rapid growth and it is used as feed source in the abalone industry as well as a thickening agent in the cosmetics and food industries. Consequently, the development of extraction conditions that permit successful extraction of phlorotannins from *M. pyrifera* is of potential industrial interest.

Further, the identification of the different phlorotannins extracted is of considerable interest for the industry. In this respect, the main objectives of this study were to determine the effect of extraction conditions: (1) use of pre-treatment, (2) type of solvent, (3) drying temperature of algae and (4) extraction parameters (particle size, time and temperature of extraction and solid/liquid ratio) on total phlorotannin concentration (TPC) and total antioxidant activity (TAA) from *Macrocystis pyrifera* as well as the identification of the various phlorotannins extracted.

2.2 Materials and Methods

2.2.1. Algal Materials

Macrocystis pyrifera were collected by scuba diving 30 km southwest of Puerto Montt, Chile. The samples were harvested in November 2011 (M1), March 2013 (M2) and June 2013 (M3). The chemical analyses of *M. pyrifera* samples were carried out in order to determine its initial composition in terms of protein, lipids, carbohydrates and ash elements. The analyses were done at the Institute of Agroindustry, University of the 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): 930.04, 978.04, 991.36, 930.05 and 962.09, respectively [AOAC, 2000]. The carbohydrate content was calculated from the difference in the initial mass and the sum of values reported for proteins, humidity, lipids and ash [Wang et al., 2014].

2.2.2. Effect of the extraction solvent on TPC and TAA

Prior to obtaining extracts, the dry seaweed (M1 dried at room temperature) was pretreated according to Koivikko, et al. [2007]. The pretreatment consisted of washing the alga with hexane (solid/liquid ratio of 1:5 w/v) three (3) times to remove pigments and lipids compound. Additionally, 5.0 g of dry algae washed with hexane was dispersed separately in 50 mL of different solvents (methanol, ethanol, water, water/methanol 50:50, hexane/ethanol 88:12, ethanol/water 25:75 or 80:20, ethyl acetate/water 50:50, water/acetone 20:80 or 30:70 and methanol/chloroform 66:33 % v/v) [Koivikko et al., 2007; Kindleysides et al., 2012; Ortiz, 2011; Lopez et al., 2011; Tanniou et al., 2013; Tierney et al., 2013], and incubated in a platform at 200 rpm and 40°C for 2h. The mixture was centrifuged at 2000 rpm at 4°C for 20 min and supernatant was stored at -20°C before further analysis for the quantification of TPC and TAA values.

2.2.3. Effect of drying temperature on TPC and TAA

The best drying conditions were determined using the fresh algae (M2) dried at four temperatures 30, 40, 50 and 60°C [Gupta et la., 2011]. The experiments were performed in a horizontal dryer with hot air flow (Tray drier, Armfield UOP8) at an air flow speed of 1.3

m/s. Once the samples were dried, they were milled (1.4-2 mm) and stored at 4°C prior extraction.

As the next step, 0.5 g of alga (dried and pretreated) was placed in test tube containing distilled water at a solid/liquid ratio of 1/10 and incubated in a platform shaker at 200 rpm and 40°C for 2h. The sample was centrifuged at 7000 rpm for 10 min at 4°C and the supernatant was stored for further analyses. The best drying temperature was determined in terms of highest TPC and TAA obtained.

2.2.4. Effect of extraction parameters on TPC and TAA using Taguchi orthogonal array

The different extraction variables, namely extraction time, extraction temperature, solid/liquid (S/L) ratio and algae particle size were simultaneously evaluated using Taguchi orthogonal array L_9, 3^4 where 9 is the number of experiments, 4 is the number of factors tested and 3 is number of work levels [Taguchi, 1990]. Seaweed M3 was previously dried at 40°C, washed with hexane and water was used as extractant. Further, Table 2.1 demonstrates the different variables evaluated and describes the conditions chosen for the different experimental runs. The best extraction conditions were determined in terms of the highest TPC and TAA values measured after the extraction.

Table 2.1. Extraction parameters and levels of work evaluated using Taguchi orthogonal array design L_9, 3^4.

Runs	Time [h]	Temperature [°C]	S/L[a] ratio	Particle size [mm]
1	2	25	1/10	< 1.4
2	2	40	1/15	2-1.4
3	2	55	1/20	> 2
4	3	25	1/15	> 2
5	3	40	1/20	< 1.4
6	3	55	1/10	2-1.4
7	4	25	1/20	2-1.4
8	4	40	1/10	> 2
9	4	55	1/15	< 1.4

[a]S/L, Solid to liquid ratio

2.2.5. Determination of TPC

The amount of total phlorotannins compounds in extracts was determined according to the Folin–Ciocalteu assay [Singleton and Rossi, 1965] adapted to 96-well plates. Standards containing gallic acid with a concentration varying from 20 to 100 mg/L were prepared to measure the amount of phlorotannins in the extracts. Samples and standard (20 µl) were introduced separately into 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 a UV–Visible spectrophotometer (Oasys, UVM 340). The phlorotannin concentration was determined by the regression equation of the calibration curve expressed as gallic acid (GAE) mg equivalent/100 g dry seaweed (DS).

2.2.6. Determination of DPPH radical scavenging activity, total antioxidant activity (TAA)

The free radical scavenging activity was measured using the modified method of von Gadow et al., [1997]. Consequently, 40 µl of 0.4 M 1,1-diphenyl-2-picryl-hydrazyl (DPPH)

solution in ethanol was added to 50 µl of the sample solution, complemented with 110 µl of ethanol. The plates were mixed and allowed to stand for 30 min in the absence of UV light to avoid decomposition. The absorbance was measured at 520 nm against an ethanol blank. Calibration curves of Trolox (0–24 mg/L) were prepared and the results were expressed as the number of equivalents of Trolox (mg TE/100 g DS).

2.2.7. Characterization of phlorotannin extract

The composition of the phlorotannin extract obtained under the best extraction conditions was characterized and the type of phlorotannin present was identified as follows.

2.2.7.1. Fourier transform infrared spectroscopy – FTIR

The sample was pre-fractioned with methanol in order to induce precipitation of the co-extracted carbohydrates (methanol was chosen among different solvents tested, not shown), concentrated in rotary evaporator at 40°C and pressure bellow 0.1 mbar and finally lyophilized at -50°C under vacuum (below 0.1 mbar) overnight.

The IR spectra obtained for the sample and phloroglucinol were recorded on an ATI Mattson Infinity Series IR spectrometer at room temperature. The samples were freeze-dried before the FTIR analyzes and blended in a 3 % w/w ratio with potassium bromide (KBr) powder, followed by tableting (10 tons for 1 min) before measurement. A region of 4.000 – 400 cm^{-1} was used for scanning.

2.2.7.2. Carbohydrate analysis

Carbohydrates in the obtained phlorotannin extract were quantified with an acid methanolysis method followed by sample silylation and GC analysis as follows: A volume of 2 mL of methanolysis reagent containing 2 M of hydrochloric acid (HCl) in methanol was added to 10 mg of freeze dried algae samples and a calibration solution containing known amounts of carbohydrates. As the next step, the tubes were inserted into an oven operating at 100°C for 3 h. Once the reaction was completed, 200 µL of pyridine was added to neutralize any excess of HCl, and 2 mL of each internal standard solutions containing 0.1 mg/mL of sorbitol and resorcinol in methanol, respectively, were added to each sample. After mixing, methanol was evaporated at 50°C under nitrogen stream and the sample was further dried under vacuum (Heraeus VTR 5022) at 42°C below 50 mbar for 20 min prior silylation of the

samples [Sundberg et al., 2003]. Once the samples were completely dry, silylation was commenced by adding 150 µL of pyridine, 150 µL of hexamethyldisilazane (HMDS) and 70 µL of chlorotrimethylsilane (TMCS), followed by a thorough mixing using a high-sheer vortex mixer. Further, the samples were kept in an oven at 70°C for 45 min and the clear liquid phase was analyzed in order to determine the carbohydrate content of the samples by gas chromatography [Sundberg et al., 2003]. Consequently, about 1 µL of a silylated sample was injected via a split injector (260°C, split ratio 1:15) into a 30 m/0.32 mm i.d. column coated with dimethyl polysiloxane (HP- 1, Hewlett Packard) with the film thickness of 0.17 µm. The column temperature program was as follows: a temperature ramp from 100 to 175°C (4°C/min), followed by a ramp of 175 to 290°C (12°C/min). The detector (FID) temperature was 290°C. Hydrogen was used as a carrier gas.

2.2.7.3. High Precision Liquid Chromatography Mass Spectrometry (HPLC-ESI-MS/MS) Identification

A volume of 14 mL of extract phlorotannins obtained under the best extraction condition was concentrated at room temperature in a vacuum concentrator (SpeedVac, Savant Instruments Inc., NY-USA). Consequently, the concentrated extract was re-suspended in 4.5 mL of water and a volume of 20 µL was analysed using a LC-ESI-MS/MS system which consisted of the HP1100 liquid chromatography (Algilent Technologies Inc., CA-USA) connected to the mass spectrophotometer (Esquire 4000 ESI-Ion Trap LC/MS(n) system, Bruker Daltonik GmbH, Germany). A Luna C18 150 x 4.6 mm, 5 µm and 100 Å analytical column (Phenomenex Inc., CA-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 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 [Ferreres et al., 2012]. 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 nebulising gas at 45 psi, drying gas at 345°C and flow rate 10 L/min. All scans were performed in the range 20-2200 m/z. The trap parameters were set in ion charge control using 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.

2.2.8. Statistical analysis

All extracts and fractions were analyzed in triplicate and the measurements were presented as average ± standard deviation. To evaluate the effect of the extraction solvent and drying temperature of phlorotannins from *M. pyrifera* were selected the best condition used as criteria higher-the-better for TPC and TAA.

The significance and relative influence of the individual extraction parameters on TPC and TAA using Taguchi orthogonal array was determined using the variance analysis (ANOVA), the significance of the parameter was determined at 5% confidence level.

2.3 Results and discussion

2.3.1. Raw material analysis

The chemical analysis of *M. pyrifera* indicated that the ash content was higher in the batch M1 (40.26% w/w), whereas the lipid content was higher in the batch M3 (6.45% w/w) and carbohydrate content highest in the batch M2 (62.14% w/w, Table 2.2). According to the literature [Deniaud-Bouët et al., 2014], alginate–phenol linkages play an essential role in the brown algal cell wall structure localized and it is expected that this correlates with high phlorotannin concentration in the brown alga. On the other hand, the differences observed in the lipid and carbohydrate content, for the alga batches harvested in November and March, could be attributed to environment factors associated with the seasonal, such as, water temperature, 12.5 and 13.5°C on average, respectively [Westermeier et al., 2012], and the level of UV radiation, UV-B 0.5 and 4 W/m^2 on average, respectively [Huovinen et al., 2006].

Table 2.2. Chemical analysis of *Macrocystis pyrifera* harvested during different periods.

Proximal analysis	**Unit**	***M. pyrifera* M1 (Nov 2011)**		***M. pyrifera* M2 (March 2013)**		***M. pyrifera* M3 (Jun 2013)**	
		Wet algae	**Dry basis**	**Wet algae**	**Dry basis**	**Wet algae**	**Dry basis**
Humidity	%	10.18	---	17.61	---	15.99	---
Protein	%	10.01	11.15	8.52	10.34	9.59	11.42
Ash	%	36.16	40.26	21.50	26.10	25.16	29.95
Lipids	%	1.03	1.15	1.17	1.42	5.42	6.45
Fiber	%	17.45	19.43	6.53	7.93	7.62	9.07
NNE[a]	%	25.16	28.01	44.67	54.22	36.22	43.11
Carbohydrates[b]	%	42.61	47.44	51.20	62.14	43.84	52.18

[a]NNE, No nitrogen elements which was calculated as 100 –(Fiber+Lipids+Protein+ash)

[b]Carbohydrate amount was calculated as 100 - (Humidity + Protein + Ash + Lipids)

2.3.2. Selection of operational conditions: Effect of the extractant type and drying temperature

The TPC and TAA levels obtained using different solvents are depicted in Figure 2.1. The two highest concentrations of TPC were attained using water and 70% v/v acetone as extraction solvents, giving 147±2.9 and 125±1.5 mg GAE /100 g DS, respectively. On the other hand, the highest TAA values were attained for 80% v/v acetone and water, 25±2.0 and 20.2±4.2 mg TE/100 g DS, respectively. The better yields of TAA in the case of aqueous acetone may be due to the fact that acetone is a less polar solvent. Consequently, acetone has a higher affinity for apolar compounds such as lipid compounds and pigments, which may have produced an increment in the TAA measured in the sample. The presence of apolar compounds present in the extract suggest that the pretreatment conditions realized is not optimized [Vidanarachchi et al., 2012; Gupta and Abu-Ghannam, 2011]. Therefore, these results suggested that water was the best solvent for the extraction of phlorotannins. Similar results were obtained by Kindleysides, et al. [2012]. Moreover, when studying the extraction strategies of phlorotannins for other brown seaweed species (*Scytosiphon lomentaria*, *Papenfussiella kuromo* and *Nemacystus decipiens*), water was considered as the best extraction solvent [Kuda et al., 2005].

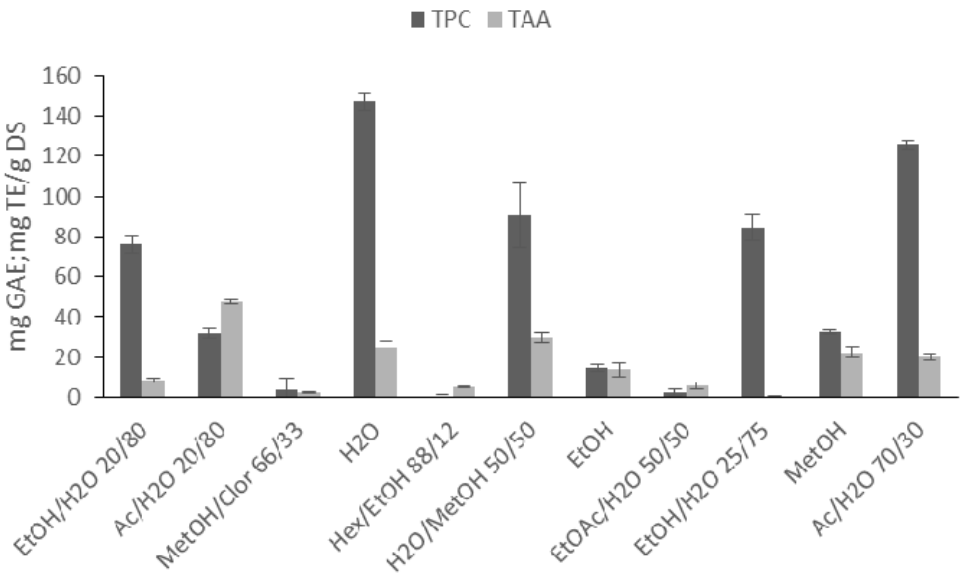

Fig 2.1. Evaluation of the best extractant for total polyphenols (TPC) and total antioxidant activity (TAA) in *Macrocystis pyrifera*. Ac, acetone.

The influence of temperature and drying time on the extraction and quality of phlorotannins extracted from *M. pyrifera* was evaluated. The drying curves are presented in Figure 2.2a. As can be observed, the drying profiles for the temperatures 40, 50 and 60 ° C are relatively similar. On the contrary, at 30°C, a much slower release of moisture was observed. In order to evaluate the best drying temperature, the extraction of phlorotannins was carried out using water as an extractant. Notwithstanding, the phlorotannin compounds are easily decomposed, as also reported earlier [Koivikko et al., 2007]. Consequently, the addition of 0.3% ascorbic acid as a preservative during extraction was evaluated. The results of the total phlorotannins content (TPC) and TAA concentration obtained are presented in Figure 2.2b. The optimal drying temperature was 40°C, giving TPC and TAA values in the extract amounting to 180±13 mg GAE/100 g DS and 62±19 mg TE/100 g DS, respectively. On the other hand, for fresh seaweed (85% average moisture content; fresh seaweed, F/S) gave 210±20 mg GAE/100 g DS of TPC and 115±36 mg TE/100 g DS of TAA. Consenquently, the TPC and TAA values obtained for non-dried alga gave better results, a 14 and 46% higher that the values of dry seaweed at 40°C, respectively, presumably due to the degradation of some phlorotannins during the drying process. Admittedly, the aim of the drying process was to reduce the moisture content in order to minimize the deterioration of the algae due to microbial growth, thus increasing the storage time of the seaweed prior any treatments. Further, the drying process also reduces the weight and volume of the seaweed, thus minimizing the packaging, storage and transportation costs [Vega-Gálvez et al., 2011].

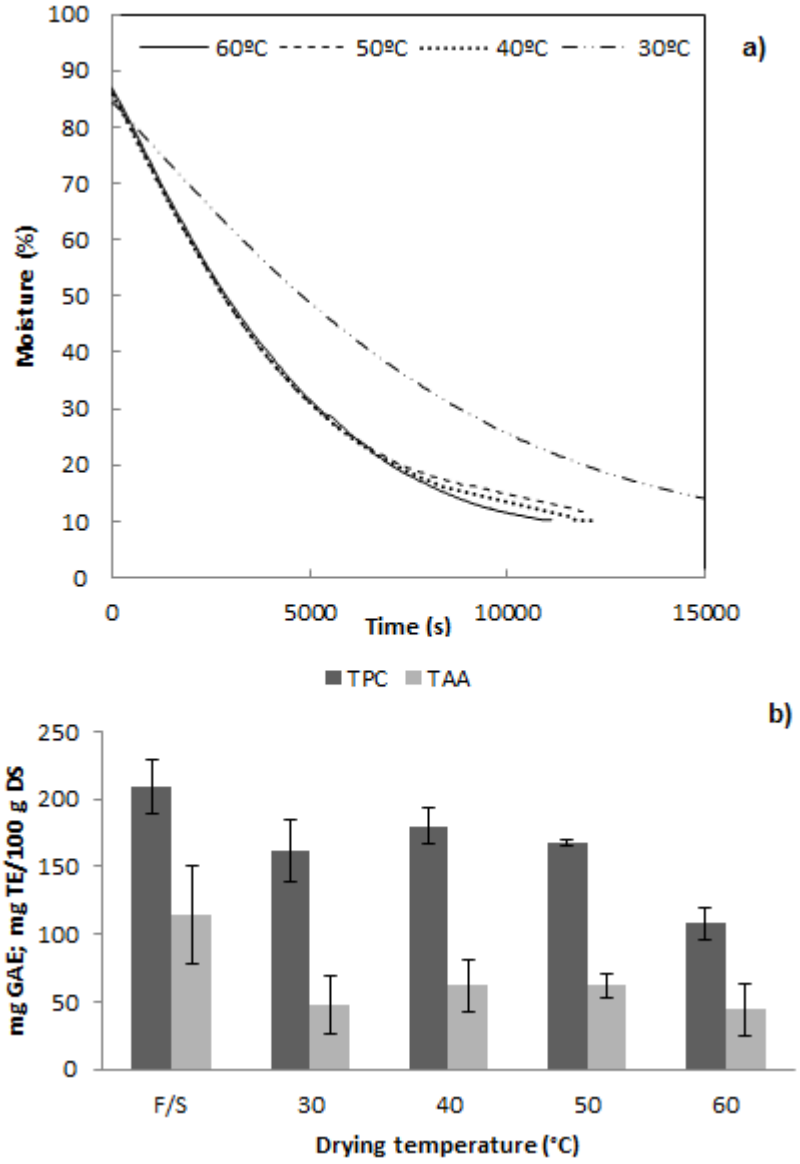

Fig 2.2. a) Drying curves for *Macrocystis pyrifera* at different drying temperatures, **b)** Evaluation of the different drying temperatures for *Macrocystis pyrifera* in terms of total concentration polyphenols (TPC) and total antioxidant activity (TAA). F/S denote fresh seaweed.

The difference observed in the concentration of TPC and TAA for the extract obtained previously, 147±2.9 mg GAE/100 g DS and 20.2±4.2 mg TE/100 g DS for the effect of extractant type and, 180±13 mg GAE/100 g DS and 62±19 mg TE/100 g DS for effect of drying temperature, can be explained as due to the effect of different drying conditions (temperature and time) and by the fact that algae samples correspond to different harvest periods [Buschmann et al., 2013], Table 2.2. Nevertheless, it is possible to minimize the

differences observed by controlling drying conditions and via standardization of biomass through changes in the form of culture [Hafting et al., 2015].

2.3.3. Optimized extraction of phlorotannins: Effect of extraction parameters

The TPC and TAA values measured upon different conditions of water extraction are recorded in Table 2.3. The best extraction conditions correspond to the entry N° 9, whereupon the extraction performed for a sample particle size below 1.4 mm, and extraction with water at 55°C for 4 hours (S/L ratio of 1/15). Consequently, TPC of 200.5±5.6 mg GAE/100 g DS and TAA of 38.4±2.9 mg TE/100 g DS were recorded, resulting in 10% higher TPC value compared with previous conditions, 180±13 mg GAE/100 g DS. The reported value is higher than the corresponding values reported for other types of brown algae. Indeed, Chandini, et al. [2008] reported a TPC value of 0.29 mg GAE/100 g DS upon the water extraction of *Sargassum maginatum,* whereas in case of *Ecklonia stolonifera*, 8.21±0.01 mg GAE/100 g DS was reported [Iwai, 2008]. Further, in an additional study of *Turbinaria conoides* and using diethyl ether extraction, 119±0.1 mg GAE/100 g DS of TPC was obtained [Devi et al., 2011]. Thus, it is evident that *M. pyrifera* has potential since much higher TPC values were attained for this worldwide relatively abundant seaweed.

Table 2.3. The influence of extraction parameters determined by Taguchi orthogonal array design L_9, 3^4 on TPC and TAA from the extraction of *Macrocystis pyrifera*.

Runs	Time [h]	Temperature [°C]	S/L[a] Ratio	Particle size [mm]	TPC [mg GAE/100 g DS]	TAA [mg TE/100 g DS]
1	2	25	1/10	< 1.4	120.8±11.3	42.7±0.4
2	2	40	1/15	2-1.4	58.5±4.0	29.1±8.4
3	2	55	1/20	> 2	39.5±3.1	14.4±4.2
4	3	25	1/15	> 2	31.4±18.1	27.3±4.3
5	3	40	1/20	< 1.4	47.2±5.9	42.0±3.2
6	3	55	1/10	2-1.4	136.9±20.8	55.6±1.9
7	4	25	1/20	2-1.4	100.0±6.3	20.8±2.0
8	4	40	1/10	> 2	89.1±9.3	46.1±4.8
9	**4**	**55**	**1/15**	**< 1.4**	**200.5±5.6**	**38.4±2.9**

[a]S/L, Solid to liquid ratio

Finally, all factors had significant effects ($p<0.05$) on the TPC and TAA (Table 2.4), the highest influence was obtained with the S/L ratio, particle size and extraction time of 29.4, 27.8 and 21.7%, respectively, the sum of these three factors accounted for 78.9% on the TPC. For the TAA the highest influence, 57%, was that of factor S/L ratio. The statistical methodology applied predicted a maximum TPC of 218.9±15.0 mg GAE/100 g DS and TAA of 64.4±2.9 mg TE/100 g DS under the optimal extraction conditions (Table 2.5).

Table 2.4. Variance analysis (ANOVA) for the total concentration of phlorotannins (TPC) and the total antioxidant activity (TAA) for *Macrocystis pyrifera*.

Response	Factors	DOF[a]	Sums of Squares	Variance	F-ratio	Pure sum	%
TPC	Time	2	9636.9	4818.4	39.1	9390.3	21.7
	Temperature	2	6137.3	3068.7	24.9	5890.8	13.6
	S/L[b] Ratio	2	12960.9	6480.5	52.6	12714.4	29.4
	Particle size	2	12266.2	6133.1	49.8	12019.6	27.8
	Other/error	18	2218.8	123.3	---	3204.9	7.4
	Total	26	43220.1	---	---	43220.1	100
TAA	Time	2	830.8	415.4	23.8	795.9	18.5
	Temperature	2	242.4	121.2	6.9	207.5	4.8
	S/L[b] Ratio	2	2490.4	1245.2	71.4	2455.5	57.0
	Particle size	2	433.2	216.6	12.4	398.3	9.2
	Other/error	18	314.0	17.4	---	453.5	10.5
	Total	26	4310.8	---	---	4310.8	100

[a]DOF, degree of freedom

[b]S/L ratio, Solid/Liquid ratio of extraction

Table 2.5. Condition giving the maximal total phlorotannins concentration (TPC) and total antioxidant activity (TAA) from *Macrocystis pyrifera*.

	TPC		TAA	
Factors	**Optimum level**	**Contribution**	**Optimum level**	**Contribution**
Time	4h	25.0	3h	6.5
Temperature	55°C	20.8	40°C	3.9
S/L[a] ratio	1:10	30.7	1:10	13.0
Particle size	<1.4	17.9	<1.4	5.9
Total contribution from all factors		94.4		29.2
Expected result at optimum ± CI[b] condition		218.9±15.0		64.4±2.9

[a]S/L, Solid/Liquid ratio of extraction

[b]CI, confidence interval

2.3.4 Characterization of phlorotannin extract

2.3.4.1. Fourier Transform Infrared (FTIR) spectrometry

As can be seen, the chemical compositions of phlorotannin extract obtained under the optimal condition, and of standard phloroglucinol were similar, see Figure 2.3. The phloroglucinol standard gave rise to twenty three major peaks within the range between 3070 and 521 cm^{-1}, meanwhile similar peaks were observed for phlorotannins extract between 1618 and 536 cm^{-1}. Indeed, in phlorotannin extract, the stretching bands around 1470-1450 cm^{-1} correspond to the aromatic nuclei. The characteristic stretch band of carboxyl groups, the peak at 536 cm^{-1}, was presumably caused by the stretching vibration of O-H whit relatively high strength in the axial position. In turn, the bands at 1026 cm^{-1} correspond to the glycosidic linkage vibrations of C-OC and C-O-H, indicating the presence of some carbohydrates in the sample.

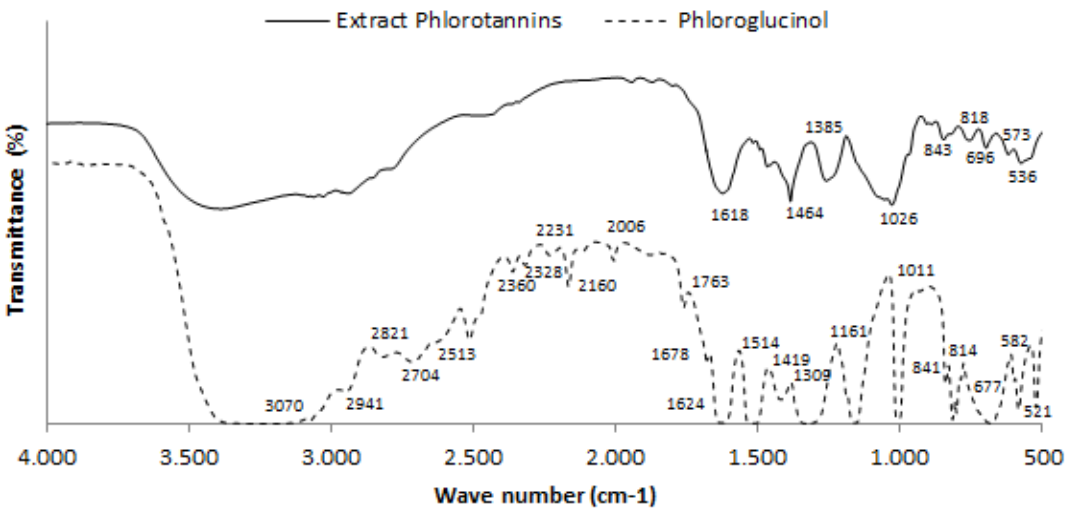

Fig 2.3. FTIR spectra of standard phloroglucinol and water phlorotannin extracts from *Macrocystis pyrifera*.

Nonetheless, the stretch bands identified for some carbohydrate compounds may reveal the presence of some phlorotannin compounds in association with some carbohydrates. The signal at 843 cm^{-1} corresponds to characteristic absorption of mannuronic acid (a monomeric unit of alginate, one of the main constituents of brown algae) [Chandía et al., 2004].

2.3.4.2. Analysis of the carbohydrate present in the phlorotannins extract

Various carbohydrates could be identified in the water extract of phlorotannins (Table 2.6), such as polysaccharide alginate, different types of monosaccharides (arabinose, fucose, fructose, galactose, glucose, mannose, rhamnose and xylose) sugar acid, (galacturonic and glucuronic acid) and a polyol (mannitol).

Table 2.6. Average concentration (±standard deviation) of carbohydrates present in alga *Macrocystis pyrifera* and in its phlorotannin extract.

Carbohydrate	Phlorotannins Extract	*M. pyrifera* (M3)
	mg/g DS	mg/g DS
Alginate	23.4±6.6	191.7±3.4
Arabinose	1.0±0.6	1.7±0.5
Fucose	39.7±5.7	73.5±0.1
Fructose	1.4±0.1	14.7±0.1
Galactose	5.7±1.3	31.7±2.7
Galacturonic acid	0.5±0.7	6.8±0.1
Glucose	2.0±0.6	11.1±0.1
Glucuronic acid	0.8±0.1	4.9±0.1
Mannitol	38.1±2.3	38.2±0.4
Mannose	2.0±0.9	11.5±1.1
Rhamnose	2.7±0.4	2.0±0.1
Xylose	1.2±0.4	9.0±1.3
Total	118.5	396.9

2.3.4.3. HPLC-ESI-MS/MS Identification

The chromatograms obtained were compared to the reported ones for phlorotannins [Martínez and Catañeda, 2013; Wang et al., 2012; Ferreres et al., 2012; Liu, 2011]. The identification was carried out using all m/z signals for each corresponding separated fragment. Additionally, an extracted ion chromatogram (EIC) of the expected precursor signal was performed. Two peaks were identified upon the analysis and compared with earlier data [Martínez and Catañeda, 2013; Wang et al., 2012; Ferreres et al., 2012]. The peak 1 (t_R 34.4 min) was detected with a signal m/z 497, (the fragmentation gave m/z values of 478.2 ($[M\text{-}H_2O+H]^+$) and m/z 245.5 ($[M\text{-}2x\text{Phloroglucinol}+H]^+$)), likely corresponding to a derivative of phloroglucinol named phloroeckol (Figure 2.4a and 2.4b). The peak 2 (t_R 52.1 min) was detected with a signal m/z of 499, (the fragmentation gave signals with m/z of 480.4

([M-H_2O+H]$^+$), m/z 245.8 ([M-2xPhloroglucinol+H]$^+$) and m/z 235.9 ([Phloroglucinol-H_2O+H]$^+$)), likely originating from a tetramer of phloroglucinol isomers: difucophloroethol, fucodiphloroethol, tetrafucol or tetraphloroethol (Figure 2.4c and 2.4d) [Martínez and Catañeda, 2013].

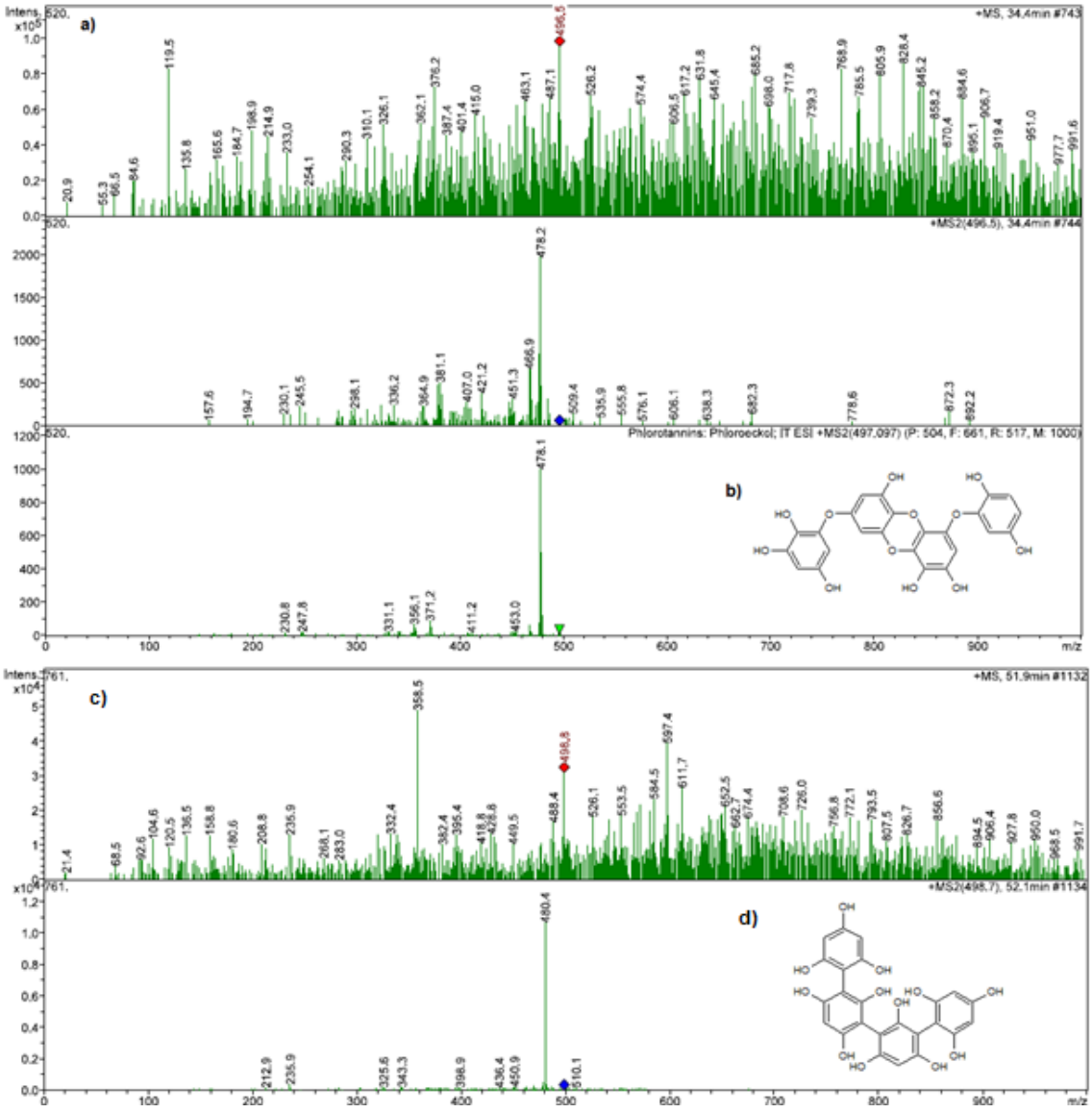

Fig 2.4. **a)** Peak 1, spectrum of mass and fragmentation obtained from HPLC-ESI MS/MS, **b)** chemical structure of phloroeckol, **c)** Peak 2, spectrum of mass and fragmentation obtained from HPLC-ESI MS/MS, **d)** chemical structure of tetramer phloroglucinol.

These phlorotannins have interesting propierties. Fucodiphloroethol exhibits free radical scavenging ability [Li et al., 2009] and antiallergic effect [Li et al., 2008], whereas phloroeckol exhibits antidiabetic effect [Le et al., 2009] and prevents Alzheimer's disease [Yoon et al., 2009]. Finally, we propose that phlorotannin extracts could find use as medicinal foods or therapeutics substrates in human health products.

2.4 Conclusions

The orthogonal design set of experiments allowed identification of the S/L ratio, particle size and time of extraction as the most important variables to obtain the highest concentrations of phlorotannins in the extract. The best extraction conditions upon extraction of phlorotannins from brown algae *Macrocystis pyrifera*, were determined: pre-treatment with hexane, extraction with water, drying temperature 40°C, particle size below 1.4 mm, at 55°C for 4 hours and solid-to-liquid ratio of 1:15. At optimal conditions, the TPC value obtained was 200.5±5.6 mg GAE/100 g DS and the TAA of 38.4±2.9 mg TE/100 g DS. Finally, two types of phlorotannins were identified in the water extract, corresponding to phloroeckol and a tetramer of phloroglucinol.

Chapter 3

Carbohydrate active enzymes from macroalgae-associated microorganisms: optimization of production using experimental design

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Abstract

Brown seaweed has complex polysaccharides in their cell walls. These polysaccharides constitute a crucial biomass to be used in bioproduct production for which very specific carbohydrate active enzymes are necessary. Therefore, the aims of this work were to (1) isolate *Macrocystis*-associated microorganisms that produce carbohydrate active enzymes, (2) optimize the production of enzymes using experimental design and (3) in parallel, determine the potential of hydrolysis of *Macrocystis*. Three microorganisms were isolated from *Macrocystis* tissue and presented high carbohydrate active enzyme preliminarily identified within the genus *Bacillus sp., Rhodotorulo sp.* and *Alternaria sp*. The carbohydrate active enzymes (CAE) were maximized using Taguchi design obtaining the following culture conditions: 1 wt.% seaweed, 8 g/L yeast extract at 25°C for 1 day for *Bacillus sp*. and 1.5 wt.% seaweed, in the absence of yeast extract at 25°C for 3 days for *Rhodotorula sp*. and *Alternaria sp*. with CAE of 84.6 ± 2.01, 105.2 ± 0.35 and 60.34 ± 4.97 U/mg, respectively. The chemical analysis of *M. pyrifera* indicated that the alga presented a high content of protein and carbohydrate, 10.3 and 62.1 wt.%, respectively, with a high proportion of mannitol, alginic acid and fucose, 321.7 ± 3.5, 284.3 ± 24.9 and 17.3 ± 3.1 mg/g DS, and a high content of salts such as potassium, sodium and calcium, 10, 4.5 and 1.8 wt.%, respectively. This emphasizes the potential use of marine microorganisms for production of specific CAE to hydrolyze the polysaccharide present in the algae that have the capacity to tolerate high salt concentrations. The purification of CAE was performed, obtaining for *Bacillus sp*. BAC a purification with a 60.7-fold increase in the specific activity with a yield of 52%. For *Rhodotorula sp*. B the CAE purification was with a 28.1-fold increase in the specific activity with a yield of 59%. Finally, the CAE of *Alternaria sp*. HN was purified with a 22.7-fold increase in the specific activity with a yield of 19%. Further work includes the characterization process of carbohydrate active enzymes produced by each strain.

Keywords: Isolation; brown seaweed; *Macrocystis*; marine microorganism; Taguchi design.

3.1 Introduction

Seaweeds are multicellular organisms extensively distributed worldwide. Up to date about 20,000 species are industrially grown and processed, and, consequently, used in the production of hydrocolloids, thickeners, gelling agents or different products among the food, cosmetic and pharmaceutical industries [Critchley et al. 1998; Roesijadi et al. 2010; Reith et al. 2009]. Seaweeds have been classified according to their pigment constituents into green algae (*Chorophyta*), brown algae (*Phaeophyta*) and red algae (*Rhodophyta*).

The Phaeophyta are algae of rapid growth with a high content of soluble carbohydrates, between 30 to 70 wt.% of dry weight [Klasing et al. 1988], mainly used as feed for abalone and hydrocolloid production. In the last few years, *Phaeophyta* have been used as a chemical platform for the production of varied biocompounds, via bioconversions to microbial fermentation [Yeon et al. 2011; Kim et al. 2011; Horn et al. 2000 a, b; Adams et al. 2009; Ge et al. 2011; Lee and Lee 2012a; Ravanal et al. 2016]. The carbohydrates present in brown algae are present, forming part of the structure of the cell wall [Deniaud-Bouët et al., 2014], supplying flexibility in the structure, maintaining the ionic balance and protection against osmotic water loss [Hahn et al. 2012; Usov and Zelinsky 2013]. Therefore, for an efficient use of these compounds a hydrolysis process is necessary in order to liberate and depolymerize these carbohydrates.

The hydrolysis process, for this type of biomass, must meet the following requirements: tolerate presence of inhibiting compounds, such as metal ions and high salt concentrations [Lee and Lee 2012b], act on different types of carbohydrates (heterogeneous), and preserve the quality of the compounds. An alternative to this equipment is the use of specific carbohydrate active enzymes for each polysaccharide type present in brown algae such as: alginate lyase (EC4.2.2.-; family PL 7, 15 and 17) which acts on alginate, linear anionic polysaccharide composed of β-D-mannuronic (M) and α-D-guluronic (G) acids, via a β-elimination mechanism to generate an unsaturated hexenuronic acid residue and a new reducing end; 1,3-β-D-glucanase (laminarinase, EC3.2.1.39; family GH16) which acts on laminarin, linear polysaccharide composed of units of 1, 3-β-D-glucans and terminal residues of mannitol, via hydrolysis of glycosidic bond between two or more carbohydrates; and

finally fucoidanase (EC3.2.1.44; not information of family type) which acts on fucoidan, sulfated polysaccharides composed mainly of sulfated L-fucose and small residues of galactose, mannose, xylose, glucose, uronic acids and rhamnose, via hydrolysis of methyl L-fucoside and fucosidic linkages of fucoidan [Cazy, 2016].

In this context, several studies have pointed out that marine microorganisms are a suitable source of carbohydrate active enzymes [Khambhaty et al. 2012; Trivedi et al. 2013; Chi et al. 2009; Alderkamp et al. 2007]. In this regard, the objectives of this study were to (1) isolate *Macrocystis*-associated microorganisms that produce carbohydrate active enzymes, (2) optimize the production of enzymes using experimental design and (3) in parallel, determine the potential of hydrolysis of *M. pyrifera*.

3.2 Material and methods

The methodology used to isolate microbial strains associated with *Macrocystis* was cultivation on brown algal carbohydrate-enriched fermentation broth and then spread on Petri dishes. The colonies were picked and screened for carbohydrate active enzymes, summation of specific activity of alginate lyase, fucoidanase, and 1,3-β-D-glucanase. The microbial active strains were selected and identified at the genus level according to 16S/18S rDNA and their culture conditions were optimized in order to get the higher carbohydrate active enzymes in the culture supernatant. In parallel, chemical composition of the algae was carried out to determine the potential of carbohydrate hydrolysis and as a source of culture medium. The analyses were performed as follows.

3.2.1 Isolation and cultivation of microbial strains associated with Macrocystis

Several microbial strains associated with *Macrocystis* were cultured. The algae *M. pyrifera* was harvested in Chiloe, 30 km southeast of Puerto Montt, Chile, in March 2013. The algae were washed with distilled water to remove external material, and cut aseptically with a scalpel. Small amounts of tissues were introduced in specific brown algal carbohydrate-enriched fermentation broth for the isolation of microbial strains. The media for the isolation of bacteria contained 70 vol.% artificial seawater (ASW; c.a. 5.8%. Salinity close to that present in the algae), 27.5 g/L NaCl; 5.38 g/L $MgCl_2 \cdot 6H_2O$; 6.78 g/L $MgSO_4 \cdot 7H_2O$; 0.72 g/L KCl; 0.2 g/L $NaHCO_3$ and 1.6 g/L $CaCl_2$ [Shene et al. 2013]. Additionally, the culture was provided with 4.0 g/L yeast extract; 5.0 g/L peptone; 4.0 g/L sodium alginate (from Brown algae by Sigma-Aldrich); 3.5 g/L laminarin (from *L. digitata* by Sigma-Aldrich); 2.5 g/L mannitol (by Merck) and 50 μg/mL cycloheximide. The media for the isolation of yeast and filamentous fungus was: 70 vol.% ASW, 10 g/L yeast extract; 5.0 g/L peptone; 8.0 g/L sodium alginate; 7.0 g/L laminarin; 5.0 g/L mannitol and 100 mg/L chloramphenicol.

The cultures were incubated at 25°C for 3-7 days taking samples on a daily basis (50 μL) and spread on Petri dishes. The medium for the Petri dishes was: marine media Difco 2216 supplemented with 15 g/L agar and 1 wt.% dry algae *M. pyrifera* a source of carbon. Different types of colonies were picked after 2-5 days of incubation and grown on new Petri

dishes to obtain pure cultures. The strains isolated were stored at -80°C in 30 vol.% glycerol. All the isolates were examined for carbohydrate active enzymes.

3.2.2 Screening for carbohydrate active enzymes (CAE)

For the screening of carbohydrate active enzyme the enzymatic extract obtained from each isolated microbial strains was analyzed after growth in liquid medium, 70 vol.% ASW, 4 g/L yeast extract and 1 wt.% dry *M. pyrifera*. For each strain, one colony isolated on Petri dish was inoculated in this liquid medium. The culture was incubated for 5 days at 25°C. Finally, the culture was centrifuged at 8,500 g at 4°C for 5 min and the supernatant was collected for determination of the carbohydrate active enzymes (secreted into the medium).

The determination of the CAE was performed via quantifying a specific activity on the polysaccharide: alginate, fucoidan and 1,3-β-d-glucan. The activity quantification was performed using a separate assay for each enzyme. The alginate lyase activity was performed in a test tube containing 200 µL sodium alginate 1 wt.% in 0.04 mol/L phosphate buffer at pH 7.0 into which 100 µL of culture supernatant was added. In turn, fucoidanase activity was determined in a test tube containing 900 µL of fucoidan (from *M. pyrifera* of Sigma-Aldrich) 0.5 wt.% in 200 mM acetate buffer at pH 4.5 into which 100 µL of culture supernatant was added [Rodríguez-Jasso et al. 2013]. Finally, for the 1,3-β-d-glucanase activity was determined in a test tube containing 40 µL of culture supernatant into which 170 µL of laminarin 0.5 wt.% in 100 mM sodium acetate buffer at pH 5.5 was added. All mixtures were incubated at 37°C for 1 hr. The enzyme activity was determined by measuring the increase of reducing sugars by the dinitrosalicylic acid (DNS) method with glucose as standard (Miller 1959). One unit of enzyme activity, U, was defined as the amount of enzyme that catalyzed the liberation of one micromole glucose equivalent reducing end 1 mg per minute under the assay conditions [An et al. 2008; Ghose 1987].

Three microbial strains that represent each one of the microorganism types (fungus, yeast and bacterial) were selected according to CAE production.

3.2.3 Microscopic analysis

The strain morphology was characterized by colony observation with an optical microscope (Olympus CX22LED) with 400x magnification image.

3.2.4 DNA extraction of active microbial strains

The genomic DNA was extracted using the Cetyltrimethyl ammonium bromide (CTAB) method. 2 mL of growth medium was centrifuged at 10,000 g for 5 min at 4°C and the resulting cell pellet was suspended in 150 µl 10 mM Tris-HCl at pH 8.0 whit 1 mM EDTA (TE buffer). Subsequently, 8 µL of 50 mg/mL lysozyme was added and gently mixed and incubated for 30 min at 37°C. In the case of yeast and fungus DNA extraction, the pellet was ground with liquid nitrogen before dissolution in TE buffer. After dissolution, 9 µL of 20% sodium dodecyl sulfate and 5 µL of 100 mg/mL proteinase-K were added, gently mixed and kept at 60°C for 1 h. Then, 54 µL deionized water and 45 µL NaCl (5 M) were incorporated in the tube, mixed in vortex and 30 µL CTAB (10 w/v.% in deionized H_2O) was added to the mixture and incubated for 30 min at 65°C. The DNA was extracted with 300 µl chloroform: isoamyl alcohol (24:1) and centrifuged at 10,000 g for 5 min at 4°C. The upper layer was collected in a microcentrifuge tube with 180 µL isopropanol. The tube was kept at -20°C and centrifuged at 10,000 g for 30 min at 4°C. The extracted DNA was washed with 500 µl 70 vol.% ethanol and centrifuged at 10,000 g for 5 min at 4°C. The tube was kept at 37°C for 2-3 min to evaporate the residual ethanol. The DNA was resuspended in 50 µL bi-distilled sterile water and stored at 4°C.

3.2.5 Identification and sequencing of active microbial strains

The specific identification of the microbial active strains was carried out using universal primers 16S, F-5′-AGAGTTTGATCCTGGCTCAG-3′and R-5′-AAGGAGGTGATCCAGCC-3′ [Singh et al. 2009], for bacterial and 18S, F-5′-TACCTGGTTGATCCTGCCAG-3′, R-5′-CCTTCCGCAGGTTCACCTAC-3′ [Gupta et al. 2012] and F-5′-ATTGGAGGGCAAGTCTGGTG-3′, R-5′-CCGATCCCTAGTCGGCATAG-3′ [Einselen et al., 1997], for yeast and fungus, respectively. All amplification reactions were performed in a 50 µL reaction volume, containing a final concentration of 0.2 µM primers, 1X Taq reaction buffer, 200 µM dNTPs, 1.25 U Taq DNA polymerase, 200 ng DNA template and sterilized water. The amplified fragments were purified by Gel Extraction Spin Column (Thermo Scientific kit). The fragments were cloned into the pGemT Easy vector and transformed into *E. coli* DH5α competent cells. The colonies were blue/white screened on Luria Broth agar with ampicillin

(Sigma), X-gal (Promega) and isopropyl-β-D-thiogalactopyranoside (IPTG, Promega). The white colonies were randomly chosen, cultivated and stored at -80°C. The plasmid DNA was extracted using a plasmid extraction kit (Thermo Scientific). The sequencing of fragment of DNA was performed by Macrogen, Ltd (Korea). The nucleotide sequences of 18S and 16S rDNA were identified by BLASTN using the GenBank nucleotide sequence databases. The 18S and 16S sequences determined in this study were uploaded to the GenBank/EMBL sequence database.

3.2.6 Optimization in the production of CAE using experimental design

The optimization of carbohydrate active enzyme production, by each microbial strain, was performed using a design of experiments (DOE), which permits simultaneous evaluation of several factors with a smaller number of experiments, time and recourses [Taguchi 1990]. The factors were simultaneously evaluated using Taguchi orthogonal array L_9, 3^4 where 9 is the number of experiments, 4 is the number of factors tested and 3 is number of work levels. The factors and levels evaluated were determined by previous assay (data not shown).

Three microbial active strains, selected previously, were pre-cultured in liquid medium: 70 vol.% ASW, 4 g/L yeast extract and 1 wt.% dry *M. pyrifera*, by 3 days at 25°C under continuous shaking at 200 rpm.

The factors evaluated were the composition of fermentation broth, seaweed and yeast extract (YE) concentration, and culture conditions: temperature and time of culture. The factors and levels were varied according to Table 3.1. The experiments were conducted in triplicate in 250 mL Erlenmeyer flasks with 100 mL fermentation broth and 5 mL of pre-cultured as inoculum (5 vol.% of total volume). Finally, the sample was centrifuged at 8,500 g at 4°C for 5 min and the supernatant was collected for determining the carbohydrate active enzymes and protein concentration by Bradford method (Bio-Rad protein assay).

Table 3.1. Factors and levels varied according to Taguchi orthogonal array design L_9, 3^4

Runs	Temperature (°C)	Seaweed (%)	YE[a] (g/L)	Time (days)
1	15	0.5	0	1
2	15	1	4	3
3	15	1.5	8	6
4	25	0.5	4	6
5	25	1	8	1
6	25	1.5	0	3
7	35	0.5	8	3
8	35	1	0	6
9	35	1.5	4	1

3.2.7 Evaluation of the CAE extract as pre-treatment in the extraction process of phlorotannins from M. pyrifera

After determining the best conditions for production of carbohydrate active enzymes by the three microbial active strains, their incorporation as an enzymatic pre-treatment in the phlorotannin extraction process from brown algae was evaluated. The phlorotannin quantification was performed by determination of the potential of hydrolysis of carbohydrate active enzymes produced by each strain, due to that, these compounds are attached to the polysaccharides present in the structure of the cell wall of brown algae [Singh and Sidana 2013]. Therefore, the polysaccharide hydrolysis would increase phlorotannin output.

For this, 0.3 g of *M. pyrifera*, previously dried and ground, was mixed with 3 mL of buffer 100 mM sodium acetate buffer (pH 5.5) and 3 mL of enzymatic extract produced by each strain and evaluated separately. The samples were then incubated at 37°C, 250 rpm for 12 hours. After this time the mixture was centrifuged at 7000 g at 4°C for 5 min and the solid fraction dried at 60°C for 12 h for subsequent phlorotannin extraction.

Phlorotannin extraction was performed at 100°C for 180 min in a ratio of alkaline solution to algal biomass of 20 [Leyton et al., 2016]. The quantification of phlorotannins in the supernatant was determined according to the Folin–Ciocalteu assay [Singleton and Rossi

1965] using phloroglucinol as standard. The phlorotannin concentration was expressed as mg equivalent phloroglucinol (PGE)/100g dry seaweed (DS).

3.2.8 Purification of carbohydrate active enzymes (CAE)

The three microbial active strains B, BAC and HN were cultivated under the best conditions of CAE production as determined in point 2.6. Afterwards, the culture was centrifuged at 6000 g, 4°C for 10 min. The supernatant solution was gradually brought up to 60% (w/v) saturation with $(NH_4)_2SO_4$ and then centrifuged at 12,000 g for 15 min and the precipitate was dissolved in 2 mL of 50 mM buffer Tris-HCl pH 7.0. Continuously, the supernatant solution was further brought to 80% (w/v) saturation with $(NH_4)_2SO_4$ and the precipitate was dissolved in 2 mL of 50 mM buffer Tris-HCl pH 7.0. The whole process was carried out at 4°C. The precipitates were dialyzed (3.5 kDa molecular weight cut off) extensively against 1 L of the same buffer for 24 hours at 4 °C, changing buffer every 12 hours. The dialyzed solution (0.5 mL) was loaded onto a column (0.5 × 6 cm) of DEAE-Sepharose (GE Healthcare, Marlborough, MA, USA) and equilibrated with 50 mM Tris-HCl buffer at a flow of 0.1 mL/min. The column was washed with the same buffer and then eluted with a linear gradient of 0–1 M NaCl (15 CV). Then, the sample was concentrated and dialyzed to 300 µL on an Amicon PM-10 membrane (Millipore Co., Billerica, MA, USA) and 50 mM buffer Tris–HCl, pH 7.0. CAE activity and protein concentration were determined in the different fractions by the Bradford method (Bio-Rad protein assay).

3.2.9 Characterization of M. pyrifera

The characterization of *Macrocystis* biomass was performed to determine the potential of carbohydrate hydrolyzation, knowing the necessary enzyme type to hydrolyze the polysaccharide and the natural conditions of microbial strains associated to macroalgae-growth. The analyses were performed as follows.

3.2.9.1 Analysis of compositional chemistry of M. pyrifera

The chemical analysis of *M. pyrifera* was carried out in order to determine the initial composition of the algae in terms of protein, lipids, carbohydrates and ash elements. The analyses were made at the Institute of Agroindustry, University of the Frontera, Chile. The moisture content as well as protein, lipid, ash and fiber contents was quantified following the

official methods of the Association of Official Analytical Chemistry (AOAC): 930.04, 978.04, 991.36, 930.05 and 962.09, respectively (AOAC 2000). The carbohydrate content was calculated from the difference in the initial mass and the sum of values reported for proteins, humidity, lipids and ash (Merril and Watt 1973).

3.2.9.2 Analysis of the carbohydrate type present in M. pyrifera

The type of carbohydrate present in *M. pyrifera* was quantified with an acid methanolysis method followed by sample silylation and gas chromatograph (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 gas chromatography [Pezoa-Conte et al. 2015].

3.2.9.3 Elementary analysis of M. pyrifera

The elemental analysis of *M. pyrifera* was done by Inductively coupled plasma optical emission spectrometry (ICP-OES) for which the biomass was previously freeze dried, ground in an Al_2O_3 mortar and weighed into Teflon ® bombs to perform the analysis. Subsequently, 5 mL nitric acid (65 vol.%) and 1.2 mL hydrogen peroxide (30 vol.%) were added to the sample and dissolved in a microwave oven (Anton Paar, Multiwave 3000). After dissolution, the sample was diluted to 100 mL with bi-distilled water and the analysis performed in a Perkin Elmer (Optima 5300 DV instrument). The analytes measured were: aluminum (Al), arsenic (As), boron (B), iron (Fe), silicon (Si), strontium (Sr), calcium (Ca), potassium (K), magnesium (Mg), sodium (Na) and sulfur (S). Due to the grinding in an Al_2O_3 mortar, the reported amount might be overestimated. In turn, the organic elemental analysis was performed to quantify the content of carbon (C), nitrogen (N), hydrogen (H) and S in the samples. The analysis was carried out in triplicates of about 2.0 mg using a standard commercial cysteine. Additionally 2.5-(Bis(5-tert-butyl-2-benzo-oxazol-2-yl) thiophene (BBOT) was used a reference. The sample was analyzed in a Flash 2000 Thermo Scientific instrument, using helium and oxygen gases in the experiments.

3.2.10 Statistical analysis

All the CAE were analyzed in triplicate. The measurements were presented as average ± standard deviation. The experimental data were processed with the higher-the-better analysis for determining the best conditions for production of carbohydrate active enzyme.

The significance and relative influence of each individual factor on the optimization of carbohydrate active enzyme production by microbial active strains was performed using Taguchi orthogonal array and employed the variance analysis (ANOVA). The significance of the factors was determined at 5% confidence level [Taguchi 1990].

3.3 Results

3.3.1 Characterization of M. pyrifera

The chemical analysis of *M. pyrifera* (Figure 3.1) harvested in March, summer season, indicated that ash content was of a 26.1 wt.%, protein of a 10.3 wt.%, lipid of a 1.4 wt.% and carbohydrate of a 62.1 wt.%. This composition was similar to that presented by other brown seaweeds, *F. vesiculosus* and *U. pinnatifida,* harvested in the same season [Hahn et al., 2012; Westermeier et al., 2012; Cazón et al., 2014]. The carbohydrates present in high proportion in the algae (Table 3.2) were mannitol, alginic acid and fucose with a 321.7 ± 3.5, 284.3 ± 24.9 and 17.3 ± 3.1 mg/g DS, respectively, being these types of carbohydrate associated with the polysaccharides laminarin, alginate and fucoidan, respectively which suggests the use of 1,3-β-D-glucanase, alginate lyase and fucoidanase, respectively, for the hydrolysis of the polysaccharide present in the algae.

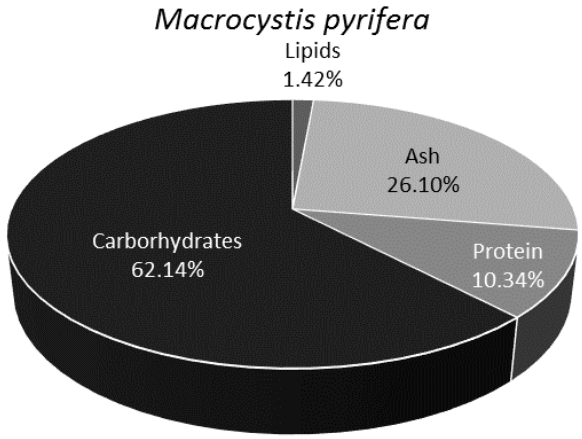

Fig 3.1. Chemical analysis of *Macrocystis pyrifera* harvested in Chiloe, 30 km southeast of Puerto Montt, Chile in March 2013.

On the other hand, the ICP-OES analysis showed a relatively high content of salts in *M. pyrifera* with an amount of potassium, sodium and calcium of 10, 4.5 and 1.8 wt.%, respectively (Table 3.3). This high salt is usually associated with the carboxylic groups of guluronic acid and mannuronic present in alginate. The results suggest the use of enzymes

capable of hydrolyzing all types of polysaccharides present in the alga, and have the ability to tolerate high salt concentration, placing marine microorganisms as a good source of such enzymes.

Table 3.2. Carbohydrate content (mg/g of dry algae) in *M. pyrifera* (March, 2013). Monosaccharides content analyzed by acid methanolysis method. Samples were analyzed in duplicate. Values represent average ± standard desviation. Total calculated as the total sum of identified monosaccharide and carbohydrate. ND: not detected.

Carbohydrate	***M. pyrifera***
	mg/g DS
Alginic acid	284.37±24.96
Arabinose	0.69±0.04
Fucose	17.36±3.15
Fructose	N.D[a]
Galactose	13.49±1.88
Galacturonic acid	10.90±1.35
Glucose	5.09±0.51
Glucuronic acid	9.76±3.11
Mannitol	321.75±3.58
Mannose	8.66±1.14
Rhamnose	0.56±0.20
Xylose	4.46±0.68
Total	677.09±41.11

Table 3.3 Inductively coupled plasma optical emission spectrometry (ICP-OES) and organic elemental analysis of fresh *Macrocystis pyrifera* biomass

ICP-OES					
Al [mg/kg]	As [mg/kg]	B [mg/kg]	Fe [mg/kg]	Si [mg/kg]	Sr [mg/kg]
260	70	130	150	640	1000
Ca [wt.%]	K [wt.%]	Mg [wt.%]	Na [wt.%]	P [wt.%]	S [wt.%]
1.8	10	0.9	4.5	0.3	1.7
Organic elemental analysis					
C	N	O	H	S	-
[wt.%]	[wt.%]	[wt.%]	[wt.%]	[wt.%]	-
27.9 ± 0.3	1.73 ± 0.02	30.1 ±0.4	3.96 ±0.06	1.55 ±0.22	-

3.3.2 Isolation and screening macroalgae-associated microorganisms with carbohydrate active enzymes (CAE)

Twenty microorganisms were isolated from the *M. pyrifera* tissue, where by microscopic observation was possible determine that the isolated strain AH, JH, HN and A-3A-H are fungi isolated; F, D, I, BAC1, M, N, L and G are bacterial isolated, and B, K, K*, A-3A-B, E, E*, AB and JB are yeast isolated. Figure 3.2 shows the 1,3-β-D glucanase, alginate lyase and total CAE for each microbial isolate. A total of three microorganisms, namely HN, B and BAC1 were selected based on the production of carbohydrate active enzymes and morphological characteristic. The microorganism HN had a total activity of 0.04 U/mL considering both alginate lyase and 1,3-β-D-glucanase activity. In turn, the microorganism B rendered a total activity of 0.039 and 0.031 U/mL for alginate lyase and 1,3-β-D-glucanase activity, respectively. Finally, the microorganism BAC1 presented a activity of 0.046 and 0.021 U/mL for alginate lyase and 1,3-β-D-glucanase, respectively. The strain F preliminary presented best results of enzymatic activity in comparison with strain BAC 1, but it was not selected because in a subsequent experiment, in order to determine the culture times, strain F did not show good results of activity (data not shown).

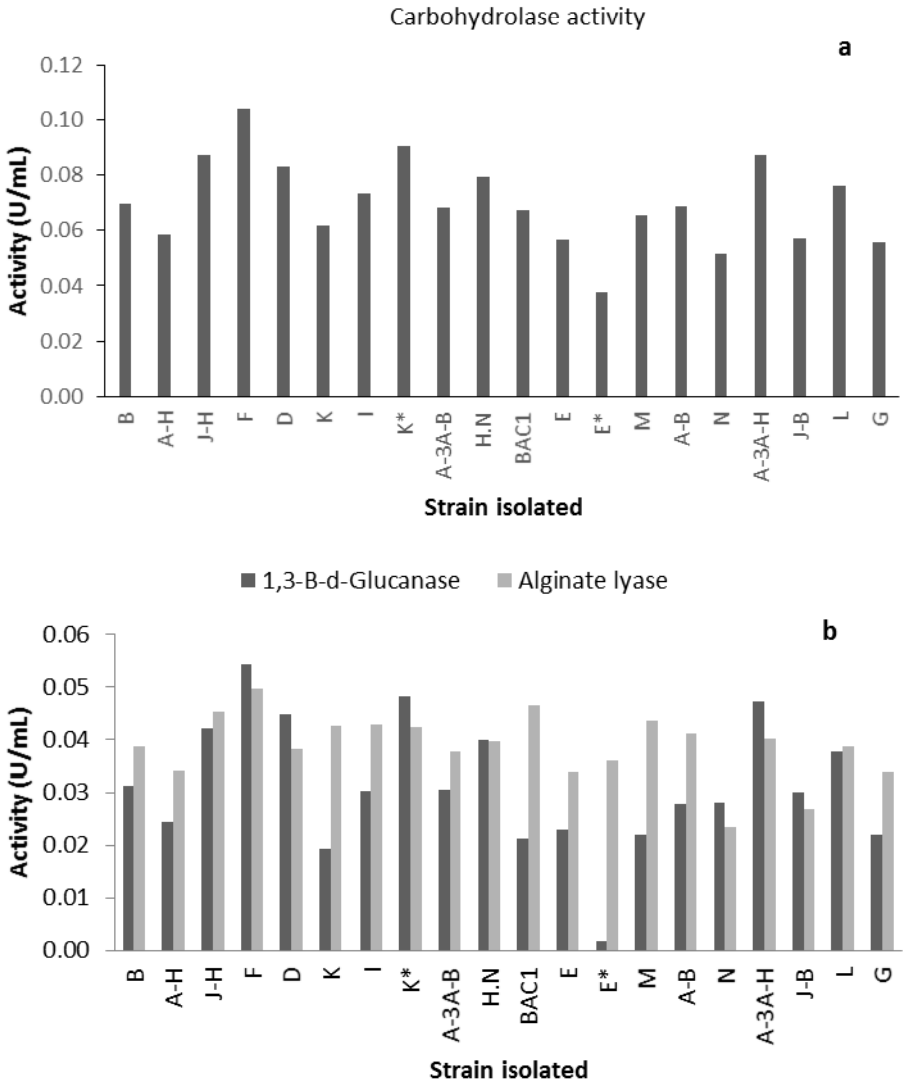

Fig. 3.2. Screening of enzymatic activity of different isolates. a. Production of carbohydrate active enzymes, b. Production of alginate lyase and 1,3-β-D-glucanase. Phylo fungi isolated: AH, JH, HN and A-3A-H; bacteria isolated: F, D, I, BAC1, M, N, L and G; yeast isolated: B, K, K*, A-3A-B, E, E*, AB and JB. Isolated select B – *Rhodotorula sp*., BAC1- *Bacillus sp*. and HN – *Alternaria sp.*

Figure 3.3 shows the microscopic images of the cultures of the isolated microorganisms that rendered the maximum growth and CAE. These microorganisms are classified into three different types, namely filamentous fungus (HN), yeast (B) and bacterial (BAC1).

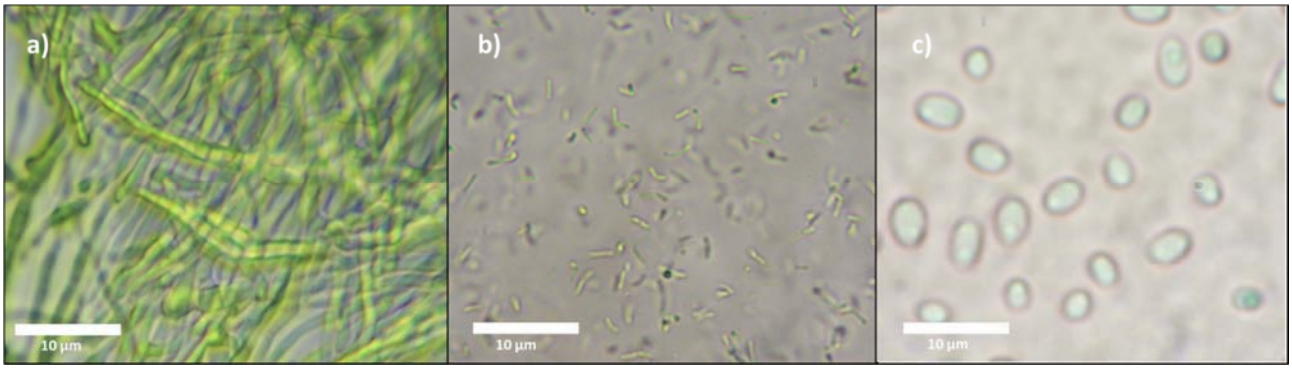

Fig. 3.3. Microscopic characteristics of strain growth for 3 days at 25°C were examined under optical microscope. a) HN, b) BAC 1 and c) B.

3.3.3 Microbial phylogenetic identification of selected isolates

The molecular identification of the strains, based on 18S and 16S rDNA gene sequence analysis was attained. The alignment of these sequences with the ones available in the NCBI database indicated that the 16S rDNA of the strain BAC1 was significantly 99% similar to the one published for a *Bacillus sp* species (accession N°: KU167713). In turn, the 18S rDNA of the strain HN and B were significantly similar in a 100 and 99% to those published for *Alternaria sp* (accession N°: KU163454) and *Rhodotorula sp* (accession N°: KU167831), respectively.

3.3.4 Optimization of the CAE *production by microbial strains*

In order to identify the conditions for improving CAE production of *Bacillus sp.* BAC, *Rhodotorula sp* B. and *Alternaria sp.* HN, separately, culture conditions were varied, using a DOE shown in Table 3.4.

Table 3.4. Influence of growth parameters determined by Taguchi orthogonal array design L_9, 3^4 on carbohydrate active enzymes for different isolated.

Runs	Temperature (°C)	Seaweed (%)	YE[a] (g/L)	Time (days)	*Bacillus* CAE[b] (U/mg)	*Rhodotorula* CAE[b] (U/mg)	*Alternaria* CAE[b] (U/mg)
1	15	0.5	0	1	26.11±8.79	84.33±17.14	14.35±12.73
2	15	1	4	3	15.48±5.97	107.5±10.03	19.94±9.43
3	15	1.5	8	6	16.4±5.97	69.89±4.27	11.7±5.84
4	25	0.5	4	6	18.89±5.04	56.11±7.78	9.3±4.8
5	25	1	8	1	84.57±2.01	89.06±19.86	20.62±4.11
6	25	1.5	0	3	73.8±13.39	105.17±0.35	60.34±4.97
7	35	0.5	8	3	18.22±5.51	66.01±2.56	16.91±6.22
8	35	1	0	6	15.97±2.12	21.49±15.3	6.45±2.38
9	35	1.5	4	1	20.75±6.15	53.85±11.12	6.84±5.03

[a]YE, yeast extract as nitrogen source.
[b]CAE, carbohydrate active enzymes.

The best production conditions of CAE for *Bacillus sp.* BAC are medium and growth conditions of: 1 wt.% seaweed, 8 g/L YE at 25°C for 1 day of growth, obtaining a CAE of 84.57 ± 2.01 U/mg (Run 5, Table 3.4). For *Rhodotorula sp.* B the best conditions of CAE production were: 1.5 wt.% seaweed, absence of YE at 25°C for 3 days of growth, with a CAE of 105.17 ± 0.35 U/mg (Run 6, Table 3.4). Finally, for *Alternaria sp.*HN, the best conditions were the same as for *Rhodotorula sp.* obtaining a CA of 60.34 ± 4.97 U/mg.

The type of CAE produced for each strain are reported in Figure 3.4. Under the best conditions of CAE production *Bacillus sp.* presented high fucoidanase activity, 20.48 ± 1.5 U/mg, followed by 1,3-β-d-glucanase, 43.50 ± 7.5 U/mg and alginate lyase activity , 20.59 ± 4.0 U/mg,. In turn, *Rhodotorula sp.* yielded the following activities: of 20.85 ± 2.3 U/mg for fucoidanase, 14.33 ± 0.17 U/mg for alginate lyase and 70.35 ± 0.15 U/mg for 1,3-β-D-glucanase. Finally, *Alternaria sp.* rendered a fucoidanase activity of 13.07 ± 1.1 U/mg, alginate lyase activity of 5.58 ± 0.15 U/mg and 1,3-β-D-glucanase activity of, 40.46 ± 5.5 U/mg.

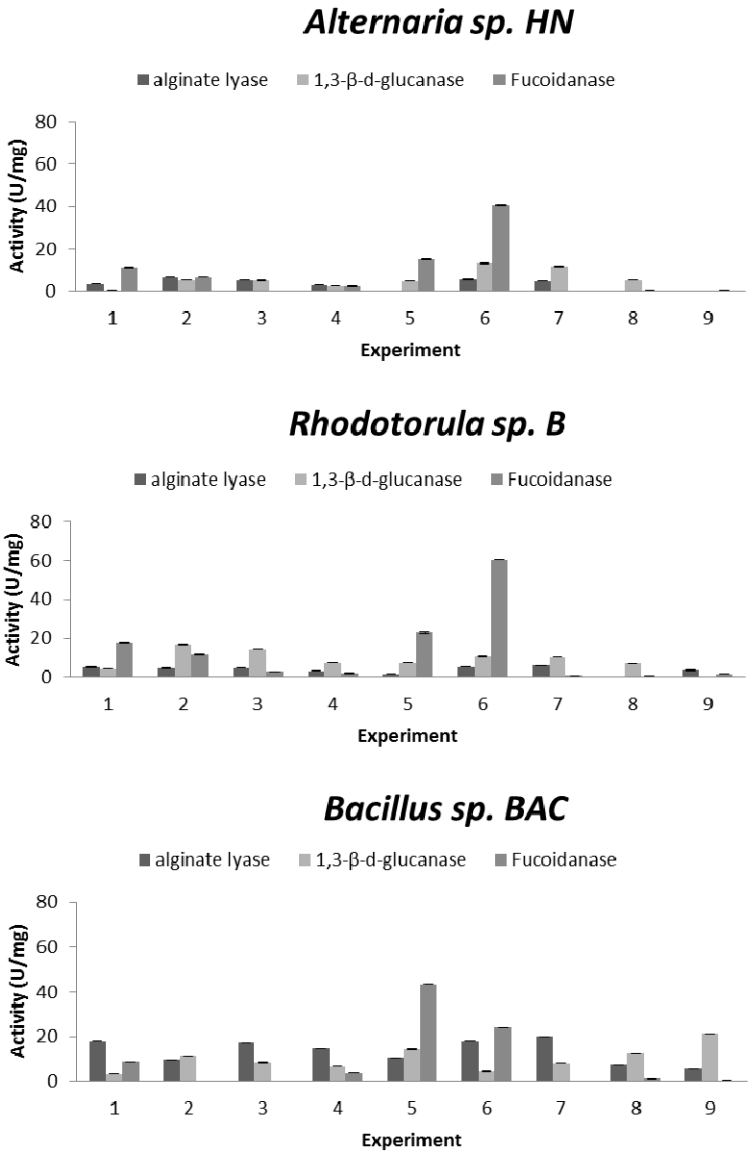

Fig. 3.4. Production of specific carbohydrate active enzymes, alginate lyase, 1,3-β-D-glucanase and fucoidanase, for different selected isolates. The conditions for each experiment are detailed in Table 3.4.

The variance analysis for different conditions evaluated for the optimization of production of CAE for each strain shown in Table 3.5. The analysis reported that all the factors evaluated in this study had significant effects ($p<0.05$) in the CAE. Still, the highest influence was obtained with the variation of temperature and time in the culture conditions

accounting for an improvement of 54.4 and 16.9% for *Bacillus sp.*, 35.7 and 32.6% for *Rhodotorula sp.*, and 25.5 and 35.5% for *Alternaria sp.,* respectively. Interestingly, the sum of these two factors was 71.3, 68.3 and 61.0%, respectively, on the CAE in each strain.

Table 3.5. Variance analysis (ANOVA) for the production of carbohydrate active enzymes from different isolates.

Response	Factors	DOF[c]	Sums of Squares	Variance	F-ratio	Pure sum	%
***Bacillus* CAE[a] (U/mg)**	Temperature	2	10013.5	5006.8	145.9	9944.9	54.4
	Seaweed	2	1610.8	805.4	23.5	1542.2	8.4
	YE[b]	2	2881.4	1440.7	42.0	2812.8	15.4
	Time	2	3167.9	1584.0	46.2	3099.3	16.9
	Other/error	18	617.5	34.3		892.0	4.9
	Total	26	18291.2			18291.2	100.0
***Rhodotorula* CAE[a] (U/mg)**	Temperature	2	23749.2	11874.6	43.2	23199.1	35.7
	Seaweed	2	9118.0	4559.0	16.6	8567.9	13.2
	YE[b]	2	5388.7	2694.3	9.8	4838.6	7.5
	Time	2	21691.3	10845.6	39.4	21141.1	32.6
	Other/error	18	4951.0	275.1		7151.5	11.0
	Total	26	64898.2			64898.2	100.0
***Alternaria* CAE[a] (U/mg)**	Temperature	2	2000.24	1000.12	21.9	1908.81	25.5
	Seaweed	2	786.80	393.40	8.6	695.38	9.3
	YE[b]	2	1121.18	560.59	12.3	1029.75	13.8
	Time	2	2745.70	1372.85	30.0	2654.28	35.5
	Other/error	18	822.86	45.71		1188.57	15.9
	Total	26	7477			7477	100.0

[a]CAE, carbohydrate active enzymes.
[b]YE, yeast extract as nitrogen source.
[c]DOF, degree of freedom

Finally, Table 3.6 reports the results obtained by the statistical methodology applied to predict the maximum CAE values under the optimal conditions for the three strains. For each microorganism, the maximum expected production of carbohydrate active enzymes was relatively similar to the one found in the experiments. Therefore, the best conditions of CAE

production search for each strain, run N° 5 for *Bacillus sp.* and N°6 for *Rhodotorula sp.* and *Alternaria sp.*, correspond the predicted by the statistical methodology.

Table 3.6. Optimum level for each factor predicted by the statistical analysis that maximizes the production of carbohydrate active enzymes for different strains. *Bacillus sp., Rhodotorula sp.* and *Alternaria sp*.

	***Bacillus sp.* BAC**		***Rhodotorula sp.* B**		***Alternaria sp.* HN**	
Factors	**Optimum level**	**Contribution**	**Optimum level**	**Contribution**	**Optimum level**	**Contribution**
Temperature	25°C	28.93	25°C	30.85	25°C	12.54
Seaweed	1%	6.46	1.5%	23.70	1.5%	8.35
YE[a]	0 g/L	7.55	0 g/L	17.73	0 g/L	7.05
Time	1 day	11.65	3 days	40.29	3 days	14.85
Total contribution from all factors		54.59		112.57		42.70
Expected result at optimum ± CI[b] condition		85.68±5.50		105.18±15.18		60.35±6.35

[a]YE, yeast extract as nitrogen source.
[b]CI, confidence interval

3.3.5 Evaluation of the effect of carbohydrate active enzymes (CAE) in the phlorotannins extraction process

The CAE obtained under the best production conditions for each microbial strain, i.e. BAC, B and HN, were used separately in the pretreatment of *M. Pyrifera* for phlorotannin improved extraction.

The enzymatic hydrolysis of brown seaweed allowed an increase in the extraction of phlorotannins, being their initial concentration of 281 ± 60 mg PGE/g DS and after the hydrolysis process, with the CAE of *Bacillus sp.* BAC, of 1395 ± 95 mg PGE/g DS, with CAE of *Alternaria sp.* HN of 1074 ± 49 mg PGE/g DS and finally, with CAE of *Rhodotorula sp*. B the concentration phlorotannins was of 1234 ± 155 mg PGE/g DS. Therefore, the pre-treatment enzymatic allowed an increase of 5-, 3.8- and 4.4-fold, respectively, compared to initial concentration.

3.3.6 Purification of carbohydrate active enzyme (CAE)

The carbohydrate active enzymes were purified from the supernatant, under the best production conditions determined for each strain, via ammonium sulfate fractioning, diethylaminoethyl (DEAE)-Sepharose Fast-Flow anion-exchange chromatography. These results, summarized in Table 3.7, indicate that the CAE of *Bacillus sp*. BAC was purified with a 60.7-fold increase in the specific activity with a yield of 52%. For *Rhodotorula sp*. B the purification of CAE was with a 28.1-fold increase in the specific activity with a yield of 59%. Finally, the CAE of *Alternaria sp*. HN was purified with a 22.7-fold increase in the specific activity, with a yield of 19%. This is the first approach in CAE purification from marine microorganisms. Future work is necessary so as to characterize the enzyme purified in estimation of the molecular mass, optimum pH and temperature for enzyme activity and stability.

Table 3.7. Purification of carbohydrate active enzyme from *Bacillus sp*. BAC, *Rhodotorula sp*. B and *Alternaria sp*. HN.

Purification step	Protein (mg/mL)	Total activity (U/mL)	Specific activity (U/mg)	Yield (%)	Purification (Fold)
		***Bacillus sp*. BAC**			
Crude extract	0.02	2.11	84.6	100.0	1
60% $(NH_4)_2SO_4$	0.04	85.45	2440.4	40	28.9
80% $(NH_4)_2SO_4$	0.04	89.73	2483.2	43	29.4
DEAE-Sepharose (60%)	0.02	109.42	5132.7	52	60.7
DEAE-Sepharose (80%)	0.02	106.42	5051.4	50	59.7
		***Rhodotorula sp*. B**			
Crude extract	0.021	2.18	105.2	100	1
60% $(NH_4)_2SO_4$	0.082	103.70	1271.9	48	12.1
80% $(NH_4)_2SO_4$	0.087	108.89	1246.9	50	11.9
DEAE-Sepharose (60%)	0.044	97.81	2243.8	45	21.3
DEAE-Sepharose (80%)	0.044	129.37	2956.8	59	28.1
		***Alternaria sp*. HN**			
Crude extract	0.039	2.35	60.34	100	1
60% $(NH_4)_2SO_4$	0.004	3.64	877.98	15	14.6
80% $(NH_4)_2SO_4$	0.004	3.64	877.98	15	14.6
DEAE-Sepharose (60%)	0.032	43.56	1354.38	19	22.4
DEAE-Sepharose (80%)	0.032	44.09	1367.04	19	22.7

3.4 Discussion

The chemical analysis of *M. pyrifera* emphasizes its possible use as fermentation broth for the production of varied compounds due to protein and carbohydrate content, 10.3 and 62.1 wt.%, it can be employed as a source of nitrogen and carbon, respectively. Besides, the high proportion of carbohydrate as mannitol, alginic acid and fucose, 321.7 ± 3.5, 284.3 ± 24.9 and 17.3 ± 3.1 mg/g DS, suggest the presence of polysaccharides as laminarin, alginate and fucoidan, respectively. Therefore, the use of specific carbohydrate active enzymes hydrolyzes these types of polysaccharides present in the algae. On the other hand, the high content of salts as potassium, sodium and calcium, 10, 4.5 and 1.8 wt.%, suggest that the carbohydrate active enzymes employed for hydrolyzing polysaccharides should be able to tolerate high salinity, being marine microorganisms a good alternative for their production. The chemical composition presented by algae is not constant in the different seasons of the year. According to Westermeier et al. [2012], variations in protein, lipid and carbohydrate levels between summer and winter were observed, where the values decreased in summer and increased in winter for proteins and lipids, and increased in summer and decreased in winter for carbohydrates. This seasonal change in the composition of alga is found correlated with changes in alga-associated bacterial populations, where the microorganisms form a biofilm on the surface of the alga, which is an effective defense mechanism against colonization by opportunistic and pathogenic bacteria [Mazure and Field 1980; Potin et al., 2002; Fernandes et al. 2012]. Gravot et al. [2010] pointed out that brown algae produce and secrete large amounts of mannitol, which has recently been shown to affect the formation of biofilms of marine bacteria such as *Pseudolatermonas spp*. and *Z. galactanivorans* [Salaün et al. 2012], a predominance of mesophilic bacteria in summer and more psychrophilic population in winter being observed in *L. saccharina*.

In our case, it was possible to isolate several microorganisms from the surface of *Macrocystis* which presented hydrolytic activity on laminarin, fucoidan and alginate. From these microorganisms, three microbial strains were selected, BAC1, HN and B, based on the production of CAE and morphological characteristics and preliminary identified within the genera *Bacillus sp*. (bacteria), *Alternaria sp.* (filamentous fungus) and *Rhodotorula sp*. (yeast), respectively.

The genus *Rhodotorula* has been described as capable of producing high concentrations of biomass in short periods of time with a high lipid yield [Almazan et al. 1981], especially *Rhodotorula mucilaginosa* that was isolated from the fish skin [Li et al. 2010; Saenge et al. 2011]. Moreover, this species is also known to produce pigments such as β-carotene, which has been demonstrated to possess anti-cancer and antioxidant properties [Pierre 1997; Laich et al. 2013]. The genus *Alternaria sp*. have been reported to play an important role on plant pathogenicity [Hong et al. 2005; Thomma 2003] and on the production of secondary metabolites with biological properties such as indole-diterpenoids, from marine *Alternaria tenuissima* [Sun et al. 2013]. Finally, marine *Bacillus* species had been used for cellulase production [Harshvardhan et al. 2013], polyhydroxybutyrate production (PHB) [Sathiyanarayanan et al. 2013], degradation of crude oil through biosurfactant production [Sakthipriya et al. 2015] and for acetoin production (natural flavor) [Dai et al. 2015]. This study present for the first time the production of CAE for degradation of brown seaweed by marine strains of *Rhodotorula sp., Alternaria sp.* and *Bacillus sp*.

The application of a design of experiment allowed the identification of the culture conditions that improved the production of CAE from *Rhodotorula sp.* B*, Alternaria sp.* HN and *Bacillus sp.* BAC, the conditions being 1 wt.% seaweed, 8 g/L YE at 25°C for 1-day growth for BAC and 1.5% seaweed, absence of YE at 25°C for 3-day growth for B and HN, obtaining under these conditions a production of CAE 84.6 ± 2.01, 105.2 ± 0.35 and 60.34 ± 4.97 U/mg for BAC, B and HN, respectively; this allowed a 30-, 29- and 31-fold increase, respectively, compared to the initial activity. It is worth noting that for the growth of the species *Alternaria sp*. and *Rhodotorula sp*. no external nitrogen source (YE) is required since *M. pyrifera* contains 10.34 wt.% protein.

The use of a variety of commercial enzymes to increase the extraction efficiency of bioactive compounds from brown algae had been used by other authors. Among the commercial enzymes used are cellulase for biofuel production, carbohydrase and proteases for extraction of carbohydrates, alcalase for the production of antioxidant extract and alginate lyase for alginate extraction [Ravanal et al., 2016; Park et al., 2004; Ahn et al.,2004; Heo et al., 2005; Siriwardhana et al., 2008; Rhein-Knudsen et al., 2015]. In particular production of carbohydrate active enzymes has been reported by other authors, e.g. production of

intracellular of fucoidanase from marine bacterium *Sphingomonas paucimobilis* PF-1, with 0.019 U/mg [Kim et al., 2015], production of extracellular alginate lyase from marine bacterium *Vibrio sp*. QY105, with 91 U/mg [Wang et al., 2013], extracellular 1,3-β-D-glucanase from *Streptomyces torulosus* PCPOK-0324, with 1.71 U/mg [Park et al., 2012], and finally, production of extracellular poly (β-D-mannuronate) lyase from *Azotobacter chroococcum* 4A1M, 1.93 U/mg [Haraguchi, and Kodama, 1996].

3.5 Conclusions

The chemical analysis of *M. pyrifera* indicated that the alga presented a protein and carbohydrate content of, 10.3 and 62.1 wt.%, respectively, with a high proportion of mannitol, alginic acid and fucose, 321.7 ± 3.5, 284.3 ± 24.9 and 17.3 ± 3.1 mg/g DS, respectively, between the carbohydrates and a high content of salts as potassium, sodium and calcium, 10, 4.5 and 1.8 wt.%, respectively. On the other hand, it was possible to isolate microorganisms that produce carbohydrate active enzymes using microbial populations associated at *Macrocystis.* Three microbial strains were selected and preliminary identified within genus *Bacillus sp, Rhodotorula sp.* and *Alternaria sp.* The application of an experimental design allowed the identification of the culture conditions that improved the production of carbohydrate active enzymes, being the culture conditions of *Bacillus sp*.: 1 wt.% seaweed, 8 g/L YE at 25°C for 1 day, and rendered 84.6 ± 2.01 U/mg product. In turn, for *Rhodotorula sp.* and *Alternaria sp*. the culture conditions were: 1.5 wt.% seaweed, in the absence of YE at 25°C for 3 days which resulted in a production of CAE of 60.34 ± 4.97and 105.2 ± 0.35 U/mg, respectively. The purification of CAE was performed, thus obtaining for *Bacillus sp*. BAC a purification with a 60.7-fold increase in the specific activity with a yield of 52%. For *Rhodotorula sp*. B, CAE purification was with a 28.1-fold increase in the specific activity with a yield of 59%. Finally, CAE of *Alternaria sp*. HN was purified with a 22.7-fold increase in the specific activity with a yield of 19%. Further work includes the characterization process of carbohydrate active enzymes produced by each strain.

Chapter 4

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 as well as, more recently, of polyphenol compounds like 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 NaOH 0.5 N 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 hours 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 hours and a S/L ratio of 1/20 thus giving a yield (EY) of 2.14±0.25 wt.%. On the other hand, the statistical analysis of both processes revealed that a maximum EY of 91.24 wt.% for carbohydrates and 3.31 wt.% EY for phlorotannins.

***Keywords**:* brown seaweed; extraction; experimental design; Taguchi design; Box-Behnken design.

4.1 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 allowing 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 fast growth rate of algae relative to any terrestrial plants and not need of requirements extra of nutrients, sweet 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, *Macrocystis 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 in seaweed 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]. On the other hand, the flora of polysaccharides found in brown seaweed mainly consists 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 the bioactive compounds, the cell wall must be disrupted by means of a pretreatment. The open literature describes that the 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 to obtain the maximum recovery of the desired compounds [Wijesinghe and Jeon, 2012].

The phenolic compounds, known as phlorotannins, are produced as secondary metabolism products 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. 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 Macrocystis 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.

4.2 Materials and Methods

4.2.1 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. The 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, carbohydrates 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): 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, ashes, lipids and fibers, respectively [Merril, 1973].

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

In our previous study, was determined that washed the algae with hexane followed by extraction with water improvement the extraction of phlorotannins [Leyton *et al.*. 2016]. Considerate these parameters, the influence of the pH in the extraction of carbohydrate and phlorotannins was evaluated. For which 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) [Briceño-Dominguez *et al*., 2014; Gomez-Ordoñez *et al*., 2014; Pham *et al*., 2013]. All of the above-mentioned procedures were tested in 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.

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

For optimize of carbohydrate and phlorotannins extraction were evaluated 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 Section 4.2.2). All these variables were simultaneously evaluated using the Box-Behnken design [Tahmouzi and Ghodsi, 2014] according to Table 4.1. At the end of the incubation time of each every experimental design run, the samples were centrifuged at 3,600 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:

$$Response = \beta_0+\beta_1*A+\beta_2*B+\beta_3*C+\beta_{11}*A^2+\beta_{12}*A*B+\beta_{13}*A*C+\beta_{22}*B^2-+\beta_{23}*B*C+\beta_{33}*C^2 \quad \textbf{(Eq.1)}$$

Where *response* represented the dependent variables of the yields obtained from the carbohydrate and phlorotannin extraction; A, B and C represented the independent variables: time, temperature and S/L ratio, respectively. B_0 was a constant coefficient in the model; β_1, β_2 and β_3 were the coefficients of the linear equation; β_{11}, β_{22} and β_{33} were 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].

4.2.4. 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 pre-cultured in 250 mL Erlenmeyer flasks with 100 mL fermentation broth: 70 vol.% artificial seawater (ASW, 27.5 g/L NaCl; 5.38 g/L $MgCl_2 \cdot 6H_2O$; 6.78 g/L $MgSO_4 \cdot 7H_2O$; 0.72 g/L KCl; 0.2 g/L $NaHCO_3$ and 1.6 g/L $CaCl_2$)

[Shene et al. 2013], 4 g/L yeast extract and 1 wt.% dry *M. pyrifera*, by 3 days at 25°C under continuous shaking at 200 rpm. Posteriorly, 5 mL of pre-cultured 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 8,500 g at 4°C for 5 min and the phase liquid (enzymatic extract) was collected for used. The activity of the enzyme extract was of 60.34±4.97 U/mg, where one unit of enzyme activity, U, was defined as the amount of enzyme that catalyzed the liberation of one micromole 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 4.2 [Taguchi, 1990]. Upon completion of the incubation time of each and every experimental design run, the mixture was centrifuged at 3,000 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 Section 4.2.3. The optimized conditions of pretreatment of the algae were determined based on the liquid phase obtained after the extraction process, and evaluated in terms of the extraction yield of phlorotannins and carbohydrates.

4.2.5. Determination of the concentration of the phlorotannins (TPC)

The amount of total polyphenol 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 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 grams of phloroglucinol per gram of dry seaweed, ie. phlorotannin extraction yield (wt.%).

4.2.6. 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 equlibriated 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) was prepared and the results were expressed as grams of glucose per gram of total carbohydrate present in the algae, i.e. the carbohydrate extraction yield (wt.%).

4.2.7. 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]. Consequently, 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) were prepared and the results were expressed as mg of the equivalent Trolox by grams of dry seaweed (mg TE/g DS).

4.2.8 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].

4.2.9. Determination of the phlorotannins 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]. 20 µL of sample was analysed using a LC-ESI-MS/MS system which consisted of the HP1100 liquid chromatography (Algilent Technologies Inc., CA-USA) connected to the mass spectrophotometer (Esquire 4000 ESI-Ion Trap LC/MS(n) system, Bruker Daltonik GmbH, Germany). A Luna C18 150 x 4.6 mm, 5 µm and 100 Å analytical column (Phenomenex Inc., CA-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 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 [40]. 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 nebulising gas at 45 psi, drying gas at 345°C and flow rate 10 L/min. All scans were performed in the range 20-2200 m/z. The trap parameters were set in ion charge control using 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.

4.2.10. Statistical analysis

All the extracts were analyzed in triplicate. The measurements were 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.

4.3. Results and Discussion

4.3.1 Characterization of Macrocystis pyrifera

The characterization of the alga was carried out to determine the instrinsic extraction potential. The chemical analyses of *M. pyrifera* (Figure 4.1a) 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 (Figure 4.1b). According to the literature [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 its correlates with a high phlorotannin concentration in the brown alga.

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.

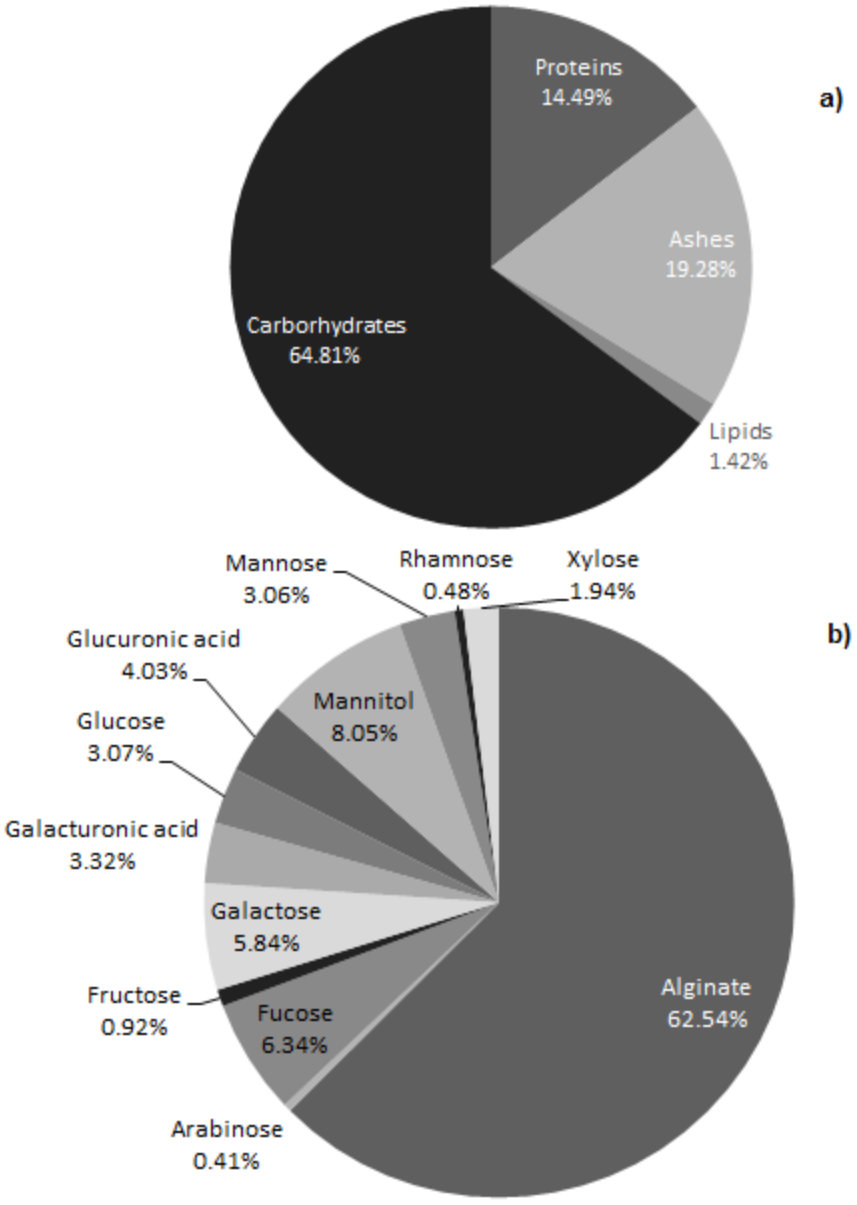

Fig 4.1. Composition of *Macrocystis pyrifera* harvested in May 2013. **a)** Chemical composition and **b)** Type of carbohydrates.

The variability of the chemical composition of *Macrocystis pyrifera* in Southern Chile was observed by Westermeier *et al*., 2012. The protein levels observed in the cultured *Macrocystis* varied in a range from 12% DW (January–February) and 15% DW (March–June). Naturally, 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].

4.3.2. 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. 4.2). 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. 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]. Consequently, since alginate is the most prominent carbohydrate present in *M. pyrifera* where phlorotannins compounds found linkage with the carbohydrate. Therefore, its hope a high EY of carbohydrate is directly relate with a high EY of phlorotannins.

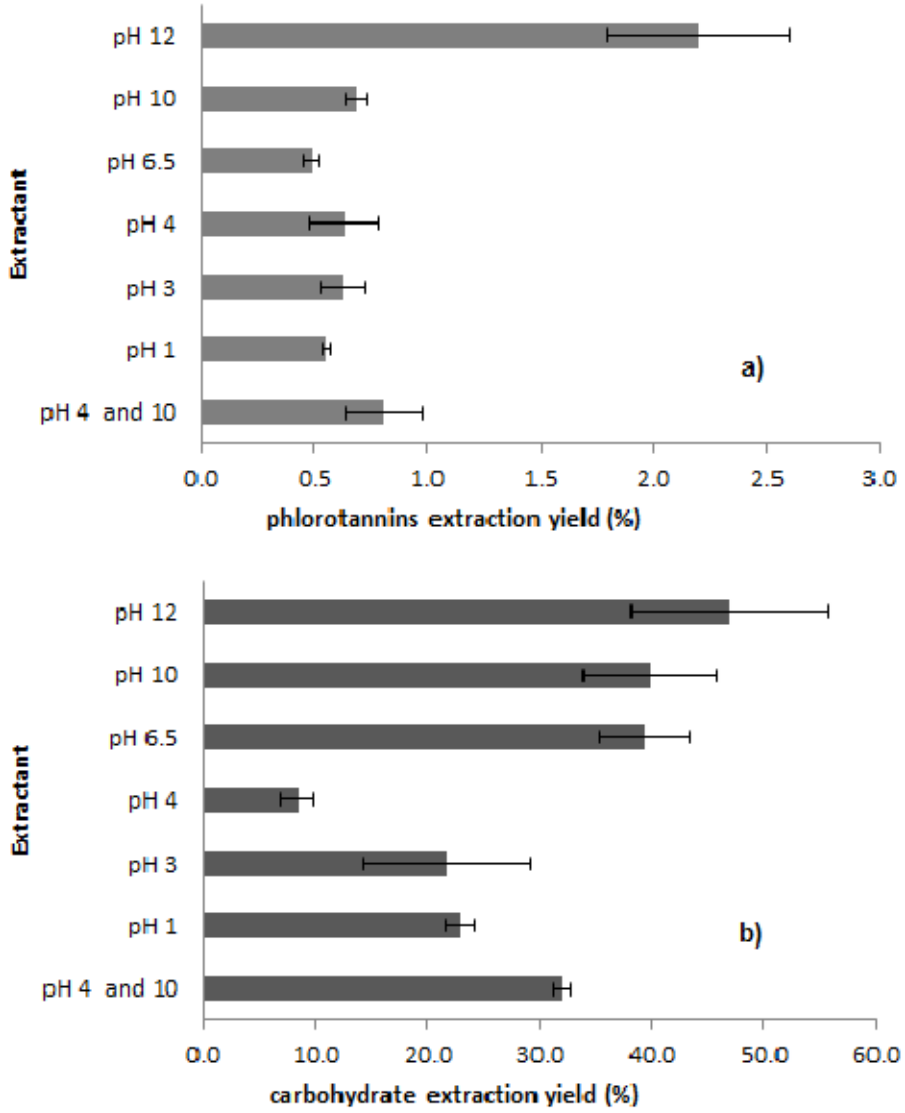

Fig. 4.2. Extraction yields of a) phlorotannins and b) carbohydrates from *Macrocystis pyrifera* using different pH of extraction.

4.3.3. 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 4.1). The condition that allowed the EY of phlorotannins to

be optimized were 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 4.1). 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.

Table 4.1. 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.

Runs	Time (min)	Temperature (°C)	S/L Ratio	CEY[b] experimental [%]	CEY[b] predicted [%]	PEY[c] experimental [%]	PEY[c] 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} [d]	88.97	R^{2}[d]	96.08

[a]S/L, solid/liquid ratio of algae/extractant
[b]CEY, carbohydrate extraction yield
[c]PEY, phlorotannin extraction yield
[d]R^2, correlation coefficient

The antioxidant activity of phlorotannins obtained under conditions of runs 2 and 4 of Table 4.1 were 227.15±3.14 and 138.61± 6.71 mg TE/g DS, respectively (Fig. 4.3). 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 4.1).

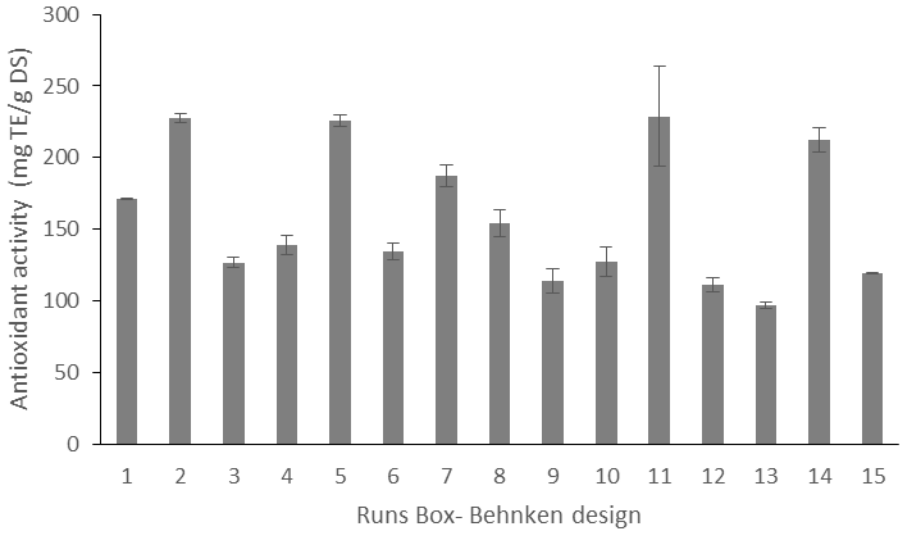

Fig 4.3. Antioxidant activity in the liquid phase obtained from each experimental condition of the Box-Behnken design. See Table 4.2 for further details with respect to each run.

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

$$EY\text{-}of\text{-}carbohydrates = -270.6 + 0.13*A + 4.51*B + 7.77*C - 0.00001*A^2 - 0.001*A*B + 0.01*A*C - 0.02*B^2 - 0.02*A*C - 0.19*C^2 \quad \textbf{(Eq.2)}$$

$$EY\text{-}of\text{-}phlorotannins = 0.03 + 0.03*A - 0.003*B - 0.13*C - 0.0001*A^2 - 0.0003*A*B + 0.001*A*C + 0.0003*B^2 - 0.001*B*C + 0.004*C^2 \quad \textbf{(Eq.3)}$$

In case of both equations (Eq. 2 and 3), it was possible to determine the predicted value for each experimental run (Table 4.1, columns 6 and 8). These predicted values

presented a correlation coefficient of 96% in case phlorotannins and 89% in case of carbohydrates, respectively. According to the statistical analysis (Table 4.3) 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 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. 4.4 and 4.5, 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.

The alkaline extraction of polysaccharide from seaweed has been reported. The EY obtained from *Salicornia brachiata* and *M. pyrifera* were of 58.1 and 33 wt.%, respectively [Sanandiya and Siddhanta, 2014; Gomez *et al*., 2009]. For 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. Being, these results lower than those obtained in this work, which could are relate with the lower temperature of extraction used, 75 °C in comparison with 100°C used for own. Same effect of temperature and type of solvent was observed in the polyphenol compound extraction from *Fucus vesiculosus* with ethanol (95 vol.%) with an EY of 0.10 wt.% [Peinado *et al*. 2014].

Table 4.3. Analysis of variance (ANOVA) for extraction process of *Macrocystis pyrifera* with carbohydrolase enzyme and subsequent extraction process of carbohydrates and phlorotannins

Extraction process							
Response	**Factors**	**DOF[c]**	**SS[d]**	**Variance**	**F-value**	**p-Value**	**-**
Phlorotannin	A:Time	1	0.076	0.076	3.57	0.1173	-
EY[a]	B:Temperature	1	1.684	1.684	79.14	**0.0003**	-
(%)	C:S/L[b] 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	A:Time	1	597.715	597.715	7.8	**0.0383**	-
EY[a]	B:Temperature	1	791.622	791.622	10.33	**0.0236**	-
(%)	C:S/L[b] ratio	1	165.347	165.347	2.16	0.2018	-
	A^2	1	0.007	0.007	0.0	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				

[a]EY, extraction yield
[b]S/L, solid/liquid ratio of algae/enzyme extract or alkaline solvent
[c]DOF, degree of freedom
[d]SS, Sums of squares

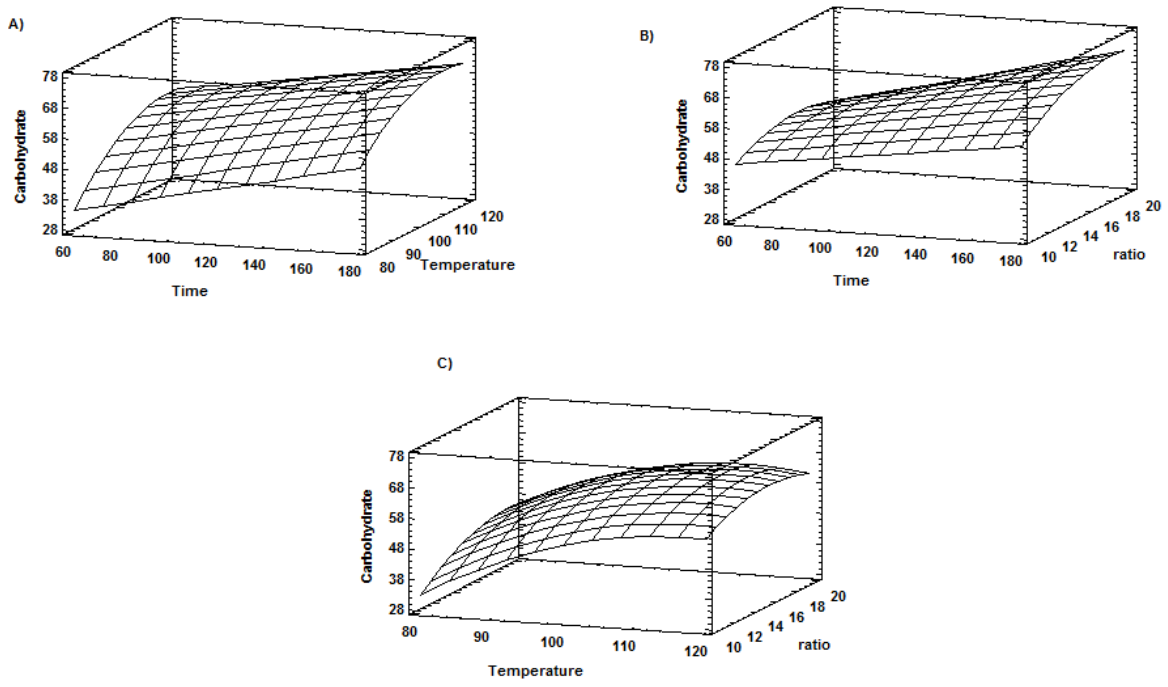

Fig. 4.4. Response surface (3-D) plots showing the effects of A) extraction time and temperature, B) extraction time and the alkaline solvent to raw material ratio, and C) extraction temperature and alkaline solvent-raw material ratio on the extraction yield of carbohydrates.

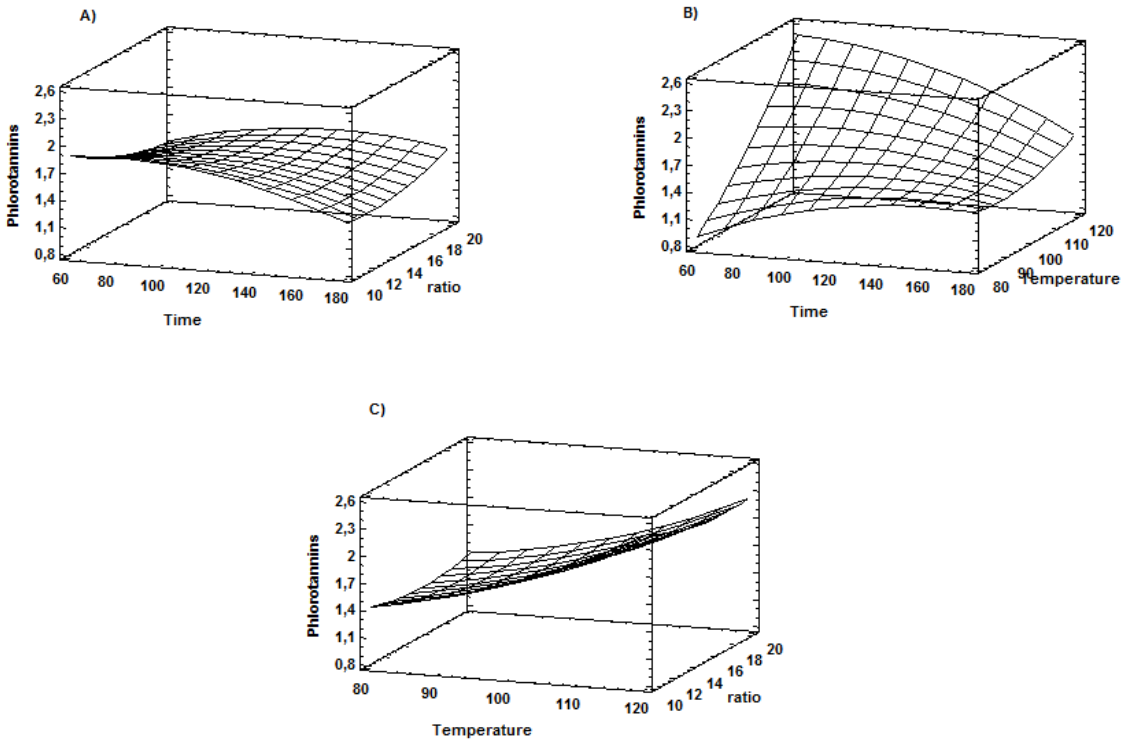

Fig 4.5. Response surface (3-D) plots showing the effects of A) extraction time and temperature, B) extraction time and the alkaline solvent to raw material ratio, and C) extraction temperature and the alkaline solvent to raw material ratio on the extraction yield of phlorotannins.

4.3.4. 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 NaOH 0.5 N 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 4.2, whereupon the incubation pretreatment was carried out for 24 hours 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 hours of incubation at 25°C, pH 7.0 and S/L ratio of 1/20, run 7 in Table 4.2, thus yielding an EY of 2.14±0.25 wt.% for the phlorotannins.

Table 4.2. Influence of the process parameters in the pretreatment of *Macrocystis pyrifera* using the Taguchi experimental design on carbohydrate and phlorotannin extraction yields.

Runs	Time (h)	Temperature (°C)	pH buffer	S/L[a] ratio	Carbohydrate EY[b] (%)	Phlorotannin EY[b] (%)
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

[a]S/L, solid/liquid ratio of algae/enzyme extract
[b]EY, extraction yield

The statistical analysis allowed determining 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 4.4). 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 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.

Table 4.4. Analysis of variance (ANOVA) for the pretreatment of *Macrocystis pyrifera* with carbohydrolase enzyme and subsequent extraction process of carbohydrates and phlorotannins.

Enzymatic pretreatment							
Response	**Factors**	**DOF[c]**	**SS[d]**	**Variance**	**F-value**	**Pure sum**	**%**
Phlorotannin	Time	2	1.07	0.54	32.17	1.04	46.0
EY[a]	Temperature	2	0.21	0.1	6.19	0.17	7.6
[%]	pH	2	0.37	0.19	11.16	0.34	15.0
	S/L[b] 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	Time	2	381.99	191	5.97	317.96	10.9
EY[a]	Temperature	2	11.84	5.92	0.18	-52.19	0.0
[%]	pH	2	911.32	455.66	14.23	847.28	29.1
	S/L[b] 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

[a]EY, extraction yield
[b]S/L, solid/liquid ratio of algae/enzyme extract or alkaline solvent
[c]DOF, degree of freedom
[d]SS, Sums of squares

Thus, an EY increase of 16% in the case of the carbohydrates and 33% in the case of the 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 type of work, albeit with a 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 a 12 wt.% for carbohydrates from *Sargassum ssp*., respectively. Olivares-Molina and Fernández [2016] realized a study comparative of carbohydrate and phlorotannins extraction from brown seaweed by enzymatic and conventional method of extraction (maceration), obtained high yield extraction with enzymatic extraction, 37.7 and for carbohydrate and phlorotannins respectively, with a better

activity biochemistry in natural inhibitors of the angiotensin I-converting enzyme. These results emphasize the use of enzyme for the extraction of biocompunds protect the bioactivity of the compounds.

4.3.5. 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 an 2.72%, glucose 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 isomers: difucophloroethol, fucodiphloroethol, tetrafucol or tetraphloroethol. These compounds has been described with propierties free radical scavenging ability and antiallergic effect for tetramer of phloroglucinol [Li et al., 2008 and 2009], and antidiabetic effect and prevents Alzheimer's disease for phloroeckol [Le et al., 2009; Yoon et al., 2009].

4.4 Conclusions

In order to increase the extraction yields of carbohydrates and phlorotannins from *Macrocystis pyrifera*, a combined enzymatic pre-treatment followed by an alkaline extraction process were evaluated. Combining both processes allowed for an increase in the extraction efficiency of phlorotannins from 1.6 to 2.1 wt.%, and in the case of the carbohydrates from 77.6 to 89.7 wt.%. Future work includes isolation process of the phlorotannins fraction and the use of extracted carbohydrates and phlorotannins in the biochemical and nutraceutical platforms, respectively.

Chapter 5

Purification of phlorotannins from *Macrocystis pyrifera* using macroporous resins.

Abstract

The phlorotannins are secondary metabolites produced by brown seaweed, which are known for their nutraceutical and pharmacological properties. The aim of this work was to determine the type of macroporous resin and the conditions of operation that improve the purification of phlorotannins extracted from brown seaweed *Macrocystis pyrifera*. For the purification of phlorotannins, six resins (HP-20, SP-850, XAD-7, XAD-16N, XAD-4 and XAD-2) were assessed. The kinetic adsorption allowed determine average adsorption time for the resins of 9 hours, the high level purification of phlorotannins was obtained with XAD-16N, 42%, with an adsorption capacity of 183 ± 18 mg PGE/g resin, desorption ratio of 38.2 ± 7.7%. According the adsorption isotherm the best temperature of operation was 25°C, being the model that better described the adsorption properties was the Freundlich model. The purification of phlorotannins might expand their use as a bioactive substance in the food, nutraceutical and pharmaceutical industries.

Keywords: Separation, bioactive compounds, brown seaweed.

5.1 Introduction

In recent decades, the chemistry of natural products of marine origin has been the subject of intense research aiming to find new compounds with pharmacological and nutraceutical properties. Phlorotannins, present only in brown seaweed, have been recognized as highly bioactive. These are polyphenolic compounds formed by oligomers and polymers of the monomer phloroglucinol [Shibata et al., 2004]. These compounds have been extensively researched for their antioxidant, antiangiogenic, antiallergic, anti-inflammatory, and antidiabetic properties [Kim and Himaya, 2011; Zubia, et al. 2008; Zhao et al., 2008; Li et al. 2011; Gupta and Abu-Ghannam, 2011].

The extraction of phlorotannins from brown seaweed and their characterization have been reported previously [Ortiz et al., 2006; Sanchez-Machado et al., 2004; Glombitza and Pauli, 2003; Tello-Ireland et al., 2011; Li et al., 2006; Gupta et al., 2011; Koivikko et al., 2007; Leyton et al., 2016a], although their purification from crude extracts has not been widely studied yet. Several techniques have been applied to purify phlorotannins, such as chromatography using a Sephadex LH-20 column [Kantz and Singleton, 1990; Nwosu et al., 2011; Koivikko et al., 2007], ultrafiltration using membranes with cut-off between 100 and 5 KDa [Wang et al., 2012] and liquid‑liquid fractionation with ethyl acetate [Cho et al., 2012; Kang et al., 2012; Shibata et al., 2004]. The main drawbacks of these techniques are the use of non food-grade solvents, such as acetone, methanol and ethyl acetate, the low selectivity due to the presence of other co-extracted compounds, the precipitation of polyphenol–protein complexes [Siebert, 1999] and the high cost of these processes [Wang et al., 2012].

An alternative that overcomes most of the limitations of these techniques is macroporous resins separation [Kim et al., 2014]. In this method, polyphenols in aqueous solutions are adsorbed on the resins due to hydrophobic binding and aromatic stacking. The adsorbed polyphenols are later desorbed using a mixture of water with an organic solvent, such as ethanol (food-grade); sugars present in the crude extract do not interact with the resins, hence, they are easily removed with water. In addition, these resins are approved by the Food and Drug Administration to produce food products [Scordino et al., 2003].

To design the process adequately, the mechanisms of adsorption and desorption of the macroporous resin should be understood. Therefore, adsorption isotherms must be

determined and fitted using isotherm models such as Langmuir or Freundlich, from where several thermodynamic parameters, such as isosteric heat, entropy and free energy, can be derived [Allen et al., 2004; Sohn and Kim, 2005]. In addition, kinetic data can be fitted to pseudo-first-order and pseudo-second-order kinetic to determine the adsorption efficiency [Ho and Mckay, 1998].

Therefore, the aim of this work was to determine the type of macroporous resin and the operating conditions that improve the purification of phlorotannins extracted from brown seaweed *Macrocystis pyrifera*. Therefore, adsorption kinetic, static adsorption and desorption, adsorption isotherm and thermodynamic adsorption parameters of phlorotannins will be evaluated.

5.2 Materials and methods

5.2.1 Preparation of phlorotannin extract

The brown seaweed employed was *Macrocystis pyrifera* collected from Chiloe, 30 km southeast of Puerto Montt, Chile, in June 2013, donated by Prof. A. Buschmann from Universidad de Los Lagos. The alga was dried at 40°C and ground size < 0.5 mm prior to the extraction. The extraction condition was optimized in our previous work [Leyton et al., 2016b]: solution 0.5 M of NaOH, solid/liquid ratio of 1/20 at 100°C for 3 h. Finally, the mixer was filtered using Whatmann N°1 paper, and the liquid phase, phlorotannin extract, was stored at 4°C until use. The phlorotannins' concentration present in the extract was of 1,800 mg phloroglucinol equivalent (PGE)/L.

5.2.2 Adsorbents

Six macroporous resins were tested to choose the one with the best adsorption/desorption behavior: Diaion HP-20, Sepabeads SP-850, Amberlite XAD-7, XAD-16N, XAD-4 and XAD-2 (from Sigma-Aldrich). Their physicochemical characteristics are shown in Table 1. Before the experiments, all resins were washed with ethanol 70% at 25 °C for 12 h. All experiments were performed in duplicate.

Table 5.1. Physicochemical properties of six resins evaluated.

Resins	**HP-20**	**SP-850**	**XAD-7**	**XAD-16N**	**XAD-4**	**XAD-2**
Structure	SDVB[a]	SDVB[a]	Acrylic ester	SDVB[a]	SDVB[a]	SDVB[a]
Porosity (mL/g)	1.3	1.2	1.14	0.55	1.0	0.65
Surface area (m^2/g)	600	930	450	800	725	330
Pore radius (A)	260	38	90	200	20	90
Particle size (mm)	0.25-0.60	0.30-0.80	0.25-0.84	0.56-0.71	0.4-0.6	0.25-0.84

[a]SDVB, styrene divinyl-benzene (Information obtained from provider)

5.2.3 Adsorption kinetics of phlorotannins

The adsorption kinetics' determination was carried out as follows: 0.2 g of each resin was mixed with 30 mL of phlorotannins extract under continuous agitation (300 rpm) at 25

°C, taking 1 mL of solution at different time intervals to determine the concentration of phlorotannins in the aqueous solution. The adsorption kinetics allowed us to determine the time of adsorption equilibrium. The adsorption capacity of phlorotannins on the resins at a given contact time was calculated with the following equation:

$$q_t = (C_0 - C_t) \times \frac{V_i}{W} \qquad (Eq.1)$$

Where q_t is the adsorption capacity at the given contact time t (mg PGE/g dry resin), C_t is the concentrations of phlorotannins in the solution at the given contact time (mg PGE/mL), C_0 is the initial concentrations (mg PGE/mL) of phlorotannins in the solution, V_i is the volume of the initial sample solution (mL), and W is the resin weight (g). Each experiment was carried out in duplicate.

To determine the adsorption efficiency of different resins, the kinetic models of pseudo-first-order (Eq 2) and pseudo-second-order (Eq 3) were adopted [Ho and McKay, 1999; Simonin, 2016; Ho, 1996]:

Pseudo first order:

$$Lg(q_e - q_t) = \frac{K_1}{2.303} t + Lg\ q_e \qquad (Eq.2)$$

Pseudo second order:

$$\frac{t}{q_t} = \frac{1}{q_e}\ t + \ \frac{1}{K_2 {q_e}^2} \qquad (Eq.3)$$

Where K_1 and K_2 are the constant rate of the pseudo-first-order-model and pseudo-second-order-model, respectively, q_e is the adsorption capacity at the adsorption equilibrium (mg PGE/g dry resin) and t is time.

5.2.4 Adsorption of phlorotannins on the resins

In order to determine the best macroporous resin for the adsorption of phlorotannins, a static adsorption was performed, where 2 g of each resin was put into a tube with 30 mL of

phlorotannin extract. The tube was shaken using a shaking incubator, 300 rpm, at 25°C to reach adsorption equilibrium. After adsorption, the resins were filtered for the subsequent desorption of phlorotannins, and the concentration of phlorotannins in the extract was measured. The adsorption capacity was calculated according to the following equation:

$$q_e = \frac{(C_0 - C_e)V_i}{W} \qquad (Eq.\ 4)$$

Where q_e is the adsorption capacity at the adsorption equilibrium (mg PGE/g dry resin), and C_e is the equilibrium concentration (mg PGE/mL) of phlorotannins in the solution. C_0, V_i and W are the same as defined above.

5.2.5 Desorption of phlorotannins from the resins

In order to determine the best macroporous resin for desorption of phlorotannins, a static desorption was performed. A first step was to determine the best ethanol concentration for desorption of phlorotannins, and different concentrations of ethanol (10, 20, 40, 60, 80, 90 and 100% v/v) were screened in XAD-7HP. Once the best ethanol concentration was determined, 30 mL of solvent was added in the different resins, and shaken at 300 rpm and 25°C to reach desorption equilibrium. After desorption, the resins were filtrated and the concentration of phlorotannins in the extract was measured. Desorption ratio, capacity and level purification were calculated according to the following equations:

Desorption ratio

$$D = \frac{C_d V_d}{(C_0 - C_e)V_i} * 100 \qquad (Eq.\ 5)$$

Desorption capacity

$$q_d = C_d \times \frac{V_d}{W} \qquad (Eq. 6)$$

Level purification

$$P = \frac{q_e}{q_d} * 100 \qquad (Eq.7)$$

Where D is the desorption ratio (%), C_d is the concentration of phlorotannins in the desorption solution (mg PGE/mL), and V_d is the volume of the desorption solution (mL). C_0, C_e and W are the same as defined above, q_d is the desorption capacity of phlorotannins (mg PGE/g) and P is the level of purification. Each experiment was carried out in duplicate.

5.2.6 Adsorption isotherms of phlorotannins

In order to determine the optimum temperature for the adsorption of phlorotannins, three adsorption isotherms were determined, for which 30 mL of extract of phlorotannins at different initial concentrations were mixed with 2 g of resin and shaken at 300 rpm at different temperatures, specifically 25, 35 and 45°C, to reach adsorption equilibrium. After the adsorption, the concentration of phlorotannins in the extract was measured. Each experiment was carried out in duplicate.

In order to select a suitable model for describing the adsorption properties of the different resins, the Langmuir and Freundlich equations were tested:

Langmuir:

$$q_e = \frac{K_L C_e q_m}{1 + K_L C_e} \qquad (Eq.8)$$

Freundlich:

$$q_e = K_F C_e^{\frac{1}{n}} \qquad (Eq.9)$$

Where q_m is the maximum adsorption capacity of the adsorbent (mg/g dry resin), K_L is the parameter related to the adsorption energy (L/mg), K_F reflects the adsorption capacity of an adsorbent (mg/g· (L/mg)1/n), and the parameter n represents the affinity of the adsorbent for a given adsorbate.

5.2.7 Thermodynamic parameters

In order to determine the thermodynamic behavior of the adsorption on the resins, the changes in thermodynamic parameters, such as enthalpy (*ΔH*), entropy (*ΔS*), and free energy (*ΔG*) were determined. The change of *ΔH* and *ΔS* can be obtained from the slope and intercept of the plot of the natural logarithm of the constant of adsorption equilibrium (K_{eq}) and 1/absolute temperature (1/T). *ΔG* was determined using the following equations:

$$\Delta G = -RTlnK_L \qquad (Eq.10)$$

$$lnK_L = -\frac{\Delta G}{RT} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \qquad (Eq.11)$$

Where *R* is the gas constant (J/mol K) and *T* is absolute temperature (°K)

5.2.8 Determining the phlorotannin content

The concentration of phlorotannins in the extracts was determined according to the Folin–Ciocalteu assay [Singleton and Rossi 1965] adapted to 96-well plates, as well as standards containing phloroglucinol with concentrations varying from 20 to 100 mg/L. Samples and standards (20 µl) were introduced separately into 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 a UV–Visible spectrophotometer. The phlorotannin concentration was determined by the regression equation of the calibration curve and expressed as mg of phloroglucinol equivalent (mg PGE/mL). Each analysis was carried out in triplicate. The measurements were presented as average ± standard deviation.

5.2.9 Statistical analysis

Each experiment was carried out in triplicate. The measurements were presented as average ± standard deviation. The significance and relative influence of each resins in the static adsorption and desorption of phlorotannins were determined using the variance analysis (ANOVA). The significance of the factors was determined at 5% confidence level.

5.3 Results

5.3.1 The adsorption kinetics of phlorotannins

The curves of the adsorption kinetics for each resin are presented in Figure 5.1. In this figure a gradual increase in the adsorption of phlorotannins with time was observed, where all resins reached adsorption equilibrium approximately after 8 and 9 h of contact time.

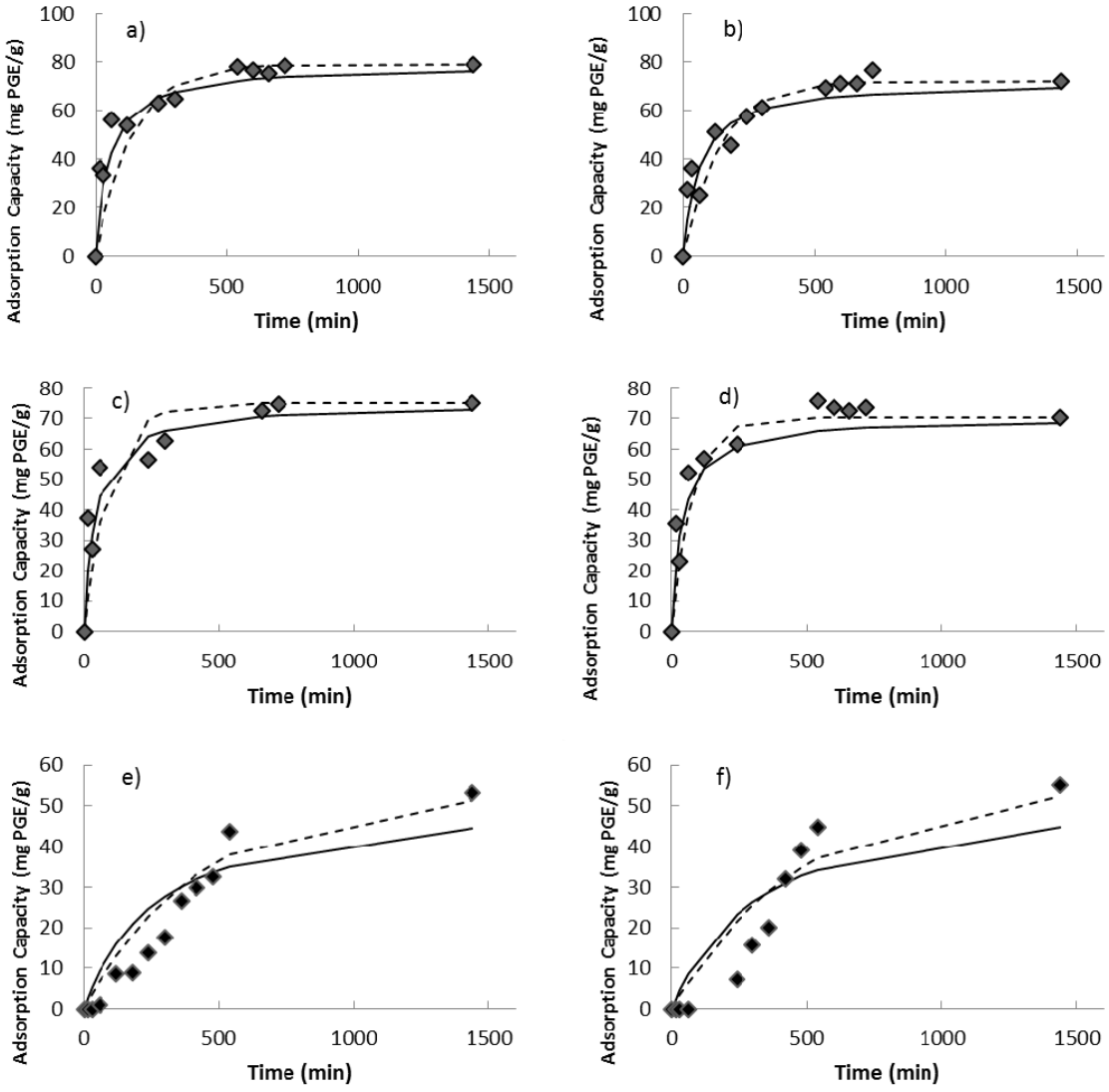

Figure 5.1. Adsorption kinetics of phlorotannins on: a) XAD-7, b) XAD-16N, c) SP-850, d) HP-20, e) XAD-4 and f) XAD-2 at 25°C. The continuous line is for pseudo-first-order and the segmented line for pseudo-second-order.

Kinetic data was fitted to pseudo-first-order and pseudo-second-order models as shown in Table 5.2. According to the correlation coefficient, the pseudo-first-order model describes the adsorption kinetics of phlorotannins best (column 8, Table 5.2).

Table 5.2. Kinetic parameters of phlorotannin adsorption on XAD-7, XAD-16N, HP-20, XAD-4, XAD-2 and SP-850 at 25°C. q_e, q_1 and q_2 are the experimental and theoretical adsorption capacity of each model, respectively.

		Pseudo first order model			Pseudo second order model		
Resin	**q_e**	**K_1**	**q_1**	**R^2**	**K_2**	**q_2**	**R^2**
HP-20	70.3	0.03	70.3	0.88	3.9E-04	68.6	0.87
XAD-7	79.0	0.02	79.0	0.92	2.5E-04	76.4	0.91
XAD-16N	72.2	0.02	72.2	0.89	2.4E-04	69.4	0.84
SP-850	75.1	0.03	75.1	0.83	3.3E-04	73.0	0.85
XAD-4	53.1	0.01	51.2	0.95	6.8E-05	44.5	0.90
XAD-2	55.1	0.01	52.4	0.90	5.5E-05	44.9	0.85

5.3.2 The static adsorption and desorption of phlorotannins

The adsorption and desorption capacity of phlorotannins obtained for the resins tested are shown in Figure 5.2. The highest adsorption capacity was obtained by HP-20 followed closely by XAD-16N and SP-850 with 190 ± 10, 183 ± 18 and 183 ± 31 mg PGE/g, respectively.

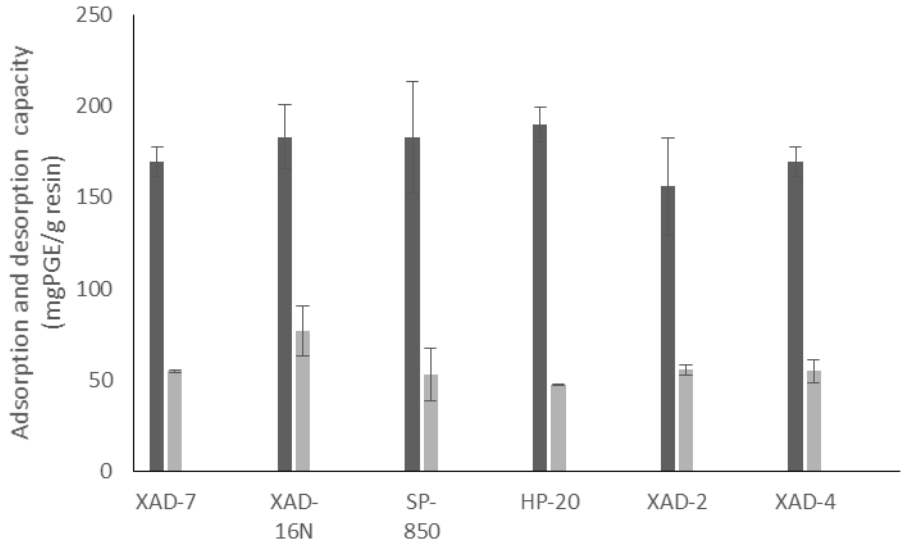

Figure 5.2. The static adsorption (dark column) and desorption (light column) capacities of different resins at 25°C.

The lowest adsorption capacity was obtained with XAD-2, (156 ± 27 mg PGE/g). The best desorption of phlorotannins was achieved (data not shown) with ethanol at 90% v/v; hence, this concentration was employed for the desorption experiments. The desorption ratios obtained for HP-20, XAD-16N and SP-850 were 32.8 ± 2.1, 38.2 ± 7.7 and 27.7 ± 4.6%, respectively, with a desorption capacity of 47.3 ± 0.2, 77 ± 13 and 53 ± 14 mg PGE/g, respectively. The purification levels reached with the resins were 24.9% for HP-20, 42.0% for XAD-16N and 29.0% for SP-850. The statistical analysis (ANOVA) showed that the resins do not present a significant effect ($p<0.05$) on the adsorption and desorption capacity (Table 5.3), therefore the criteria for selecting the best resin was the level of purification being XAD-16N with high value of purification an 42%.

Table 5.3. Analysis of variance (ANOVA) for static adsorption and desorption capacity of phlorotannins.

Adsorption capacity					
Factors	**DOF[a]**	**Sums of Squares**	**Variance**	**F-ratio**	**P- value**
Resins	5	1552	310	0.85	0.56
Others	6	2181	363		
Total	11	3733			
Desorption capacity					
Factors	**DOF[a]**	**Sums of Squares**	**Variance**	**F-ratio**	**P- value**
Resins	5	1031	206	2.85	0.12
Others	6	435	724		
Total	11	1465			

[a]DOF, degree of freedom

5.3.3 The phlorotannin adsorption isotherm

The phlorotannin adsorption isotherm for different resins at 25, 35 and 45°C are shown in Figure 5.3.

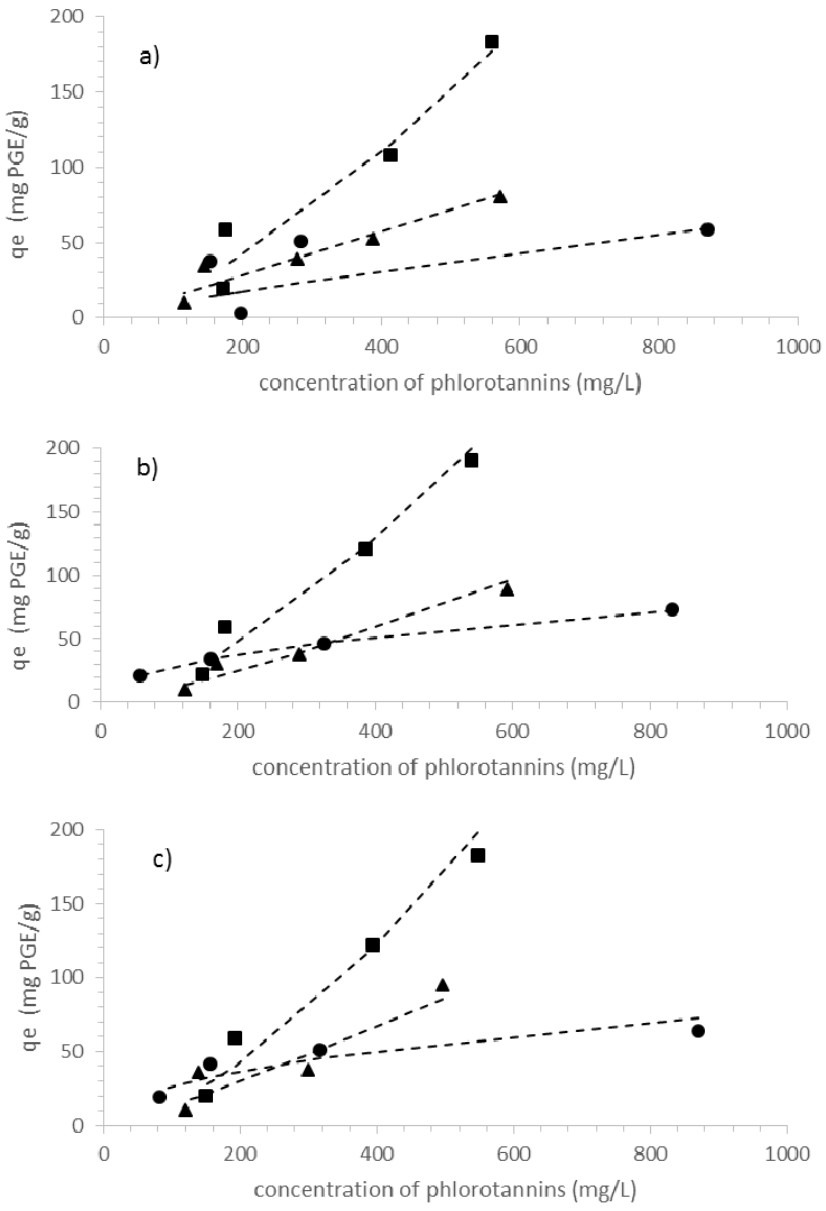

Figure 5.3. Equilibrium adsorption isotherm of phlorotannin adsorption on: a) XAD-16N, b) HP-20 and c) SP-850 at 25°C (square symbol), 35°C (triangle symbol) and 45°C (circle symbol). The segmented line is for the Freundlich model. q_e is the adsorption capacity of phlorotannins.

The phlorotannin data for adsorption was fited to the Langmuir and Freundlich isotherm equations; the equation constants and correlation coefficients obtained for each models are listed in Table 5.4. According to the correlation coefficient obtained by each

model and adjusting curves in the chart, the model that better described the adsorption properties was the Freundlich model; the correlation coefficients obtained were 0.80 for XAD-16N, 0.90 for HP-20 and 0.91 for SP-850. The best temperature for adsorption of phlorotannins by different resins was 25°C.

Table 5.4. Isotherm parameters of phlorotannin adsorption on XAD-7, XAD-16N, HP-20, XAD-4, XAD-2 and SP-850 at 25, 35 and 45°C.

		Langmuir equation			**Freundlich equation**		
Resin	**Temperature (°C)**	**K_L**	**q_m**	**R^2**	**K_F**	**n**	**R^2**
XAD-16N	25	1.2E-03	117	0.59	0.02	0.70	0.80
	35	9.4E-04	110	0.71	0.13	0.98	0.79
	45	5.9E-04	79	0.10	0.20	1.18	0.21
HP-20	25	1.5E-03	98	0.79	0.02	0.68	0.90
	35	1.5E-03	51	0.80	0.02	0.74	0.89
	45	7.7E-03	69	0.96	3.59	2.25	1.00
SP-850	25	1.6E-03	77	0.81	0.01	0.65	0.91
	35	1.1E-03	96	0.60	0.06	0.86	0.72
	45	2.8E-03	111	0.94	3.05	2.13	0.84

5.3.4 Thermodynamic parameters

The thermodynamic parameters were calculated using the constant of adsorption equilibrium of Freundlich model (K_F) for each resin, the value of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) are presented in Table 5.5. Adsorption with XAD-16N, HP-20 and SP-850 show positive enthalpy values of 87, 200 and 215 kJ/mol, respectively, which suggests an endothermic adsorption process [Özcan et al., 2005; Montgomery, 1985]. The free energy of all resins was negative, which suggests spontaneous and physical adsorption [Han et al., 2009; Karakaya, 2011]. The entropy values for XAD-16N, HP-20 and SP-850 were positive at 262, 632 and 683 KJ/moLK respectively, which suggests increased

randomness at the solid/solution interface during the adsorption of phlorotannins [Özcan et al., 2005].

Table 5.5. Thermodynamic parameters for phlorotannin adsorption on different resins.

Resins	T (K)	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS (KJ/molK)
XAD-16N	298.15	87	-78174	262
	308.15		-80799	
	318.15		-83424	
HP-20	298.15	200	-188294	632
	308.15		-194616	
	318.15		-200939	
SP-850	298.15	215	-203472	683
	308.15		-210303	
	318.15		-217135	

5.4 Discussion

Macroporous adsorption resins have been used to purify bioactive compounds from food and plant extracts [Ma et al., 2009; Alexandratos, 2009; Kim et al., 2014]. The macroporous resins screened for adsorption and desorption of phlorotannins were XAD-7, HP-20, XAD-16N, XAD-2, XAD-4 and SP-850, which have been used in the purification of plant polyphenols in previous studies [Hui et al., 2010; Lin et al., 2012; Soto et al., 2012; Bretag et al., 2009]. Only [Kim et al., 2014] have done prior studies of the purification of phlorotannins from brown seaweed with macroporous resins.

From the macroporous resins tested in our work, we found that XAD-16N was the best to purify phlorotannins extracted from *M. pyrifera.* In addition, the optimum operating conditions found were 25°C and 9 hours of adsorption and 9 hours of desorption. Under these conditions, the adsorption capacity for XAD-16N was 183 ± 18 mg PGE/g resin, desorption ratio was 38 ± 8% and the purification level was 42%. These values are higher than those reported by Kim, et al., [2014], using HP-20 for the purification of phlorotannins from *E. cava,* with an adsorption and desorption capacity of 38 and 35 mg PGE/g resin, respectively. This difference could be attributable to a higher surface area and pore size of XAD-16N in comparison to HP-20 (see Table 5.1), which could allow the migration of polymeric units of phloroglucinol that form the different types of phlorotannin compounds present in *M. pyrifera* [Leyton et al., 2016a]. On the other hand, the purification level can be further improved by employing a continuous or dynamic desorption processes [Kim et al., 2014].

The model that best described the adsorption isotherms for XAD-16N, HP-20 and SP-850 resins was the Freundlich model. This model assumes that the surface of the resin is heterogeneous and characterized by sorption sites at different energy levels [Duran et al., 2011]. Figure 5.3 shows that the capacity of the resins to adsorb phlorotannins decreases with temperature, suggesting a physisorption mechanism defined by Van der Waals forces [Duran et al., 2011; Montgomery, 1985]. The thermodynamic parameters provide information on the inherent energy change of adsorbents after adsorption, and also the mechanism involved in the adsorption process. The positive value of ΔS obtained for XAD-16N, HP-20 and SP-850, suggests that some structural changes occur on the solid/liquid interface where randomness increases as the adsorption process progresses [Gupta, 1998]. The value of ΔG for XAD-16N, HP-20 and SP-850 increases with temperature, indicating that the adsorption becomes

more favorable at lower temperatures, which is consistent with the behavior shown in Fig. 5.3 [Gao et al., 2013].

5.5 Conclusion

The best macroporous resin for the purification of phlorotannins extracted from *M. pyrifera* was XAD-16N. The optimum operating conditions determined from kinetic and isotherm of adsorption were 25°C for 9 hours of adsorption followed by desorption with ethanol 90% v/v at 25°C for 9 hours. Under these conditions, the adsorption capacity was 183 ± 18 mg PGE/g resin, with desorption ratio of 38 ± 8% and a purification level of 42%. To improve the purification level, further studies applying dynamic adsorption and desorption are needed. The purification of phlorotannin compounds from crude extracts could expand their use as nutraceuticals and increase the commercial value of the product.

Chapter 6

Main Conclusions

It was possible to design and optimize an extraction process of phlorotannins and carbohydrate from *Macrocystis pyrifera*, using marine enzymes in the step of carbohydrate hydrolysis. The drying conditions of *M. pyrifera* that allow alga storage minimizing damage of phlorotannins were at 40°C. The best extraction conditions of phlorotannins and carbohydrates were a combined alkaline extraction process followed by an enzymatic pretreatment, where the optimum conditions maximizing the extraction yields were: a pretreatment with carbohydrate-active enzyme from *Alternaria sp*. HN (with an activity of 60.34 ± 4.97 U/mg), at 37°C, pH 7.0 for 24 hours and an S/L ratio of 1:10 in the case of carbohydrates and, at 25°C, pH 7.0 for 36 hours and an S/L ratio of 20 in the case of the phlorotannins; following for both compounds an alkaline extraction with NaOH 0.5 N, at 100°C, 180 min and an algae-to-alkaline solvent ratio of 1:20. Combining both processes allowed for an increase in the extraction efficiency of the phlorotannins of 21.4% and, in the case of the carbohydrates, of 89.67%.

Further, it was possible to identify two phlorotannins through HPLC-ESI-MS analyses: phloroeckol and a tetrameric phloroglucinol. These phlorotannins have been reported in the literature to have an antidiabetic effect and prevention of Alzheimer's disease for phloroeckol, and free radical scavenging ability and antiallergic effect for tetrameric phloroglucinol. The types of carbohydrates present in the extract were polysaccharide alginate, different types of monosaccharides as arabinose, fucose, fructose, galactose, glucose, mannose, rhamnose and xylose, sugar acid as galacturonic and glucuronic acid, and a polyol mannitol.

Finally, a subsequent process of phlorotannins purification was carried out using macroporous resin XAD-16N, the operation conditions that improve the purification of phlorotannins were at 25°C for 9 hours of adsorption and desorption. Under these conditions, the adsorption capacity was of 183.19 mg PGE/g resin, with a desorption ratio of 38.2% and a purification level of 42.0%.

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Appendices

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Identification and efficient extraction method of phlorotannins from the brown seaweed *Macrocystis pyrifera* using an orthogonal experimental design

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ABSTRACT

The brown seaweed contains a type polyphenol compound characteristic of its species, the phlorotannins, which are produced from the polymerization of phloroglucinol units. They have been extensively studied due to their pharmacological and nutraceutical properties, but there is still a need for an optimized extraction protocol. In this study, the brown seaweed *Macrocystis pyrifera* was employed to determine the best conditions for extraction of phlorotannins. A set of different variables were evaluated such as the use of pre-treatment, type of solvent, drying temperature, particle size, temperature and extraction time as well as the solid/liquid ratio upon extraction. The optimal conditions for the extraction of phlorotannins were: pre-treatment with hexane, extraction with water, drying temperature 40 °C, particle size below 1.4 mm, at 55 °C for 4 h and a solid-to-liquid ratio of 1:15. Under these conditions, the concentration of phlorotannins achieved in the extract was 200.5 ± 5.6 mg gallic acid equivalent (GAE)/100 g dry seaweed (DS) and total antioxidant activity of the extract of 38.4 ± 2.9 mg trolox equivalent (TE)/100 g DS. Further, it was possible to identify two phlorotannins through HPLC-ESI-MS analyses: phloroeckol and a tetrameric phloroglucinol. These phlorotannins have been reported in the literature to have an antidiabetic effect and prevention of Alzheimer's disease for phloroeckol, and free radical scavenging ability and antiallergic effect for tetrameric phloroglucinol. Therefore, the extract of phlorotannins has potential as medicinal foods or therapeutics for human health applications.

1. Introduction

In recent decades, the quest to identify new natural products with antioxidant potential has been increasing as several studies have recognized synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), as substrates with potential health hazards [1,2]. Consequently, natural antioxidants of marine origin have been the subject of intense research, and new substances with pharmacological and nutraceutical properties have been identified [3]. Interestingly, seaweed is one of the main sources of these compounds [4,5].

The macroalgae that occupy the littoral zone can be classified as red (*Rhodophyta*), brown (*Phaeophyta*) or green (*Chlorophyta*) types, depending on pigment present and chemical composition [6,7]. The brown algae contain a substantial amount of soluble carbohydrates and they completely lack lignin, which is important when aiming for an extraction process. Also, they contain unique compounds known as phlorotannins [8,9]. These compounds are polyphenols produced by seaweed as secondary metabolites and biosynthesized via the acetate malonate pathway [10]. Further, they are derived from the polymerization of phloroglucinol units, which constitute up to 15% of the dry weight of the seaweeds [11,12]. Depending on the types of interlinkage present, phlorotannins can be classified into various subclasses, i.e., phlorotannins with phenyl linkages, ether linkages or ether and phenyl linkages that are characteristics for different types of phlorotannins [13,14].

The phlorotannins are present in the algae in soluble form, stored in physodes, and insoluble form forming part of the structure of the cell walls of algae, forming complexes with proteins and alginic acid [15]. Concentration and molecular size of these compounds vary according to intrinsic factors (reproductive condition, age and size of the algae), as well as by extrinsic factors (environmental and ecological stimuli) [15–17]. The molecular size of phlorotannins reported varies between 126 Da (phloroglucinol) and 650 kDa (phlorofucoroeckol) [18] and has been extensively studied due to their pharmacological and nutraceutical properties exhibiting antioxidative, antiangiogenic, antiallergic, anti-inflammatory, and antidiabetic effects [15–23].

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The extraction of phlorotannins has been performed from different brown seaweeds (*Himanthalia elongata*, *Stypocaulon scoparium*, *Ascophyllum nodosum*, *Ecklonia stolonifera*, *Fucus vesiculosus* between others). Also, the influence of temperature, drying time and nature of the extractant as well as the quality of antioxidant compounds, phlorotannins, has been studied [16–18,24–27]. However, there is only limited information on the extraction of phlorotannins from *Macrocystis pyrifera* [28,29]. This brown seaweed, *M. pyrifera* is abundantly present in the Pacific Ocean [8,29,30] and is very abundant in the coastal areas of Chile. *M. pyrifera* is an alga with rapid growth and it is used as feed source in the abalone industry as well as a thickening agent in the cosmetics and food industries. Consequently, the development of extraction conditions that permit successful extraction of phlorotannins from *M. pyrifera* is of potential industrial interest.

Further, the identification of the different phlorotannins extracted is of considerable interest for the industry. In this respect, the main objectives of this study were to determine the effect of extraction conditions: (1) use of pre-treatment, (2) type of solvent, (3) drying temperature of algae and (4) extraction parameters (particle size, time and temperature of extraction and solid/liquid ratio) on total phlorotannin concentration (TPC) and total antioxidant activity (TAA) from *M. pyrifera* as well as the identification of the various phlorotannins extracted.

2. Materials and methods

2.1. Algal materials

M. pyrifera were collected by scuba diving 30 km southwest of Puerto Montt, Chile. The samples were harvested in November 2011 (M1), March 2013 (M2) and June 2013 (M3). The chemical analyses of *M. pyrifera* samples were carried out in order to determine its initial composition in terms of protein, lipids, carbohydrates and ash elements. The analyses were done at the Institute of Agroindustry, University of the 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): 930.04, 978.04, 991.36, 930.05 and 962.09, respectively [31]. The carbohydrate content was calculated from the difference in the initial mass and the sum of values reported for proteins, humidity, lipids and ash [32].

2.2. Effect of the extraction solvent on TPC and TAA

Prior to obtaining extracts, the dry seaweed (M1 dried at room temperature) was pretreated according to Koivikko, et al. [27]. The pretreatment consisted of washing the alga with hexane (solid/liquid ratio of 1:5 w/v) three (3) times to remove pigments and lipids compound. Additionally, 5.0 g of dry algae washed with hexane was dispersed separately in 50 ml of different solvents (methanol, ethanol, water, water/methanol 50:50, hexane/ethanol 88:12, ethanol/water 25:75 or 80:20, ethyl acetate/water 50:50, water/acetone 20:80 or 30:70 and methanol/chloroform 66:33% v/v) [27–30,33–35], and incubated in a platform at 200 rpm and 40 °C for 2 h. The mixture was centrifuged at 2000 rpm at 4 °C for 20 min and supernatant was stored at −20 °C before further analysis for the quantification of TPC and TAA values.

2.3. Effect of drying temperature on TPC and TAA

The best drying conditions were determined using the fresh algae (M2) dried at four temperatures 30, 40, 50 and 60 °C [25]. The experiments were performed in a horizontal dryer with hot air flow (Tray drier, Armfield UOP8) at an air flow speed of 1.3 m/s. Once the samples were dried, they were milled (1.4–2 mm) and stored at 4 °C prior extraction.

As the next step, 0.5 g of alga (dried and pretreated) was placed in test tube containing distilled water at a solid/liquid ratio of 1/10 and incubated in a platform shaker at 200 rpm and 40 °C for 2 h. The sample was centrifuged at 7000 rpm for 10 min at 4 °C and the supernatant was stored for further analyses. The best drying temperature was determined in terms of highest TPC and TAA obtained.

2.4. Effect of extraction parameters on TPC and TAA using Taguchi orthogonal array

The different extraction variables, namely extraction time, extraction temperature, solid/liquid (S/L) ratio and algae particle size were simultaneously evaluated using Taguchi orthogonal array L_9, 3^4 where 9 is the number of experiments, 4 is the number of factors tested and 3 is the number of work levels [36]. Seaweed M3 was previously dried at 40 °C, washed with hexane and water was used as extractant. Further, Table 2 demonstrates the different variables evaluated and describes the conditions chosen for the different experimental runs. The best extraction conditions were determined in terms of the highest TPC and TAA values measured after the extraction.

2.5. Determination of TPC

The amount of total phlorotannins compounds in extracts was determined according to the Folin–Ciocalteu assay [37] adapted to 96-well plates. Standards containing gallic acid with a concentration varying from 20 to 100 mg/l were prepared to measure the amount of phlorotannins in the extracts. Samples and standard (20 μl) were introduced separately into 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 a UV–visible spectrophotometer (Asys, UVM 340). The phlorotannin concentration was determined by the regression equation of the calibration curve expressed as gallic acid (GAE) mg equivalent/100 g dry seaweed (DS).

2.6. Determination of DPPH radical scavenging activity, total antioxidant activity (TAA)

The free radical scavenging activity was measured using the modified method of von Gadow et al., [38]. Consequently, 40 μl of 0.4 M 1,1-diphenyl-2-picryl-hydrazyl (DPPH) solution in ethanol was added to 50 μl of the sample solution, complemented with 110 μl of ethanol. The plates were mixed and allowed to stand for 30 min in the absence of UV light to avoid decomposition. The absorbance was measured at 520 nm against an ethanol blank. Calibration curves of Trolox (0–24 mg/l) were prepared and the results were expressed as the number of equivalents of Trolox (mg TE/100 g DS).

2.7. Characterization of phlorotannin extract

The composition of the phlorotannin extract obtained under the best extraction conditions was characterized and the type of phlorotannin present was identified as follows.

2.7.1. Fourier transform infrared spectroscopy — FTIR

The sample was pre-fractioned with methanol in order to induce precipitation of the co-extracted carbohydrates (methanol was chosen among different solvents tested, not shown), concentrated in rotary evaporator at 40 °C and pressure bellow 0.1 mbar and finally lyophilized at −50 °C under vacuum (below 0.1 mbar) overnight.

The IR spectra obtained for the sample and phloroglucinol were recorded on an ATI Mattson Infinity Series IR spectrometer at room temperature. The samples were freeze-dried before the FTIR analyzes and blended in a 3% w/w ratio with potassium bromide (KBr) powder,

Table 1
Chemical analysis of *Macrocystis pyrifera* harvested during different periods.

Proximal analysis	Unit	*M. pyrifera* M1 (Nov 2011)		*M. pyrifera* M2 (March 2013)		*M. pyrifera* M3 (Jun 2013)	
		Wet algae	Dry basis	Wet algae	Dry basis	Wet algae	Dry basis
Humidity	%	10.18	–	17.61	–	15.99	–
Protein	%	10.01	11.15	8.52	10.34	9.59	11.42
Ash	%	36.16	40.26	21.50	26.10	25.16	29.95
Lipids	%	1.03	1.15	1.17	1.42	5.42	6.45
Fiber	%	17.45	19.43	6.53	7.93	7.62	9.07
NNE[a]	%	25.16	28.01	44.67	54.22	36.22	43.11
Carbohydrates[b]	%	42.61	47.44	51.20	62.14	43.84	52.18

[a] NNE, no nitrogen elements which was calculated as 100−(Fiber + Lipids + Protein + ash).
[b] Carbohydrate amount was calculated as 100−(Humidity + Protein + Ash + Lipids).

followed by tableting (10 tons for 1 min) before measurement. A region of 4,000–400 cm^{-1} was used for scanning.

2.7.2. Carbohydrate analysis

Carbohydrates in the obtained phlorotannin extract were quantified with an acid methanolysis method followed by sample silylation and GC analysis as follows: a volume of 2 ml of methanolysis reagent containing 2 M of hydrochloric acid (HCl) in methanol was added to 10 mg of freeze dried algae samples and a calibration solution containing known amounts of carbohydrates. As the next step, the tubes were inserted into an oven operating at 100 °C for 3 h. Once the reaction was completed, 200 μl of pyridine was added to neutralize any excess of HCl, and 2 ml of each internal standard solution containing 0.1 mg/ml of sorbitol and resorcinol in methanol, respectively, was added to each sample. After mixing, methanol was evaporated at 50 °C under nitrogen stream and the sample was further dried under vacuum (Heraeus VTR 5022) at 42 °C below 50 mbar for 20 min prior silylation of the samples [39]. Once the samples were completely dry, silylation was commenced by adding 150 μl of pyridine, 150 μl of hexamethyldisilazane (HMDS) and 70 μl of chlorotrimethylsilane (TMCS), followed by a thorough mixing using a high-sheer vortex mixer. Further, the samples were kept in an oven at 70 °C for 45 min and the clear liquid phase was analyzed in order to determine the carbohydrate content of the samples by gas chromatography [39]. Consequently, about 1 μl of a silylated sample was injected via a split injector (260 °C, split ratio 1:15) into a 30 m/0.32 mm i.d. column coated with dimethyl polysiloxane (HP-1, Hewlett Packard) with the film thickness of 0.17 μm. The column temperature program was as follows: a temperature ramp from 100 to 175 °C (4 °C/min), followed by a ramp of 175 to 290 °C (12 °C/min). The detector (FID) temperature was 290 °C. Hydrogen was used as a carrier gas.

2.7.3. High Precision Liquid Chromatography Mass Spectrometry (HPLC-ESI-MS/MS) identification

A volume of 14 ml of extract phlorotannins obtained under the best extraction condition was concentrated at room temperature in a vacuum concentrator (SpeedVac, Savant Instruments Inc., NY-USA). Consequently, the concentrated extract was re-suspended in 4.5 ml of water and a volume of 20 μl was analyzed using a LC-ESI-MS/MS system which consisted of the HP1100 liquid chromatography (Algilent Technologies Inc., CA-USA) connected to the mass spectrophotometer (Esquire 4000 ESI-Ion Trap LC/MS(n) system, Bruker Daltonik GmbH, Germany). A Luna C18 150 × 4.6 mm, 5 μm and 100 Å analytical column (Phenomenex Inc., CA-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 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 [40]. 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 nebulising gas at 45 psi, drying gas at 345 °C and flow rate 10 l/min. All scans were performed in the range 20–2200 m/z. The trap parameters were set in ion charge control using manufacturer's default parameters. The collision induced dissociation (CID) was performed by collisions with the helium

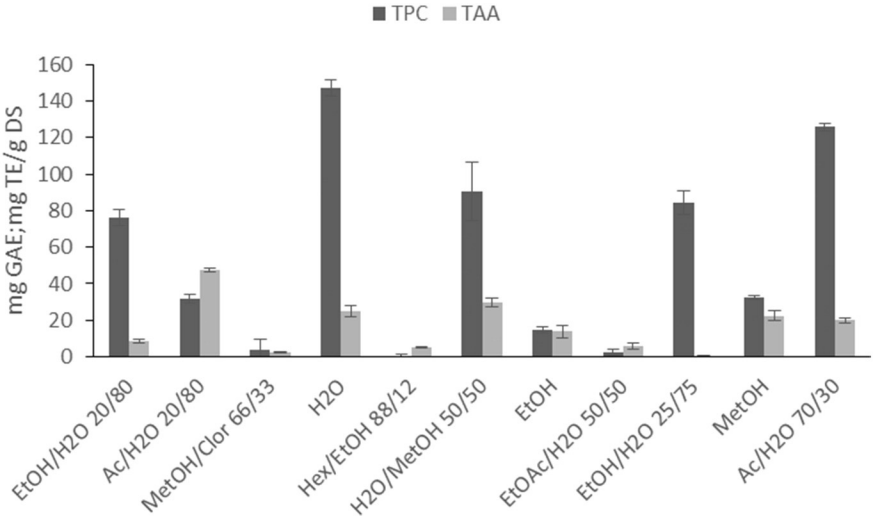

Fig 1. Evaluation of the best extractant for total polyphenols (TPC) and total antioxidant activity (TAA) in *Macrocystis pyrifera*. Ac, acetone.

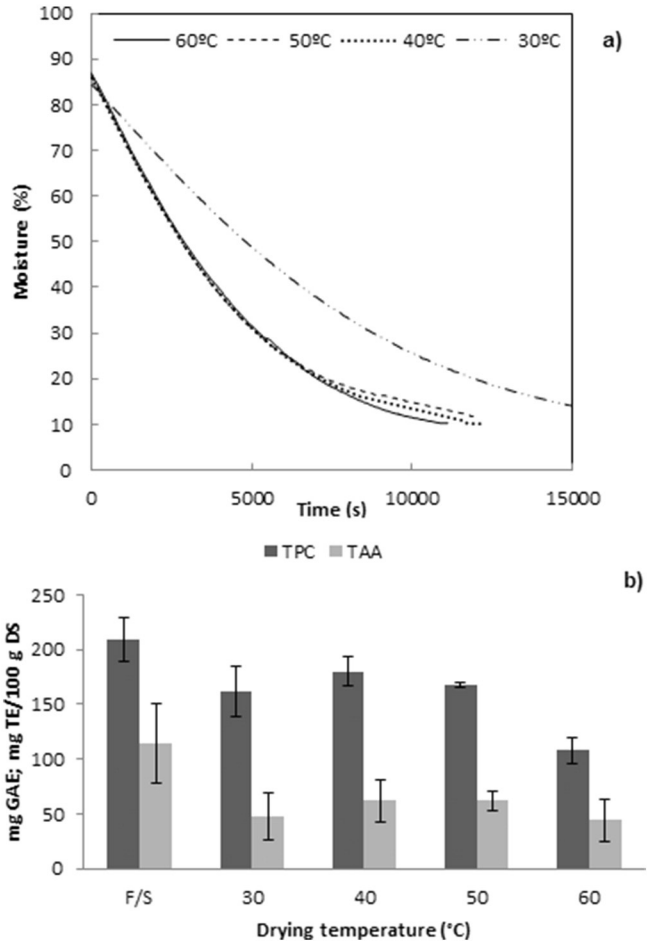

Fig 2. a) Drying curves for *Macrocystis pyrifera* at different drying temperatures, b) evaluation of the different drying temperatures for *Macrocystis pyrifera* in terms of total concentration polyphenols (TPC) and total antioxidant activity (TAA). F/S denotes fresh seaweed.

background gas present in the trap and automatically controlled trough SmartFrag option.

2.8. Statistical analysis

All extracts and fractions were analyzed in triplicate and the measurements were presented as average ± standard deviation. To evaluate the effect of the extraction solvent and drying temperature of phlorotannins from *M. pyrifera* were selected the best condition used as criteria higher-the-better for TPC and TAA.

Table 3
Variance analysis (ANOVA) for the total concentration of phlorotannins (TPC) and the total antioxidant activity (TAA) for *Macrocystis pyrifera*.

Response	Factors	DOF[a]	Sums of squares	Variance	F-ratio	Pure sum	%
TPC	Time	2	9636.9	4818.4	39.1	9390.3	21.7
	Temperature	2	6137.3	3068.7	24.9	5890.8	13.6
	S/L[b] ratio	2	12,960.9	6480.5	52.6	12,714.4	29.4
	Particle size	2	12,266.2	6133.1	49.8	12,019.6	27.8
	Other/error	18	2218.8	123.3	–	3204.9	7.4
	Total	26	43,220.1	–	–	43,220.1	100
TAA	Time	2	830.8	415.4	23.8	795.9	18.5
	Temperature	2	242.4	121.2	6.9	207.5	4.8
	S/L[b] ratio	2	2490.4	1245.2	71.4	2455.5	57.0
	Particle size	2	433.2	216.6	12.4	398.3	9.2
	Other/error	18	314.0	17.4	–	453.5	10.5
	Total	26	4310.8	–	–	4310.8	100

[a] DOF, degree of freedom.
[b] S/L ratio, solid/liquid ratio of extraction.

The significance and relative influence of each extraction parameter in TPC and TAA using the orthogonal Taguchi array were determined using the ANOVA variance analysis with a 5% confidence level.

3. Results and discussion

3.1. Raw material analysis

The chemical analysis of *M. pyrifera* indicated that the ash content was higher in the batch M1 (40.26% w/w), whereas the lipid content was higher in the batch M3 (6.45% w/w) and the carbohydrate content was highest in the batch M2 (62.14% w/w, Table 1). According to the literature [41], alginate–phenol linkages play an essential role in the brown algal cell wall structure and it is expected that this correlates with high phlorotannin concentration in the brown alga. On the other hand, the differences observed in the lipid and carbohydrate content, for the alga batches harvested in November and March, could be attributed to environmental factors associated with the season, such as, water temperature, 12.5 and 13.5 °C on average, respectively [42], and the level of UV radiation, UV-B 0.5 and 4 W/m^2 on average, respectively [43].

3.2. Selection of operational conditions: effect of the extractant type and drying temperature

The TPC and TAA levels obtained using different solvents are depicted in Fig. 1. The two highest concentrations of TPC were attained using water and 70% v/v acetone as extraction solvents, giving 147 ± 2.9 and 125 ± 1.5 mg GAE/100 g DS, respectively. On the other hand, the highest TAA values were attained for 80% v/v acetone and water, 25 ± 2.0 and 20.2 ± 4.2 mg TE/100 g DS, respectively. The better yields of TAA in the case of aqueous acetone may be due to the fact that

Table 2
The influence of extraction parameters determined by Taguchi orthogonal array design L_9, 3^4 on TPC and TAA from the extraction of *Macrocystis pyrifera*.

Runs	Time [h]	Temperature [°C]	S/L[a] ratio	Particle size [mm]	TPC [mg GAE/100 g DS]	TAA [mg TE/100 g DS]
1	2	25	1/10	<1.4	120.8 ± 11.3	42.7 ± 0.4
2	2	40	1/15	2–1.4	58.5 ± 4.0	29.1 ± 8.4
3	2	55	1/20	>2	39.5 ± 3.1	14.4 ± 4.2
4	3	25	1/15	>2	31.4 ± 18.1	27.3 ± 4.3
5	3	40	1/20	<1.4	47.2 ± 5.9	42.0 ± 3.2
6	3	55	1/10	2–1.4	136.9 ± 20.8	55.6 ± 1.9
7	4	25	1/20	2–1.4	100.0 ± 6.3	20.8 ± 2.0
8	4	40	1/10	>2	89.1 ± 9.3	46.1 ± 4.8
9	**4**	**55**	**1/15**	**<1.4**	**200.5 ± 5.6**	**38.4 ± 2.9**

Bold values in this table highlights the best conditions of TPC and TAA extraction.
[a] S/L, solid to liquid ratio.

Table 4
Condition giving the maximal total phlorotannins concentration (TPC) and total antioxidant activity (TAA) from *Macrocystis pyrifera*.

Factors	TPC		TAA	
	Optimum level	Contribution	Optimum level	Contribution
Time	4 h	25.0	3 h	6.5
Temperature	55 °C	20.8	40 °C	3.9
S/L[a] ratio	1:10	30.7	1:10	13.0
Particle size	<1.4	17.9	<1.4	5.9
Total contribution from all factors		94.4		29.2
Expected result at optimum ± CI[b] condition		218.9 ± 15.0		64.4 ± 2.9

[a] S/L, solid/liquid ratio of extraction.
[b] CI, confidence interval.

Table 5
Average concentration (±standard deviation) of carbohydrates present in alga *Macrocystis pyrifera* and in its phlorotannin extract.

Carbohydrate	Phlorotannins extract	*M. pyrifera* (M3)
	mg/g DS	mg/g DS
Alginate	23.4 ± 6.6	191.7 ± 3.4
Arabinose	1.0 ± 0.6	1.7 ± 0.5
Fucose	39.7 ± 5.7	73.5 ± 0.1
Fructose	1.4 ± 0.1	14.7 ± 0.1
Galactose	5.7 ± 1.3	31.7 ± 2.7
Galacturonic acid	0.5 ± 0.7	6.8 ± 0.1
Glucose	2.0 ± 0.6	11.1 ± 0.1
Glucuronic acid	0.8 ± 0.1	4.9 ± 0.1
Mannitol	38.1 ± 2.3	38.2 ± 0.4
Mannose	2.0 ± 0.9	11.5 ± 1.1
Rhamnose	2.7 ± 0.4	2.0 ± 0.1
Xylose	1.2 ± 0.4	9.0 ± 1.3
Total	118.5	396.9

acetone is a less polar solvent. Consequently, acetone has a higher affinity for apolar compounds such as lipid compounds and pigments, which may have produced an increment in the TAA measured in the sample. The presence of apolar compounds present in the extract suggests that the pretreatment conditions used are not optimized [44,45]. Therefore, these results suggested that water was the best solvent for the extraction of phlorotannins. Similar results were obtained by Kindleysides, et al. [28]. Moreover, when studying the extraction strategies of phlorotannins for other brown seaweed species (*Scytosiphon lomentaria*, *Papenfussiella kuromo* and *Nemacystus decipiens*), water was considered as the best extraction solvent [46].

The influence of temperature and drying time on the extraction and quality of phlorotannins extracted from *M. pyrifera* was evaluated. The drying curves are presented in Fig. 2a. As can be observed, the drying profiles for the temperatures 40, 50 and 60 °C are relatively similar. On the contrary, at 30 °C, a much slower release of moisture was observed. In order to evaluate the best drying temperature, the extraction of phlorotannins was carried out using water as an extractant. Notwithstanding, the phlorotannin compounds are easily decomposed, as also reported earlier [27]. Consequently, the addition of 0.3% ascorbic acid as a preservative during extraction was evaluated. The results of the total phlorotannins content (TPC) and TAA concentration obtained are presented in Fig. 2b. The optimal drying temperature was 40 °C, giving TPC and TAA values in the extract amounting to 180 ± 13 mg GAE/100 g DS and 62 ± 19 mg TE/100 g DS, respectively. On the other hand, for fresh seaweed (85% average moisture content; fresh seaweed, F/S) gave 210 ± 20 mg GAE/100 g DS of TPC and 115 ± 36 mg TE/100 g DS of TAA. Consequently, the TPC and TAA values obtained for non-dried alga gave better results, 14 and 46% higher than those values of dry seaweed at 40 °C, respectively, presumably due to the degradation of some phlorotannins during the drying process. Admittedly, the aim of the drying process was to reduce the moisture content in order to minimize the deterioration of the algae due to microbial growth, thus increasing the storage time of the seaweed prior any treatments. Further, the drying process also reduces the weight and volume of the seaweed, thus minimizing the packaging, storage and transportation costs [47].

The difference observed in the concentration of TPC and TAA for the extract obtained previously, 147 ± 2.9 mg GAE/100 g DS and 20.2 ± 4.2 mg TE/100 g DS for the effect of extractant type and, 180 ± 13 mg GAE/100 g DS and 62 ± 19 mg TE/100 g DS for effect of drying temperature, can be explained as due to the effect of different drying conditions (temperature and time) and by the fact that algae samples correspond to different harvest periods [48], Table 1. Nevertheless, it is possible to minimize the differences observed by controlling drying conditions and via standardization of biomass through changes in the form of culture [49].

3.3. Optimized extraction of phlorotannins: effect of extraction parameters

The TPC and TAA values measured upon different conditions of water extraction are recorded in Table 2. The best extraction conditions correspond to the entry N°9, whereupon the extraction performed for a sample particle size below 1.4 mm, and extraction with water at 55 °C for 4 h (S/L ratio of 1/15). Consequently, TPC of 200.5 ± 5.6 mg GAE/100 g DS and TAA of 38.4 ± 2.9 mg TE/100 g DS were recorded, resulting in 10% higher TPC value compared with previous conditions, 180 ± 13 mg GAE/100 g DS. The reported value is higher than the corresponding values reported for other types of brown algae. Indeed, Chandini,

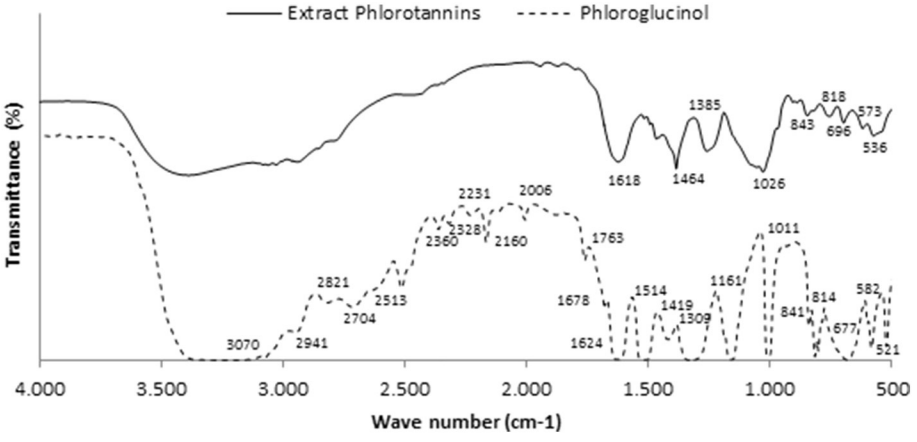

Fig 3. FTIR spectra of standard phloroglucinol and water phlorotannin extracts from *Macrocystis pyrifera*.

et al. [50] reported a TPC value of 0.29 mg GAE/100 g DS upon the water extraction of *Sargassum maginatum*, whereas in case of *E. stolonifera*, 8.21 ± 0.01 mg GAE/100 g DS was reported [51]. Further, in an additional study of *Turbinaria conoides* and using diethyl ether extraction, 119 ± 0.1 mg GAE/100 g DS of TPC was obtained [52]. Thus, it is evident that *M. pyrifera* has potential since much higher TPC values were attained for this worldwide relatively abundant seaweed.

Finally, all factors had significant effects ($p < 0.05$) on the TPC and TAA (Table 3), the highest influence was obtained with the S/L ratio, particle size and extraction time of 29.4, 27.8 and 21.7%, respectively, the sum of these three factors accounted for 78.9% of the TPC. For the TAA the highest influence, 57%, was that of factor S/L ratio. The statistical methodology applied predicted a maximum TPC of 218.9 ± 15.0 mg GAE/100 g DS and TAA of 64.4 ± 2.9 mg TE/100 g DS under the optimal extraction conditions (Table 4).

3.4. Characterization of phlorotannin extract

3.4.1. Fourier transform infrared (FTIR) spectroscopy

As can be seen, the chemical composition of phlorotannin extract obtained under the optimal condition, and of standard phloroglucinol was similar, see Fig. 3. The phloroglucinol standard gave rise to twenty

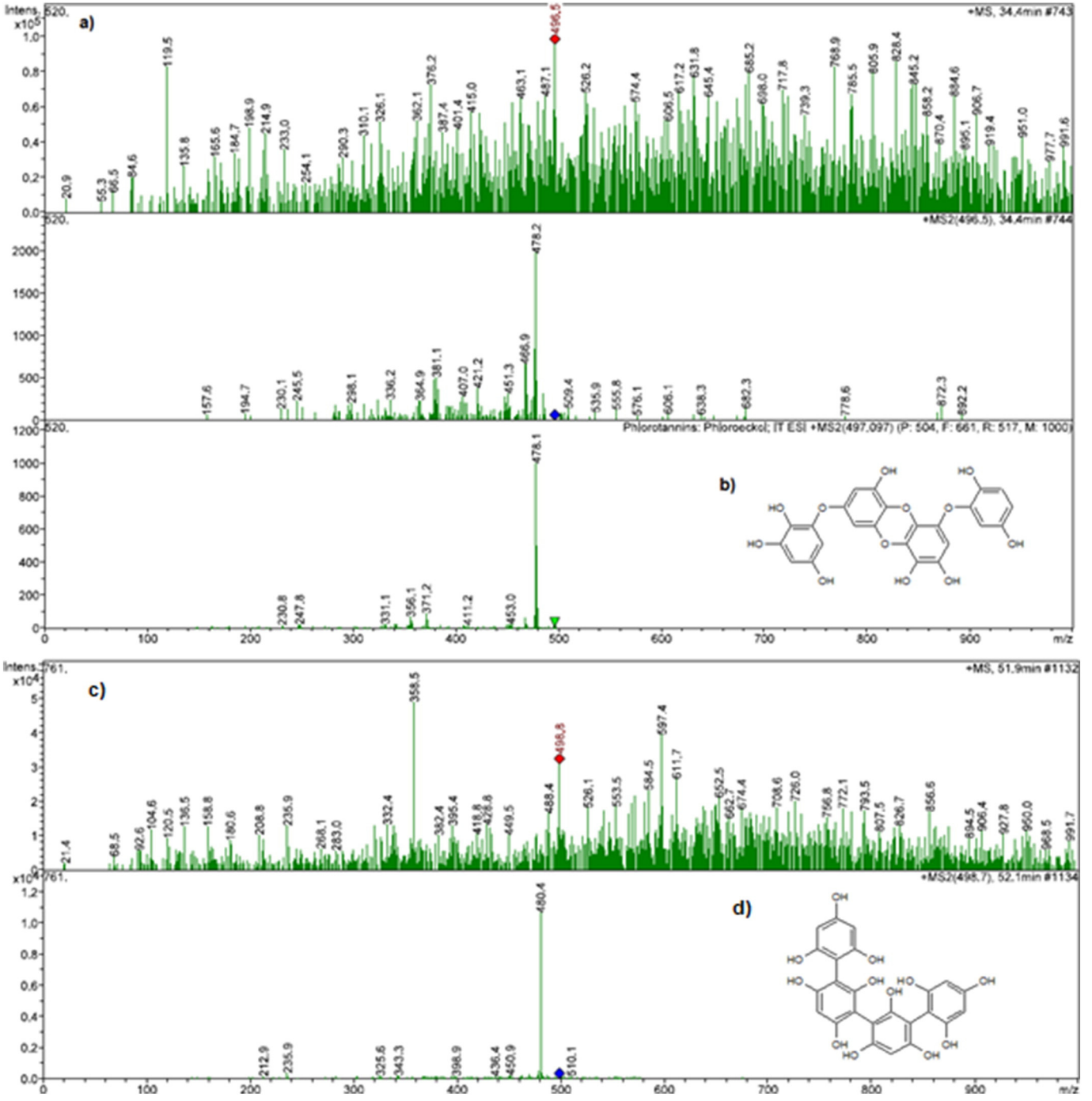

Fig. 4. Identification of phlorotannins detected in *Macrocystis pyrifera*. A, mass spectrum (MS) for peak 1 (superior) and fragmentation spectrum (MS2) for m/z 497 (inferior); B, chemical structure and library fragmentation spectrum for phloroeckol. C, mass spectrum for peak 2 (superior) and fragmentation spectrum for m/z 499; B, chemical structure for phloroglucinol tetramer.

three major peaks within the range between 3070 and 521 cm^{-1}, meanwhile similar peaks were observed for phlorotannins extract between 1618 and 536 cm^{-1}. Indeed, in phlorotannin extract, the stretching bands around 1470–1450 cm^{-1} correspond to the aromatic nuclei. The characteristic stretch band of carboxyl groups, the peak at 536 cm^{-1}, was presumably caused by the stretching vibration of O—H with relatively high strength in the axial position. In turn, the bands at 1026 cm^{-1} correspond to the glycosidic linkage vibrations of C—OC and C—O—H, indicating the presence of some carbohydrates in the sample.

Nonetheless, the stretch bands identified for some carbohydrate compounds may reveal the presence of some phlorotannin compounds in association with some carbohydrates. The signal at 843 cm^{-1} corresponds to characteristic absorption of mannuronic acid (a monomeric unit of alginate, one of the main constituents of brown algae) [53].

3.4.2. Analysis of the carbohydrate present in the phlorotannins extract

Various carbohydrates could be identified in the water extract of phlorotannins (Table 5), such as polysaccharide alginate, different types of monosaccharides (arabinose, fucose, fructose, galactose, glucose, mannose, rhamnose and xylose) sugar acid, (galacturonic and glucuronic acid) and a polyol (mannitol).

3.4.3. HPLC-ESI-MS/MS identification

The chromatograms obtained were compared to the reported ones for phlorotannins [54–57]. The identification was carried out using all m/z signals for each corresponding separated fragment. Additionally, an extracted ion chromatogram (EIC) of the expected precursor signal was performed. Two peaks were identified upon the analysis and compared with earlier data [54–56]. The peak 1 (t_R 34.4 min) was detected with a signal m/z 497, (the fragmentation gave m/z values of 478.2 ($[M\text{-}H_2O + H]^+$) and m/z 245.5 ($[M\text{-}2xPhloroglucinol + H]^+$)), likely corresponding to a derivative of phloroglucinol named phloroeckol (Fig. 4a and b). The peak 2 (t_R 52.1 min) was detected with a signal m/z of 499, (the fragmentation gave signals with m/z of 480.4 ($[M\text{-}H_2O + H]^+$), m/z 245.8 ($[M\text{-}2xPhloroglucinol + H]^+$) and m/z 235.9 ($[Phloroglucinol\text{-}H_2O + H]^+$)), likely originating from a tetramer of phloroglucinol isomers: difucophloroethol, fucodiphloroethol, tetrafucol or tetraphloroethol (Fig. 4c and d) [54].

These phlorotannins have interesting properties. Fucodiphloroethol exhibits free radical scavenging ability [58] and antiallergic effect [59], whereas phloroeckol exhibits antidiabetic effect [60] and prevents Alzheimer's disease [61]. Finally, we propose that phlorotannin extracts could find use as medicinal foods or therapeutics substrates in human health products.

4. Conclusions

The orthogonal design set of experiments allowed identification of the S/L ratio, particle size and time of extraction as the most important variables to obtain the highest concentrations of phlorotannins in the extract. The best extraction conditions upon extraction of phlorotannins from brown algae *M. pyrifera*, were determined: pre-treatment with hexane, extraction with water, drying temperature 40 °C, particle size below 1.4 mm, at 55 °C for 4 h and solid-to-liquid ratio of 1:15. At optimal conditions, the TPC value obtained was 200.5 ± 5.6 mg GAE/100 g DS and the TAA of 38.4 ± 2.9 mg TE/100 g DS. Finally, two types of phlorotannins were identified in the water extract, corresponding to phloroeckol and a tetramer of phloroglucinol.

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