UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería, Ciencias y Administración
Programa de Doctorado en Ciencias de Recursos Naturales

PROTEINS FROM DEFATTED OIL CAKE FROM MICROALGAE: EXTRACTION, CHARACTERIZATION AND STUDY FOR POTENTIAL APPLICATIONS

DOCTORAL THESIS IN FULFILLMENT OF THE REQUERIMENTS FOR THE DEGREE DOCTOR IN SCIENCES OF NATURAL RESOURCES

MARCELA VIRGINIA VERDUGO JARA

TEMUCO – CHILE
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Esta tesis fue dirigida por la Dra. MONICA OLGA RUBILAR DIAZ, perteneciente al Departamento de Ingeniería Química de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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Thesis outline

In the present work the defatted oil cake from microalgae from biodiesel production process was studied as source of protein to the potential applications troughout the elaboration of two different protein based products. First, the protein extraction from defatted oil cake from microalgae *Botryococcus braunii* was studied by factorial design; later isoelectric precipitation was carried out to obtain a protein concentrate. Following, protein concentrate was used as biopolymer for nanofibers formation by electrospinning technique, with potential focus on the development of materials for food or biomedical area. Subsequently, a protein extract was prepared from defatted oil cake from *Nannochloropsis gaditana* and their enzymatic hydrolysis was carried out with three different enzymes proteases. From the screening of the proteases and according to the degree of hydrolysis reached, the only one was chose to maximize the degree of hydrolysis and antioxidant activity by Response Surface Methodology (RSM). Finally, the protein hydrolysate obtained under optimum conditions was characterized by physicochemical, techno-functional properties and antioxidant potential.

In the first Chapter of this thesis a General Introduction is presented. In this Chapter general objective of the thesis, with regards to defatted oil cake from microalgae as protein source, is addressed. In Chapter 2, an overview about the main characteristics, potential applications and availability of vegetable oil cake and defatted oil cake from microalgae are discussed to know the current scenario and their projection in the future as protein source.

The protein extraction by alkaline method was studied in Chapter 3. The operational factors pH, temperature, and solid/liquid ratio involved in the extraction of the proteins

from defatted oil cake from *B. braunii* were studied by factorial design to maximize the protein extraction. Subsequently, the protein concentrate obtained was characterized through the chemical composition including their amino acid profile. In Chapter 4, the protein concentrate was used as biopolymer to the fibers formation by electrospinning technique. The fibers obtained were made from a polymer blend of protein concentrate and Poly(ethylene oxide), being characterized by scanning electronic microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FTIR).

In Chapter 5 a protein extract was prepared from defatted oil cake from *N. gaditana*. Subsequently the enzymatic hydrolysis of protein extract was optimized using Response Surface Methodology (RSM). A central composite design (CCD) was used to study the influence of the independent variables, hydrolysis temperature, and hydrolysis time and enzyme/substrate ratio on the degree of hydrolysis and antioxidant activity. In addition, characterization of protein hydrolysate was carried out by chemical composition, amino acid profile, techno-functional properties, SDS-PAGE electrophoresis, zeta-potential and antioxidant activity by ORAC and DPPH method.

Finally, in Chapter 6 the general discussion and conclusions of the thesis are shown. The utilization of defatted oil cake from microalgae for fibers elaboration by electrospinnig and protein hydrolysate is discussed. Concluding that proteins present in defatted oil cake can be used as biopolymer and also as raw material for protein hydrolysate preparation, having these products potential for applications, for example, as novel food ingredient for food industry or as part of the novel biomaterial for biomedical applications.

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Chapter 1

General Introduction

1.1 Introduction

Microalgae are a large and diverse group of photosynthic eukaryotes with a simple cellular structure, ranging from unicellular to multicellular forms; they can be found on water and sunlight co-occur, soils, ice, lakes, rivers, hotsprings and ocean [1]; and they have the ability to capture carbon dioxide and convert energy of sunlight to chemical energy. The first use of microalgae by humans for food dates back 2000 years to the Chinese, who used edible blue-green algae such as *Spirulina* and *Aphanizomenon* species. However, microalgal biotechnology only really began to develop in the middle of the last century [2]. Nowadays, there are numerous commercial applications of microalgae and currently appear to be one of the most promising renewable sources for biodiesel production that is capable of meeting the global demand for transport fuels.

The production of biodiesel from microalgal biomass appears to be an alternative natural resource mainly because of oil productivity, from microalgae exceeds production of oil crops [3]. Many microalgae species can be induced to accumulate substantial quantities of lipids thus contributing to a high oil yield, becoming to 10-20 times higher compare with oleaginous seeds. Some species of microalgae studied in the literature for the biodiesel production process are *Chlorella vulgaris* [4-11], *Haematococcus pluvial* [12], *Botryococcus braunii* [13-23], *Scenedesmus sp.*[24-26], C*hlorella protothecoides* [27], *Dunaliella tertiolecta* [21], *Synechocystis sp.*[19] and *Nannochloropsis gaditana* [28]. Generally microalgae also have some other advantages compare with more traditionally energy crops, including a high biomass production, short growth time and low land use, without compromising the production of food, fodder and other products derived from terrestrial crops [24, 29, 30]. However to design a sustainable biodiesel production process,

total component from microalgae should be recovered adding-value to the overall process. The main components present in microalgae biomass are those related to photosynthesis such as carotenoids, chlorophyll and protein, lipid compounds such as polyunsaturated fatty acid, glycolipids, sterols and other compounds like sulfated polysaccharides [31]. The composition of microalgae is quite variable; among the mayor component typically are present lipids, proteins and carbohydrates. Therefore the most important by-product after oil extraction for biodiesel production is the defatted oil cake with high content of proteins and carbohydrates. Until now only a few applications have been described in the literature for defatted oil cake from microalgae, such as ethanol and methane production, livestock feed, used as organic fertilizer due to its high N:P ratio, or simply burned for energy co-generation (electricity and heat) [32].

In relation to the microalgae biomass composition, not only highlights the oil content in microalgae, also the high protein content of various microalgal species is remarkable and has been one of the main reasons to consider them as a source of proteins [33]. Although, nutritional supplements derived from plants have been used and have predominated in the market, health benefits of aquatic microorganisms, such as microalgae, are being investigated and more recognized and appreciated within the last three to four decades [34]. Respect to microalga biomass as source of proteins some works have been published mainly about isolation and characterization of the proteins and bioactive peptides [35-42], but neither information was found related to the protein extraction and applications for proteins from defatted oil cake from biodiesel production. This by-product represents an important opportunity to adding value to the microalgae processing and obtaining a new protein source. Although studies on defatted oil cake from microalgae as a source of protein

are scarce, there are an interesting number of jobs from different plant materials. Most of works are based on proteins from by-products from agro-industrial sector, such as oil cakes, being their use focused on the production of protein hydrolysates for food applications [43-48], for the development of proteins-based films for food packaging applications [49] and more scarcely, soybean protein isolate extracted from defatted soy flakes during oil production, is being used in the production of nanofibers by electrospinnig technique for food and biomedical applications[50-52]. As has already been reviewed in the literature by [53], unicellular organisms such as microalgae and yeast, have been proposed as protein sources and procedures for protein isolation have been developed for yeast and the protein-producing microalga *Spirulina platensis* [54, 55]. In these cases, microorganisms were grown to produce protein as the main product and the protein isolates from *Spirulina platensis* was recovered from hexane defatted biomass by similar procedure as for soy isolate production[54]. Defatted oil cake from microalgae from biodiesel production can contain an interesting amount of proteins. In order to improve the nutritional value and functional properties of this by-product, its protein fraction should be isolated from the rest of the biomass components and different applications could be evaluated. Therefore, the objective of this thesis was to study the defatted oil cake from microalgae, from biodiesel production, as protein source for obtaining proteins product with potential for food applications, as functional ingredient or in the food packaging scope as biomaterial.

1.2 Hypotheses

- The defatted oil cake from green microalgae *B. braunii* can be used as feedstock for the protein concentrate preparation by alkaline extraction with nutritional quality acceptable y comparable to the FAO protein pattern.
- The protein concentrate from defatted oil cake from *B. braunii* can be used as biopolymer in the fabrication of electrospun fiber being compatible with the synthetic polymer allowing the preparation of fibers with a morphology and diameter defined.
- The enzymatic hydrolysis of a protein extract from defatted oil cake from *N.gaditana*, carried out under optimal combination of operational factor of the process, will allow obtaining a protein hydrolysate with higher antioxidant potential than those observed in the initial protein extract.

1.3 General objective

The objective of this thesis is to evaluate the potential of the defatted oil cake from microalgae as source of proteins, to be use as feedstock for the elaboration of protein products for potential applications.

1.4 Specific objectives

1. To study the protein extraction process from defatted oil cake from green microalgae *B. braunii* to obtain a microalgae protein concentrate to be used as feedstock in the fibers formation by elecrospinnig technique.
2. To study the electrospinnability of *B. braunii* protein concentrate, to produce fibers and to evaluate the effect of different polymer formulations on the morphology and diameter of the fibers obtained.
3. To optimize the enzymatic hydrolysis of protein extract prepared from defatted oil cake from *N. gaditana* to maximize degree of hydrolysis and antioxidant activity.

Chapter 2

Theoretical background.

Defatted oil cakes from biodiesel production: a high potential as proteins source

2.1 Introduction

In recent times there has been an important depletion of the fossil feedstock, with an increasing rise of the crude oil price, and the environment problems associated with CO_2 emissions. These reasons have forced the search and the development of the new alternative "green resources" for energy, transport fuels and chemicals: the replacement of fossil resources with CO_2 neutral biomass. In this context, many studies have assessed the main merits of use different natural resource for biodiesel production. Therefore, the current impetus of the research in the biofuel scope is towards the development of the bio-based economy. The use of green resources for biofuel production can make a significant contribution from the ecological and economic point of view. From green resources the production of food, feed, fuel and chemicals will be carried out together thinking in a biorefinery process, because some parts of plants are required for human food or animal feed production, while other parts have less potential for that purpose and are more useful for the production of fuels. Then, considering the vegetable oil is the most used feedstock for the biodiesel production, an important amount of vegetable oil cake is generated after oil extraction, and can reach up to 50% of the initial feedstock [56]. In relation to the by-products from oil processing for biodiesel productions, vegetable oil cakes can be utilized as raw materials in different processes for the production of chemicals and extraction of value-added products such as amino acids, enzymes, vitamins, antibiotics and biopesticides, according has been described in the literature [57, 58]. Besides, vegetable oil cakes may contain bioactive compounds with potential applications as functional products or protective agents against several diseases [25, 56, 59-61]. However, animal feed has been the main application of vegetable oil cakes until now, due to the composition and

nutritional value of the proteins contained in this by-product. For example, in the agro-industrial scope, defatted oil cakes have been studied for feed applications to poultry, fish and swine industry [62]. Therefore, under the biorefinery perspective, in the oil extraction process for fuel purposes, there is a big potential for conversion of this by-product into useful products or even as raw material for other industries.

In the United States, biodiesel is produced mainly from soybeans. Other sources of commercial biodiesel include canola oil, animal fat, palm oil, corn oil, waste cooking oil [63, 64], and jatropha oil [65]. However, the production of biodiesel from microalgal biomass appears to be a promising alternative because oil productivity exceeds production of oil crops, in addition to not compete with the production of food, fodder and other products derived from crops [3]. Therefore, in relation to the production of biodiesel from microalgae is expected an increasing market of production, thus in the short term defatted oil cake from microalgae also should have a destination and use. If alternative use of added-value is developed for this by-product the global cost of the biodiesel production from microalgae will be lowered. However, despite its inherent potential as a biofuel resource and the varied composition of its biomass for biorefinery, the co-production of multiple products from microalgae remains being a challenge.

Unlike of the vegetable oil cakes, the information about defatted cake oil from microalgae and their possible applications is scarce. However, it is well known the microalgae biomass mainly contain pigments, proteins, lipid compounds and sulfated polysaccharides. Consequently defatted oil cake from microalgae could be used potentially as a source of these compound present in the biomass and especially as source of protein, considering the

proteins correspond to one of the major fractions in this by-product from biodiesel production [32].

This review will focus on the oil cakes from oilseeds and defatted oil cake from microalgae from oil industry as potential source of proteins. In this context, the protein content and potential applications of these oil cakes with special emphasis on feed and food applications are discussed. Moreover current and future volume of production of vegetable and microalgae oil cakes is reviewed, because is a key issue to the potential uses that the food or feed industry could make of them.

2.2 Residual protein content in oil cakes post-oil extraction

As is mentioned above, vegetable oil cakes are by-product obtained after the extraction of oil from the plant part such as oilseeds, by expelling or solvent extraction [66]. Vegetable oil cakes have a recognized nutritional value; due to their protein content and amino acid profile they have been used as animal feed, especially for ruminants and fish. Their chemical composition has been widely studied by [62] and the protein content is presented in Table 2.1 with some microalgae species, comparing them it would be suggested a high potential for defatted oil cake from microalgae as source of protein and for elaboration of protein based products, in spite of the exact amount of protein differs from one type of microalgae to another as can be observed in Table 2.1.

On the other hand, in Table 2.1 it is possible to observe the protein content in different vegetable oil cakes, ranging from 18% to 47%, where soybean oil cake possesses the highest protein content (47.5%), followed by rapeseed oil cake (42.8%), sunflower oil cake

(34.1%), coconut oil cake (25.2%) and palm kernel oil cake (18.6%). Likewise from the Table 2.1 can be noted some species such as *Chlamydomonas rheinhardii, Chlorella pyrenoidosa, Chlorella vulgaris, Scenedesmus obliquus, Spirulina platensis and Synechococcus sp.* have an important protein content that even overcome the most popular source of protein of vegetable origin as is soybean.

Table 2.1. Protein content from different source expressed as % of dry matter.

Source	**% Crude protein**
Sunflower oil cake*	34.1
Soybean oil cake*	47.5
Rapeseed oil cake*	42.8
Palm kernel oil cake*	18.6
Coconut oil cake*	25.2
Chlamydomonas rheinhardii	48
Chlorella vulgaris	51-58
Euglena gracilis	39-61
Scenedesmus obliquus	50-56
Spirogyra sp.	6-20
Spirulina platensis	46-63
Synechococcus sp.	63

*Adapted from [62]Microalgae data are adapted from[33].

Another interesting feature of proteins is the amino acid profile, 20 amino acids exist of which 8 are essential. Human and animals require these essential amino acids sufficiently in their nutrition. Table 2.2 shows the comparison between main vegetable oil cakes and three different species of microalgae respect their amino acid profile. It is shown that a balanced diet can be made using microalgae instead of or in combination with another source of protein, such as vegetable oil cakes.

In general, the amino acidic profile of these vegetables oil cakes is comparable to the WHO/FAO protein reference (World Health Organization/Food and Agriculture Organization). Sunflower oil cake amino acid profile is high in sulfur-containing amino

acids, but low in lysine. Coconut oil cake is deficient in amino acids such as lysine, methionine, and threonine but high in arginine. The amino acid profile of palm kernel cake is poor in lysine, methionine and tryptophan but high in arginine. Soybean oil cake has highest protein content of about 48% and their amino acid composition compares favorably with WHO/FAO protein reference. The amino acid profile of soybean oil cake, shows is a good source of amino acids such as tryptophan, threonine and lysine in compare to WHO/FAO requirement. Although the amino acid profile of the proteins will be different according to the harvest or location, this data gives a good idea of the amino acid distribution in different vegetable sources.

Table 2.2. Amino acid composition of vegetable oil cakes in comparison with different microalgas species (% crude protein).

Source	Arg	Gly	His	Ile	Leu	Lys	Met + Cys	Phe + Tyr	Thr	Try	Val
Sunflower oil cake	9.1	5.6	2.8	4.2	6.9	3.5	4.0	6.5	3.4	1.4	5.8
Palm kernel cake	13.9	4.8	2.5	3.8	6.4	3.7	4.6	6.3	3.5	2.8	5.7
Coconut oil cake	11.0	4.2	2.1	3.0	6.0	2.5	1.9	7.2	3.0		5.8
Soybean oil cake	7.4	4.5	2.4	4.6	7.8	6.1	3.0	9	3.8	1.3	5.2
Chlorella vulgaris	6.4	5.8	2.6	3.8	8.8	8.4	3.6	8.4	4.8	2.1	5.5
Scenedesmus obliquus	7.1	7.1	2.1	3.6	7.3	5.6	2.1	8.0	5.1	0.3	6.0
Spirulina platensis	7.3	5.7	2.2	6.7	9.8	4.8	3.4	10.6	6.2	0.3	7.1
Egg	6.2	4.2	2.4	6.6	8.8	5.3	5.5	10	5.0	1.7	7.2
WHO/FAO	-	-	1.9	2.8	6.6	5.8	2.5	6.3	3.4	1.1	3.5

Adapted from (2007) [62]. Microalgae data are adapted from[33].

Likewise it can be seen that the amino acid profile of almost all microalgae is favorable compared with that of the reference protein (WHO/FAO). In general can be observed in Table 2.2 that microalgae contain adequate amounts of essential amino acids and thus constitutes a suitable protein source for the elaboration of protein based products.

2.3 Potential applications for protein based products from residual oil cakes

Due to the protein content and amino acid profile of vegetable oil cakes, some studies have investigated the protein fractions contained in this by-product from oil industry and the same time the opportunity of adding value them throughout of protein recovery. Until now, an important number of oil cakes from different oilseeds have been studied as source of proteins and as starting material for the development of different protein based products, mainly focused on feed, food and biomedical applications. Table 2.3 summarized research works based on proteins from various vegetable oil cakes, showing that some vegetable oil cakes were used as a source of protein for feed and food applications.

Table 2.3. Applications based on proteins from vegetables oils cakes.

Oil cake	**Description**	**Reference**
Rapeseed, canola, mustard, sesame	Nutritional value and digestibility of crude protein crude fibre and crude fat for feed applications	[67] [68] [69, 70]
Pumpkin, soybean wheat, palm	Production of protein hidrolysates with antioxidant activity for food applications	[44, 45, 48, 71]
Corn, wheat, soybean	Protein-based edible films for food packaging applications	[49]
Soybean	As substitute of synthetic polymer in electrospinnig for food industry and biomedical applications	[50-52]

2.3.1 Feed applications

The effect of feeding different levels of sesame oil cake on the intake and digestibility of crude protein, crude fibers and crude fat in Awassi lambs were investigated and the addition of sesame oil cake resulted in more daily gain and better feed conversion efficiency compared with the control without addition of sesame oil cake [70]. In similar work, canola meal was studied as feed ingredient in the diet of fish *Pagrus auratus*. The study demonstrated that canola meals used did not cause problems such as declining feed intakes or changes to blood levels of thyroid hormones when the meal was included in fish diets at 30% level [68]. In addition, groundnut oil cake is normally used on the growth performance of growing lambs, and mustard oil cake was evaluated as a cheaper alternative. The study suggested groundnut oil cake could completely be replaced with mustard oil cake without affecting feed intake, feed efficiency, nitrogen balance of lambs [69].

2.3.2 Food applications

In addition, Table 2.3 shows the production of enzymatic protein hydrolysate with antioxidant capacity from pumpkin oil cake, soybean oil cake, wheat and palm kernel cake. These hydrolysates with antioxidant properties have attracted great attention as potential natural replacers for artificial antioxidants (BHA, BHT, TBHQ) in food applications, as well as exogenous antioxidants in human nutrition.

As it was reviewed previously by [49] (Table 2.3), protein-based edible films, such us proteins from corn, wheat and soybean have received considerable attention in recent years because of their advantages, including their use as edible packaging materials, over the

synthetic films. In addition, protein-based edible films can also be used for the individual packaging of small portions of food, particularly products that are not currently individually packaged for practical reasons, such as beans, nuts and cashew nuts. In addition, protein-based edible films can be applied inside heterogeneous foods at the interfaces between different layers of components. They can be tailored to prevent the deterioration of inter-component moisture and solute migration in foods such as pizzas, pies and candies. Moreover, protein-based edible films can function as carriers for antimicrobial and antioxidant agents. Through a similar application they also can be used at the surface of food to control the diffusion rate of preservative substances from the surface to the interior of the food. Another possible application for protein-based edible films could be their use in multilayer food packaging materials together with non-edible films. In this case, the protein-based edible films would be the internal layers in direct contact with food materials. Through functions relating to mechanical and barrier properties, protein-based edible films may be able to substitute synthetic polymer films.

2.3.3 Electrospinning for food and biomedical applications

Electrospinning is a versatile fiber-forming technique that uses electrostatic force to produce submicron fibers. Due to their large surface area, electrospun fibers have unique properties well suited for various applications, including encapsulation of bioactives or drugs, tissue scaffolds, filtration, cosmetics, and protective clothing [50, 72, 73]. But electrospinning from residual protein from green material like deffated oil cake from oilcrops is scarce. In fact, a limited number of works has used electrospinning technique for the nanofibers formation from soybean proteins. In Table 2.3 a little works are summarized, one of them [50] hypothesized that ‘green’ materials, derived from renewable and

biodegradable natural sources, may confer bioactive properties to enhance wound healing and tissue regeneration.They optimized and characterized fibrous scaffolds electrospun from soy protein isolate (SPI). Fibrous mats electrospun from this plant protein remained intact without further cross-linking, possessing a skin-like pliability. Soy-derived scaffolds supported the adhesion and proliferation of cultured primary human dermal fibroblasts. These results suggest that 'green' proteins, such as soy, are promising as a platform for organotypic skin equivalent culture, as well as implantable scaffolds for skin regeneration. Future studies will determine specific mechanisms of their interaction with skin cells and their efficacy in wound-healing applications.

On the other hand, electrospun fibers from blends composed of soy protein isolate (SPI), poly(ethylene oxide) (PEO)and poly(lactic acid) were used for controlled release of a naturally occurring antimicrobial compound, allylisothiocyanate (AITC) [52]. AITC was encapsulated in β-cyclodextrin or added directly into the fiber-forming solutions. Scanning electron micrographs showed that the resulting electrospun SPI/PEO and PLA fibers possessed smooth morphology with diameters ranging from 200 nm to 2 μm. Release of AITC was negligible under dry conditions, but increased dramatically as relative humidity increased. The interactive behavior of these antimicrobial nanofibers may be promising in active packaging applications for foods.

2.4 Defatted cake oil from microalgae, a promising unexplored by-product

Currently, the utilization of microalgae as oil source for biodiesel production is in developing and optimization stage, therefore the alternative uses for defatted oil cake from microalgae have generated great interest in the last five years. The interest is justified due to the concurrent extraction of other valuable products in addition to lipids from algal

biomass may result in optimal extraction of value products and economically beneficial for algal technology [74]. The application of defatted oil cake from microalgae has been investigated by several research groups for different objectives. The main focus of these works can be divided in two main categories: first for energy production by utilizing the remaining carbon and hydrogen, and secondly for extracting products for their nutritional and economical values. In the energy production scope, [75] studied the hydrothermal liquefaction potential of defatted oil cake from microalgae for their conversion to liquid fuels. Similarly, [76] developed a two-stage process to produce hydrogen and methane gases from defatted oil cake from microalgae and [77] investigated the application of this by-product for bioethanol production. In relation to the extracting bioproducts from defatted oil cake from microalgae for their nutritional and economical values, the number of works is scarce. In this section the focus is put on the protein fraction of the defatted oil cake from microalgae. Table 2.4 summarizes the main work focused on microalgal protein. Worth mentioning that in this Table the main information in relation to the investigation developed in microalgal biomass and defatted oil cake from microalgae is presented. In Table 2.4 can be observed that, until now, the main focus about proteins from microalgae have been put on; evaluating the alkaline extraction from different microalgae species, by using different disruption cell technique. At the same time the nitrogen to protein conversion factor has been analyzing. On the other hand some authors have focused on studying the possibilities of application of these proteins, such as the production of enzymatic protein hydrolizates from biomass and also from a protein waste from *Chlorella vulgaris* for biomedical and food applications. [40-42] Have developed the enzymatic hydrolysis of protein waste which is normally discarded as low-cost animal feed, is a by-

product during production of algae essence from the microalgae *Chlorella vulgaris* in Taiwan. The results of these works suggests that the peptides from the protein hydrolysis could be potentially useful adjuncts in the treatment of gastric cancer and also the protein hydrolysate is an attractive alternative for producing a high value product for blood pressure regulation. On the other hand, [36, 37, 78] studied the enzymatic hydrolysis of the *Chlorella vulgaris* biomass focused in the developed of the functional ingredient for food applications. The findings of these works indicated that the protein hydrolysate was characterized by its high protein quality, mainly balanced amino acid content and a high in vitro protein digestibility, which accounted for its possible use as an additive for special food. Later studies demonstrated that *Chlorella* protein hydrolysate can be used for developing physiologically functional foods with immunopotentiating activity.

In the food scope, other authors have studied the functional properties of microalgal protein such as water and oil absorption capacities, emulsification and foaming capacity and stability, whose results have been compared with that of soybean meal. For instance, [79] investigated the defatted oil biomass with the objective of determining the appropriate use as functional food ingredients of three different species of microalgal biomass for use in human nutrition. They concluded that defatted oil biomass had functional properties comparable to those of soybean flour and their inclusion in spaghettis was globally good evaluated by the sensory panel.

Table 2.4. Research about proteins from microalgae

Microalga	Description	Reference
Amphidiniumcarterae, Dunaliella tertiolecta, Hilleasp., Isochrysis galbana and Skeletonema costatum	Protein extraction study (solvent/cake ratio time, grinding)	[80]
Chlorella, Chlamydomonas, Dunaliella, Rhodomonas, Synechococcus	Protein extraction study (NaOH concentration time, ultrasonic/vortex)	[81]
Chlorella sorokoniana	Protein extraction pH NaCl concentration Temperature	[82]
Asterionella formosa *Scenedesmus acutus* *Synechococcus spec.*	Time Temperature NaOH concentration Ultrasonic/peroxide	[83]
Nannochloropsis salina *Chlorella vulgaris* *and Haematococcus pluvialis*	protein electroextraction	[84]
Arthrospira platensis *Chlorella vulgaris* *Nannochloropsis oculata* *Haematococcus pluvialis*	Evaluating the effect of different cell disruption techniques on protein extractability in water	[85-87]
Chlorella vulgaris	Production of enzymatic protein hydrolysates	[36-38]
Chlorella vulgaris	Production of bioactive peptides by enzymatic hydrolysis.	[40-42]
Spirullina platensis *Porphyridium cruentum* *Nannochloropsis spp.* *Phaeodactylum tricornutum*	Functional properties	[54, 55, 79]
Haematococcus pluvialis *Nannochloropsis spp.*	Feed applications	[88, 89]

The potential of using defatted oil cake from microalgae as a protein source in animal feeds was also evaluated by [80, 81]. Overall, the results from both works are promising; for defatted oil cake from *Nannochloropsis spp.and Chlorella spp.* as a protein feedstuff in ruminant diets and defatted oil cake from *Haematococcus pluvialis* could be a valuable alternative protein and pigmentation ingredient in shrimp feed.

An important consideration in the extraction of all valuable products, from microalgae or defatted oil cake, is the effect of various treatments on the individual yields of such products. The algal biomass undergoes many processes during lipid extraction which invariably affects these coproducts. However, under realistic conditions, some damage or loss of these compounds is unavoidable as cell disruption would unbind them from cell mass to some degree by using different solvent during lipid extraction.

As has been described here, most of the research works developed until now in relation to protein extraction from microalgal biomass or defatted oil cake from microalgae have been focus on the cell disruption y extraction methods from microalgae biomass or defatted oil cake. However, studies investigating the possibility of using defatted oil cake from microalgae as source of protein for the development of food or food ingredients with functional or bioactive properties are still scarce.

Although the idea of using microalgae as a source of fuel is not new [90, 91], microalgae research is relatively young and many improvements can still be made to improve production and extraction efficiency. Especially taking account that the main bottleneck lies in the scale of production and the total production and processing costs. These costs are largely caused by the energy consumption of the different process steps in the total

production and extraction chain. Therefore when more products can be obtained from one microalgae production process, the value of algae will increase significantly and will make algae production economically feasible [92]. Consequently there is a big challenge in relation to the extraction of co-products from microalgae biomass and defatted oil cake and also there is a big challenge respect to the possible applications that can be projected to these co-products.

2.5 Potential market for proteins from defatted oil cakes

Although, the most of the carbon-based compounds currently manufactured by the chemical industry are derived from petroleum, the increasing cost and supply depletion of oil have made to pay attention on alternatives routes to making biorefinery from green biomass instead. In this context, many recent studies have assessed the relative merits to take advantage of the residual biomass available for the production of various products. Here, we highlight the volume of residual biomass as potential source of proteins from oilcrops and microalgae processing for biodiesel production. Hence, based in this information it will be possible to determine which sources are available at sufficient volumes to provide an opportunity for the protein recovery.

It is important to mention that the major imported sources for lipids and proteins in Europe are palm oil and soybeans and cake of soybeans (www.faostat.fao.org). Both these sources lead to deforestation in their region of origins [93, 94], thus further increasing the production of these crops is highly unwanted. Alternative sources for biofuels, vegetable oils or proteins derived from terrestrial crops, e.g. maize, sugarcane, rapeseed, contribute to water scarcity, forest devastation and they impose pressure on the food market[95].

Microalgae have much higher areal oil productivities and therefore less area will be necessary to produce similar quantities [96]. Moreover microalgae are not bound to arable land and can be grown in sterile places or even on the sea. Therefore, microalgal production is not competitive with other food production sources and a sustainable source for lipids and proteins.

2.5.1 Defatted oil cakes from oil crops: availability

Four by-products streams from the production of vegetable oil or biodiesel are the most important according to the volume of production. These are the oil cakes of rapeseed, sunflower, soybean, and oil palm. There has been a considerable increase in vegetable oil production during past decades, from 84 million tons in 2000 to 145 million tons in 2014 (FAO, 2012). As byproducts of vegetable oil production, large amounts of oil cakes are produced. Soybean oil cake is by far the largest available oil meal. In 2014, 179 Mt of soybean oil cake was produced worldwide [51], all of it being used for feed purposes [47]. The total potential of crude protein from soybean oil cake would be 85 Mt. Soybean meal is very suitable for feeding purposes because of the high protein content (a mass fraction of about 50%) and the amino acid profile which is relatively high in essential amino acids such as lysine.

Rapeseed meal is available at lower quantities than soybean meal, but worldwide production in 2014 was 64 Mt, so there is a potential of about 24 Mt of crude protein [52]. It is almost exclusively used as feed, although it is less rich in proteins than soybean meal.

Of sunflower meal, 15 Mt was produced worldwide in 2014, with a crude protein potential of about 6 Mt [53]. Sunflower meal is also used as feed, although it has a lower feed value

than soybean, because of the lower protein content.

Worldwide available amount of palm kernel meal in 2014 was about 6 Mt, giving a total crude protein potential of about 1 Mt [48,49]. The current application of palm kernel meal is as feed, but it is known to have only moderate nutritional value, making other applications interesting.

2.5.2 Defatted oil cake from microalgae: potential availability

There are no reliable worldwide production data about microalgae production for biodiesel or biocompound production. However, a research group of Wageningen University [92], estimated that the theoretical algal biomass needed to supply 0.4 billion m^3 of biodiesel (replacing diesel for the complete European transportation market) would provide us with about 0.3 billion of algal protein, considering the defatted oil cake about 40% rich in proteins. In addition the production of microalgae for fuels would place no pressure on the availability of rich agricultural areas for production of proteins; on the contrary, there is even the possibility of an over production of proteins[92]. This is a highly speculative number, but it shows that if there will be a sustainable and economically viable process for the production of microalgae in 15 years from now, the potential of proteins as co-products from algal biodiesel is very large. Hence, it is interesting the knowledge about the potential use for the by-product from microalgae as a source of compounds such as proteins, which may be recoverable to be used in food or feed industry. Moreover the search of alternative applications for a by-products stream, not only involves adding value to the main process, but also results in reducing the environmental impact.

2.6 Conclusions and future prospects

The main problem that has to be solved by biodiesel industry is the process feasibility. Nowadays to develop a profitable biodiesel industry it is necessary to be able to compete with fossil fuels, from an economical point of view. In this context, a biorefinery concept must be developed, using the totality of by- and bio-products. On the other hand, the increased global demand to meet the protein requirement in animal and human nutrition has given an impetus to the search for new nutritional sources. This review discussed the potential of proteins from biodiesel production as feedstock for feed or food industry. In this context, there is a diversity of species of crops and microalgae, which possess different content and types of proteins that are described and discussed here. This review is focused mainly on the proteins that different oil cakes from oil industry can provide, specially centered on potential applications. In fact, if only one-half of the protein present in the stream by-products from biodiesel production each year in the worldwide could be recovered, world protein supplies would be increased by several percent. However, it should be possible to produce both food and fuel from currently feedstock available such as oil crops or microalgae. Growing world needs for both food (especially protein) and fuel increase the possibility that such an integrated biomass-processing scheme will eventually become a commercial reality.

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Chapter 3:

Study of the protein extraction process from defatted oil cake from green microalgae *Botryococcus braunii*

Study of the protein extraction process from defatted oil cake from green microalgae *Botryococcus braunii*

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Abstract

Currently microalgae are a promising source of lipids for biofuel production. However recent studies have shown that an integrated biorefinery approach is needed to make the microalgae biofuel production profitable. In this context, protein recovery from defatted oil cake from microalgae, present an important opportunity of use for this by-product. The main objective of the present work was to study the protein extraction from defatted oil cake from green microalgae *Botryococcus braunii* and obtaining a protein concentrate. Through preliminary experiments, suitable levels of independent factors pH, temperature and solvent/cake ratio were fixed to be studied in a factorial design. A 2^3 factorial design was performed to study the effect of the factors on the soluble protein extraction. Once the experiments were carried out, a significance test was performed to the data obtained. The *F*-test and *p*-value indicated that the effect of pH was the most significant on the protein extraction, followed by the interaction of the temperature and solvent/cake ratio, the

temperature, and finally the solvent/cake ratio. Besides, ANOVA confirmed that the factors above mention were the only statistically significant ($p < 0.05$). Finally a maximum of 25.3 % w/w of protein was solublilized at pH 12, 40 ºC, and solvent/cake ratio of 90 mL/g with 30 min of extraction. Later, the protein was recovered by isolectric precipitation at pH 4. These conditions allowed the preparation of protein concentrate with a protein content of 74% w/w. Thus protein yield in the protein contentrate preparation was of 33% w/w using defatted oil cake from *B. braunii* as the starting material. In adittion protein concentrate showed an important amount of essentials amino acid (41.65%) in relation to the reference pattern of FAO for human nutrition, which demonstrated its potential as source of proteins.

3.1 Introduction

Microalgae are photosynthetic microorganisms which use light energy and CO_2 for production of high-value compounds [97]. For this reason, several species of microalgae have been studied for a number of purposes: as source of protein for food applications [36-39, 54, 55, 98]; for rearing of mollusks, crustaceans and fish, and also for the culture of zooplankton [99-102]; production of various chemical compounds, such as lipids[103-106], polysaccharides [107, 108], natural coloring pigments [109-112], antioxidants [41, 42, 113-115], antimicrobial agents [115-117], production of hydrogen and hydrocarbons [13, 14, 118]; conversion of the residual biomass into gas (methane) by fermentation[118-120]; depuration of waste water [121, 122].

In recent times, there has been an increasing interest in oil processing from microalgae for biodiesel production process. In current literature, microalgae appear to be one of the most important sources of renewable biodiesel that is capable of meeting global demand for

transport fuels. However, the use of this oil for biodiesel production generates a solid residue “defatted oil cake from microalgae”, which has been poorly described. In order biodiesel from microalgae can become environmentally friendly and economically feasible, alternative uses for such by-product should be sought. It is known that defatted oil cake from microalgae is rich in protein and carbohydrates, but until now it has been mainly used for animal feed. To date, only a few papers have been published in relation to possible applications to this by-product and no report has been found related to microalgae oil cake from *B. braunii*, which is studied in this work.

On the other hand, the alarming rate of population growth has increased the demand for food production in third-world countries leading to a gap between demand and supply. This has led to demand for formulating innovative foods from new proteinaceous sources. In this regard, numerous studies and patents have been focused on the use of protein content available in agroindustrial residues from oil industry. Traditionally, nutritional supplements derived from plants have been used and have predominated in the market. Nevertheless, health benefits of aquatic microorganisms, such as microalgae, are being investigated and more recognized and appreciated in the last three to four decades [34]. *Botryococcus braunii* is a green colonial microalga belonging to the family Chlorophyceae and is regarded as a potential source of renewable fuel because of its ability to produce large amounts of hydrocarbons. Depending on the strain and growth conditions, up to 75% of algal dry mass can be hydrocarbons and is grouped into three different races, A, B, and L, depending on the type of hydrocarbons they synthesize. This alga is mostly known for the production of hydrocarbons, exopolysaccharides, and carotenoids [123]. Because of these characteristics of *B. braunii*, this microalga was chosen to be studied as part of a

consortium project, developed in Chile, for the development of biodiesel from microalgae. Even though a considerable amount of works have been carried out on the characterization of *B. braunii*; lipid composition [13, 22], effect of culture condition on hydrocarbon production [13, 14, 18, 20, 23, 124] extraction methods for recovery of lipids [19, 125-127], there is no works developed about protein content and/or amino acid profile of *B. braunii*. Therefore the challenge of studying the defatted oil cake after oil extraction as a source of protein is an opportunity to provide more knowledge about this species and to propose an alternative use for defatted oil cake from green microalgae *B. braunni*. Certain strains of microalgae have been studied as a potential source of proteins. Some works about protein content and quality have been developed on species such as *Chorella vulgaris*, *Spirullina platensis*, *Chlorella sp.* (CS-247), *Chlorella sp.* (CS-195), *Stichococcus sp.* suggesting their use as food supplement [37, 40, 100, 102, 128, 129]. According to the literature, the protein extraction from microalgal cell is commonly achieved by treating the samples for a range of times at a fixed temperature with dilute sodium hydroxide. However, researchers argue for the need that extraction method has to be optimized for each algal species [81, 83].

In relation to the protein extraction to obtain products rich in proteins, concentrates or isolates, from meal, cake or any protein feedstock, is a process for eliminating non protein compounds [130]. The mostly used methods for protein extraction are selective processes based on solubility of protein as pH function. These techniques take advantage of the solubility of proteins, which is normally high at very alkaline pH (9-11) or very acid pH (2-3) [131]. It is also possible to observe a minimum solubility in the vicinity of the isoelectric pH, where the net charge of the protein is zero, and therefore reduces the electrostatic

repulsion which promotes aggregation. Hence, the most common method consists of blending feedstock with water at alkaline pH, the supernatant is recovered by centrifugation and later dried. In this case, the product obtained is a protein concentrate, where undesired compounds are still present (fiber, sugars, fats, phenols, antinutritional factors, etc.). The non-protein compounds can be eliminated in a further stage by means of isoelectric precipitation or ultrafiltration and the final product it is a protein isolate [130]. Some authors, including Vioque et al. (2001) classify protein concentrates and isolates products, according to the methodology used in their preparation. However, in the international Codex Alimentarius (International Food Standards) these products require at least 70% and 90% of protein (N x 6.25) on a dry basis for the classification of protein concentrates and protein isolates respectively. These two classifications are not always coincident, being possible to have protein concentrates and isolates with lower protein content than stipulated by the Codex Alimentarius. In this thesis it was decided to denominate the protein products according to the Codex Alimentarius definition. Finally, in the elaboration of the protein products is important to consider that the protein content in the final product is strongy influenced by the solubilization of the protein reached during alkaline extraction. Some of the parameters most studied that are involved in the protein extraction by alkaline extraction are pH, extraction temperature, and extraction time and solvent/cake ratio. In relation to the background described, the specific objective of this work was to study the factors involved in the protein extraction process from defatted oil cake from *B. braunii* to find out the conditions under which the protein solubilization is maximal.

3.2 Material and methods

3.2.1 Material: defatted oil cake from green microalgae B. braunii

The green microalga *Botryococcus braunii* LB572 race B from UTEX was provided by the unit of microbiology from the Universidad de Antofagasta, Antofagasta, Chile. For the development of experimental activities defatted oil cake from *B. braunii* was used. First, the microalgae biomass was dried in a tray dryer, then ground in a grinder and finally sieved to reserve the fraction < 425μm. Dry biomass was defatted by soxhlet methodology using petroleum ether as solvent. The defatted oil cake from *B. braunii* was preserved in plastic boxes for further use.

3.2.2 Preliminary experiments

The protein extraction from defatted oil cake from *B. braunii* was carried out by alkaline extraction using water as solvent medium and NaOH 4M to adjust pH. In preliminary experiments the effect of the pH, time, temperature and solvent/cake ratio was studied. Each factor was evaluated at different levels, keeping the rest of the factors constant. The protein extraction (P_e) was expressed as the ratio between soluble protein and dry weight of defatted microalgae cake oil from *B. braunii*, according to Eq. 3.1,

$$P_e = \frac{C_p V}{w_d} * 100 \qquad \text{(Eq. 3.1)}$$

Where C_p is protein concentration (mg mL^{-1}) determined by Lowry [132], V is the volume of water used for the extraction (mL), w_d is mg of dry defatted oil cake from *B. braunii*.

i) Effect of pH on protein extraction: 0.5 g of defatted oil cake from *B. braunii* were weighed and suspended in 9.5 mL of water. Twelve experiments were prepared in the same way and pH was adjusted from 1 to 12. The pH was adjusted with NaOH 4M and HCl 4M, and the samples were shaken for 30 min, 150 rpm at 25ºC. Afterwards, each tube was centrifuged and the soluble protein content of the supernatant was measured by method of Lowry (1951).

ii) Effect of extraction time on protein extraction: 0.5 g of defatted oil cake from *B. braunii* were weighed and suspended in 9.5 mL of water. The pH was adjusted at 9.5 with NaOH 4M. Nine experiments were prepared in the same way and extraction time was evaluated at 10, 20, 30, 40, 50, 60 and 70 min. The experiments were performed at 25ºC and 150 rpm. Afterwards, the samples were centrifuged and the soluble protein content of the supernatant was measured by the method of Lowry (1951).

iii) Effect of temperature on protein extraction: 0.5 g of defatted oil cake from *B. braunii* were weighed and suspended in 9.5 mL of water. The pH was adjusted at 9.5 with NaOH 4M. Four experiments were prepared in the same way and the temperature was evaluated at 25, 40, 55, 60 ºC, respectively. The experiments were performed at 150 rpm. Afterwards, the samples were centrifuged and the soluble protein content of the supernatant was measured by the method of Lowry (1951).

iv) Effect of solvent/cake ratio on protein extraction: 0.5 g of defatted oil cake from *B. braunii* were weighed and suspended in the increasing water quantity, 5, 10, 15, 20, 25, 30, 35, 40, 45 mL. The pH was adjusted at 9.5 with NaOH 4M. The samples were shaken for

30 min, at 150 rpm and 25ºC. Afterwards, the samples were centrifuged and the soluble protein of the supernatant was measured by method of Lowry (1951).

3.2.3 Study of the effect of the factors on protein extraction by factorial design

Based on preliminary experiments, the levels for the factors pH (x_{pH}), temperature (x_T), and solvent /cake ratio ($x_{s/c}$) were determinedand and fixed. A factorial design 2^3 (three factors and each one at two levels) was developed for the study of the effect of the factors and its interactions on the protein extraction (P_e). The complete design consisted of 35 experimental points including three replications in the center point and the experiment was carried out in a random order, as it can be seen in the design matrix in Table 3.2, where it describes the run order, standard order and the coded and real values of the factors. The response protein extraction is also included in the Table 3.2

Once the experiments were carried out, a data analysis was made to find the coefficient of the factors on the protein extraction calculated by Yates algorithm, determining the statistical significance using statistical $t_{\alpha,ab(n-1)}$, which is *p*-value coming from *t* distribution for α=0.05 and *ab(n-1)* degree of freedom. Besides, a fit of the values of response can be obtained according to the Statistical Model of Fixed Effects. Finally, a validation of the prediction model was made by ANOVA [133].

Table 3.2. Design matrix for the study of the protein extraction from defatted oil cake from microalgae: Standard order, run order, coded and real values of the factors and the experimental and fitted value extraction.

Run order	St. order	x_{pH}	x_T (ºC)	$x_{s/c}$ (mL/g)	P_e *-observed* (%)	P_e *-predicted* (%)
1	5	- 1 (8)	- 1 (40)	- 1 (40)	10.5	13.14
2	26	+1 (12)	- 1 (40)	- 1 (40)	22.0	22.26
3	8	- 1 (8)	- 1 (40)	- 1 (40)	11.4	13.14
4	12	+1 (12)	- 1 (40)	- 1 (40)	21.3	20.46
5	1	- 1 (8)	+1 (60)	- 1 (40)	12.9	13.93
6	4	+1 (12)	+1 (60)	- 1 (40)	16.2	21.25
7	27	- 1 (8)	+1 (60)	- 1 (40)	15.7	13.93
8	9	+1 (12)	+1 (60)	- 1 (40)	24.5	21.25
9	30	- 1 (8)	- 1 (40)	+1 (90)	15.0	14.94
10	18	+1 (12)	- 1 (40)	+1 (90)	22.0	20.46
11	34	- 1 (8)	- 1 (40)	+1 (90)	13.4	14.94
12	17	+1 (12)	- 1 (40)	+1 (90)	25.3	22.26
13	28	- 1 (8)	+1 (60)	+1 (90)	9.0	9.14
14	21	+1 (12)	+1 (60)	+1 (90)	14.7	16.46
15	29	- 1 (8)	+1 (60)	+1 (90)	11.3	9.14
16	31	+1 (12)	+1 (60)	+1 (90)	15.9	16.46
17	7	- 1 (8)	- 1 (40)	- 1 (40)	12.8	13.14
18	6	+1 (12)	- 1 (40)	- 1 (40)	21.0	20.46
19	24	- 1 (8)	- 1 (40)	- 1 (40)	14.6	13.14
20	25	+1 (12)	- 1 (40)	- 1 (40)	19.5	20.46
21	23	- 1 (8)	+1 (60)	- 1 (40)	13.5	13.93
22	33	+1 (12)	+1 (60)	- 1 (40)	21.1	21.25
23	16	- 1 (8)	+1 (60)	- 1 (40)	13.7	13.93
24	11	+1 (12)	+1 (60)	- 1 (40)	21.8	21.25
25	22	- 1 (8)	- 1 (40)	+1 (90)	13.4	14.94
26	3	+1 (12)	- 1 (40)	+1 (90)	20.2	22.26
27	13	- 1 (8)	- 1 (40)	+1 (90)	15.2	14.94
28	19	+1 (12)	- 1 (40)	+1 (90)	23.0	22.26
29	20	- 1 (8)	+1 (60)	+1 (90)	8.9	9.14
30	15	+1 (12)	+1 (60)	+1 (90)	15.1	16.46
31	10	- 1 (8)	+1 (60)	+1 (90)	10.7	9.14
32	32	+1 (12)	+1 (60)	+1 (90)	15.5	16.46
33	35	0 (10)	0 (50)	0 (65)	17.7	16.45
34	2	0 (10)	0 (50)	0 (65)	18.6	16.45
35	14	0 (10)	0 (50)	0 (65)	18.5	16.45

3.2.4 Preparation of protein concentrate from defatted oil cake from B. braunii

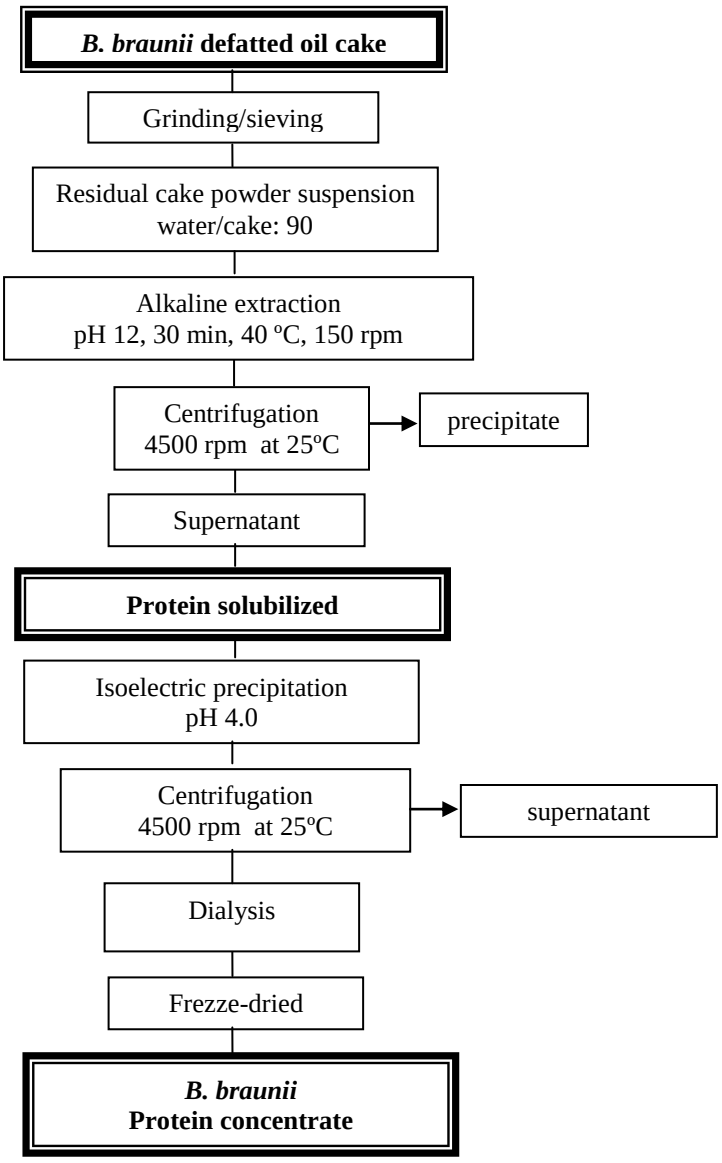

Figure 3.1. Schematic diagram of alkaline extraction and isoelectric precipitation process for production of protein concentrate from *B. braunii* defatted oil cake.

The alkaline extraction process for the proteins from defatted oil cake from *B. braunii* was studied by factorial desing as was described above. From the results of the factorial design a combination of the operational factors was determined to obtain a maximum of protein extraction within the range of conditions studied. These conditions were used for the solubilization of proteins and subsequently the precipitation at isoelectric ponit was carried out for obtaining a protein concentrate from defatted oil cake from *B. braunii*. Thus, protein solubilization was performed under the conditions; pH 12, 40ºC and solvent/cake ratio of 90 mL/g, using 30 min of extraction in an orbital skaker at 150 rpm. Later, supernatant was collected by centrifugation at 4400 rpm for 15 minutes, and was adjusted at pH 4 with 4M HCl to precipitate the protein. The precipitate was recovered by centrifugation (4500 rpm for 15 min), washed with distilled water at isoelectric pH and finally freeze-dried. The steps for the protein concentrate preparation are presented in Figure 3.1.

For the protein concentrate preparation, the protein extraction yield, was calculated as the ratio betwen the protein content in the protein concentrate and the protein content in the defatted oil cake from *B.braunii* (protein content determined by Kjeldalh method).

3.2.5 Chemical characterization of protein concentrate

The chemical composition (moisture content, lipids, crude protein, crude fibre, and ash) of the protein concentrate from defatted oil cake from *B. braunii*, was analyzed according to the AOAC (1995) procedures [134].

The amino acid profile was determined using high-performance liquid chromatography (HPLC) with an AccQ-Tag column and coupled to a UV detector (626-LC System) at 254

nm. The samples were hydrolyzed by acid hydrolysis for 18 h at 115 °C with 6M HCl containing 0.1% phenol in the absence of O_2. The samples were filtered and then derivatized using buffer and reagent (Kit AccQ-Fluor). Finally, concentrations of amino acids were calculated with respect to the curve under the area obtained for the internal standard (2.5 mM α-aminobutyric acid). For elution conditions, the sample injection volume was 5 µL, and the eluents used were AccQ-Tag, acetonitrile and Milli-Q water.

3.2.6 Mass balance calculations for protein concentrate preparation

The residual fractions of either step were quantified and mass balance was calculated on a moisture-free basis in order to estimate the recovery of proteins from the defatted oil cake from microalgae. The protein content of the *B. braunii* deffated oil cake and protein concentrate was determined by kjeldahl method to calculate the protein yield for the protein concentrate preparation.

3.3 Results and Discussion

3.3.1 Preliminary experiments for the protein extraction

For obtaining a vegetable protein isolate, for instance soya, the following steps are required, solubilization and extraction of proteins at alkaline pH followed by isoelectric precipitation, concentration and drying [135]. The same steps have been described by Chronakis to obtain a protein isolate from microalgae [54]. However to maximize the protein content in the final product, the extraction or solubilization at alkaline pH can be very influential [136, 137]. Therefore, the first step in this work was to develop preliminary experiments where

the factors pH, time, temperature, and solvent/cake ratio on protein extraction from defatted oil cake from *B. braunii* were studied.

i) Effect of pH on the protein extraction

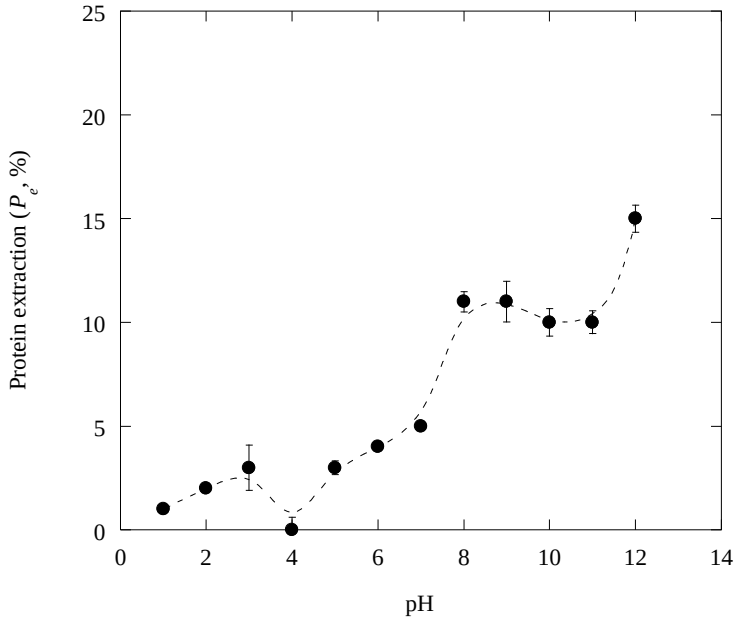

Figure 3.2. The effect of pH (x_{pH}) on the protein extraction (P_e) from defatted oil cake from *B. braunii*. Conditions of extraction: 30 min, 150 rpm, 25 ºC, s/c 20 mL/g.

As it can be observed in Figure 3.2, the results revealed that the region of minimum solubility (isoelectric point) for the proteins presents on defatted oil cake from *B. braunii* is at pH 4.0. After reaching the isoelectric point, the protein extraction showed a progressive rise with further pH increase, achieving a maximum extraction at pH 12. According to these results, a range of the maximum solubility was observed between pH 8-12, values that are very close to the range reported for the maximum extractability of proteins from *Spirulina platensis*: pH 8 to 10 [39]. Therefore, with the objective to carried out experiments that allow knowing the possible interactions of pH with other operational factors, the levels to

be studied in the factorial design, for the factor pH were chosen for the low and high level 8 and 12 respectively (see Table 3.2).

ii) Effect of time on the protein extraction

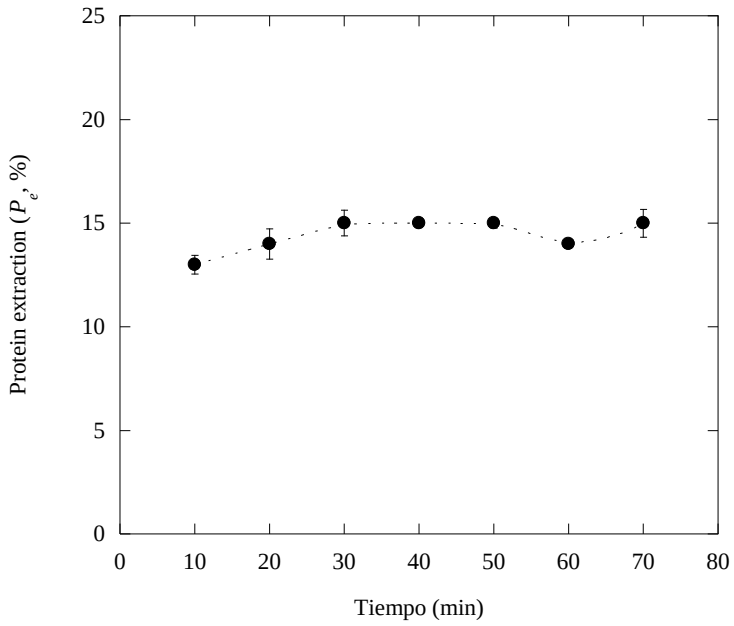

Figure 3.3. The effect of time (x_t) on the protein extraction (P_e) of defatted oil cake from *B. braunii*. Conditions of extraction: pH 9.5, 150 rpm, 25 ºC, s/c 20 mL/g.

Previous work carried out by Raush et al. (1981) on different microalgae species, proved that the protein extraction from microalgae depended on time. The study developed by Raush showed important differences in between species studied; for instance, to achieve an optimum protein extraction from *Scenedesmus acutus*, 90 min of extraction at 100ºC was necessary. In contrast, the maximum protein extraction from *Synechococcus sp* was observed betwen 5-15 min of extraction. Similar results were observed for the protein extraction from defatted oil cake from *B. braunii* as can be seen from Figure 3.3, where the

protein extraction seems to have occurred at 5-10 minutes of extraction. According to the protein extraction and the time required, it could argue that the protein extracted correspond to the soluble protein, which readily diffuse into the aqueous phase (alkaline medium in this case) in the first 5-10 min. This would explain why a greater time of treatment no showed an increase of protein extraction under the conditions studied. Therefore, in the range studied (10 - 70 min) can be noted that the protein extraction is not affected by the time, thus this factor was not included to be studied in the factorial design.

iii) Effect of temperature on the protein extraction

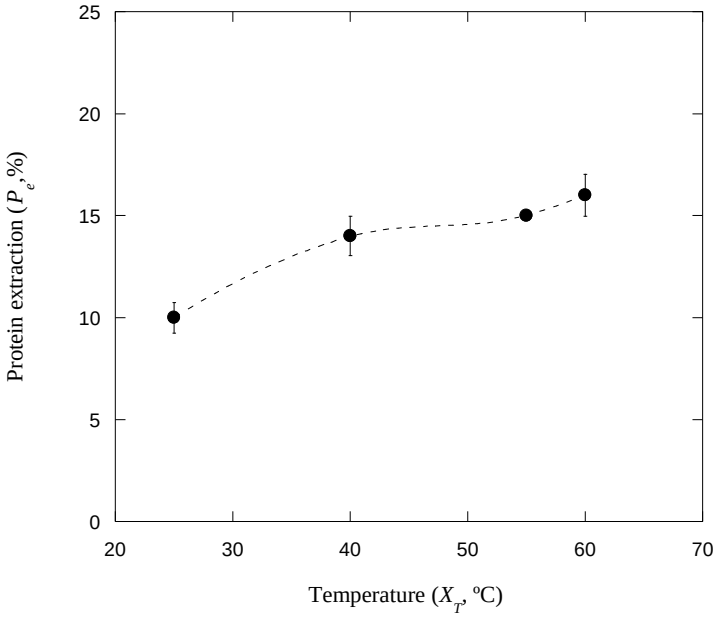

Figure 3.4. The effect of temperature (x_T) on the protein extraction (P_e) from defatted oil cake from *B. braunii*. Conditions of extraction: pH 9.5, 30 min, 150 rpm, s/c 20 mL/g.

As the results show in Figure 3.4, the experiments carried out in the orbital shaker showed an increase in the protein extraction as the temperature increased from 25 ºC to 60 ºC. *Raush* (1981) evaluated the effect of the temperature on protein extraction for three microalga species: *Scenedesmus acutus, Synechococcus sp. and Asterionella Formosa*.

Raush found different protein extraction yield for temperatures between 50 to 100ºC in between the species, which was explained because of differing cell wall structures of the species. For species with thicker walls, more temperature was required to increase the protein extraction, showing extraction temperatures about 100ºC. In similar way in our experiments, the effect of the temperature showed the maximum protein extraction at 60ºC. Although this temperature seem to be very high for the extraction of proteins, taking into account a possible denaturation, this result would reflect the structural characteristic of *B. braunii* wall which is especially resistant to disruption [138] [139, 140]. Based on the results presented here, and considering the possible damage of the proteins at temperatures over 60 ºC, it was defined to study the effect of temperature at a range of levels from 40 to 60 ºC (see Table 3.2)

iv) Effect of solvent/cake ratio on the protein extraction

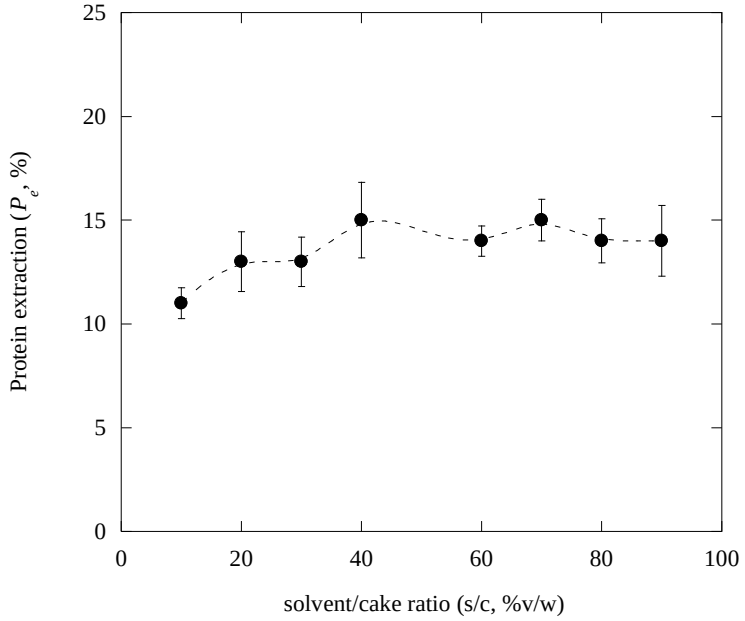

Figure 3.5. The effect of solvent/cake ratio ($x_{s/c}$) on the protein extraction (P_e) from defatted oil cake from *B. braunii*. Conditions of extraction: pH 9.5, 30 min, 150 rpm, 25 ºC.

The results of the effect of the solvent/cake ratio from the experiments carried out with defatted oil cake from *B. braunii* are shown in Figure 3.5. As can be seen increasing the ratio of solvent to sample can lead to higher protein extraction as was reported in the literature for protein extraction from fish waste [141]. In the experiments with defatted oil cake from *B. brauni*, the protein extraction seems to have a maximum at solvent/cake ratio between 40 and 70, with a tendency to the leveling off in this range. Therefore this factor was to include in the factorial design to elucidate its influence and studying possible interaction with other variables. It was defined to study the effect of this factor at solvent/cake ratio values between 40 and 90 for low and high level respectively (see Table 3.2), focused on obtain a maximum protein extraction between the levels chose.

3.3.2 Study of the effect of the operational factors on protein extraction process by factorial design.

Once the experiments were carried out, the values of the protein extraction were analyzed to find the main effects of the factors on the responses, determining those factors statistically significant with $\alpha = 0.05$. The values of the protein extraction for the set of experiments (from 1 to 35) are given in Table 3.2. The effects of the factors and their interaction on the protein extraction it is present in Table 3.3, where *t*-test and *p*-value statistical parameters were used to confirm the significance of the factors studied. These results showed that the effect of x_{pH} was the most significant, followed by the interaction of $x_T * x_{s/c}$, x_T and $x_{s/c}$ which also can be observed in the Pareto chart (Figure 3.7).

Table 3.3. Effect of the factors and interactions on the response protein extraction. Coefficient, Standar error, *t*-value and *p*-value are included.

Factor	Effect	Coefficient	Std. err.	*t*-value	*p*-value
Constant	-	16.284	0.3010	54.09	0.000
x_{pH}	**7.319**	**3.659**	**0.3010**	**12.16**	**0.000**
x_T	**-2.506**	**-1.253**	**0.3010**	**-4.16**	**0.000**
$x_{s/c}$	**-1.494**	**-0.747**	**0.3010**	**-2.48**	**0.020**
$x_{pH}\, x_T$	-1.81	-0.591	0.3010	-1.96	0.061
$x_{pH}\, x_{s/c}$	-0.469	-0.234	0.3010	-0.78	0.441
$x_T\, x_{s/c}$	**-3.294**	**-1.647**	**0.3010**	**-5.47**	**0.000**
$x_{pH}\, x_T x_{s/c}$	-0.344	-0.172	0.3010	-0.57	0.573
x_{Ct}		1.982	1.0282	1.93	0.065

Among the factors that were statistically significant (Table 3.3, bold numbers), pH showed to be the most influent operational factor on the protein extraction. The positive value of this effect means that the highest value of the response occurs at the highest level of the factor (pH=12). In the same way, for the factors with a negative effect, the highest value of the response protein extraction (P_e) is observed at the lowest level of the factor (-1), as is the case of the temperature with a negative effect (-2.506).

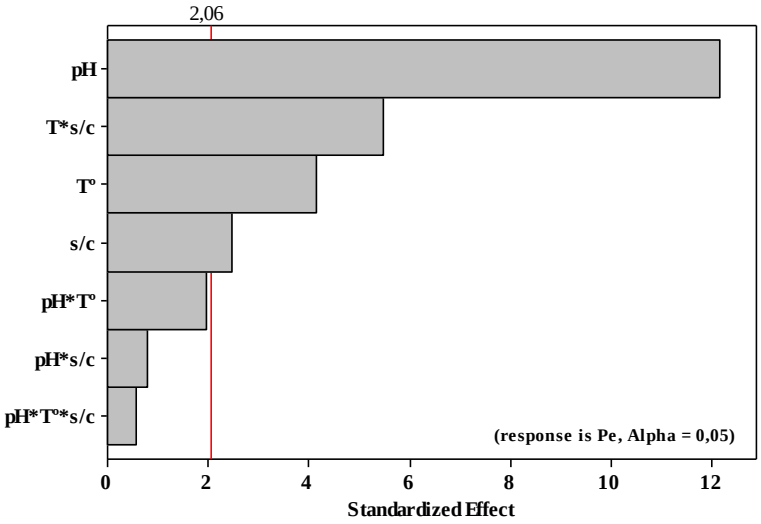

Figure 3.6. Pareto chart for protein extraction at a 0.05 significant level.

Respect to the effect of the factor s/c ratio on the protein extraction, the results shown a negative effect of magnitude 1.494, which means that the experiments carried out at the lowest level of the factor (40 mL/g) would achieve the highest value for the protein extraction. However, the effect of the T*s/c interaction is negative (-3.294) and higher in magnitude than the effect of s/c ratio. Therefore, the interaction T*s/c can increase the protein extraction value only when the factor s/c ratio take the highest value, in this case 90 mL/g. In addition, from Table 3.2 the best conditions for protein extraction according to the real and fit values were pH 12, 40 ºC and 90 mL/g, achivieng values for the protein extraction of 25.3%. This fact can be understood on base of the mass transfer occurred in the process of protein extraction corresponding to the solid-liquid extraction. Mass transfer, proteins in this case, from the defatted oil cake, is favored with a highest concentration gradient, thus will be governed by the amount of liquid available, being the mass transfer greater at greater liquid presence. In adittion pareto chart shows the plot for main effects of individual and interations of the factors. Figures 3.7 and 3.8 shown the effect of the individual factors and the bifactorial interaction on protein extraction, respectively. The effect of the pH on the protein extraction in compare to the factors temperature and solven/cake can be observed from Figure 3.7, where it is evident that the magnitude of the effect of pH is greater than the effect of the other two factors may even mask them.

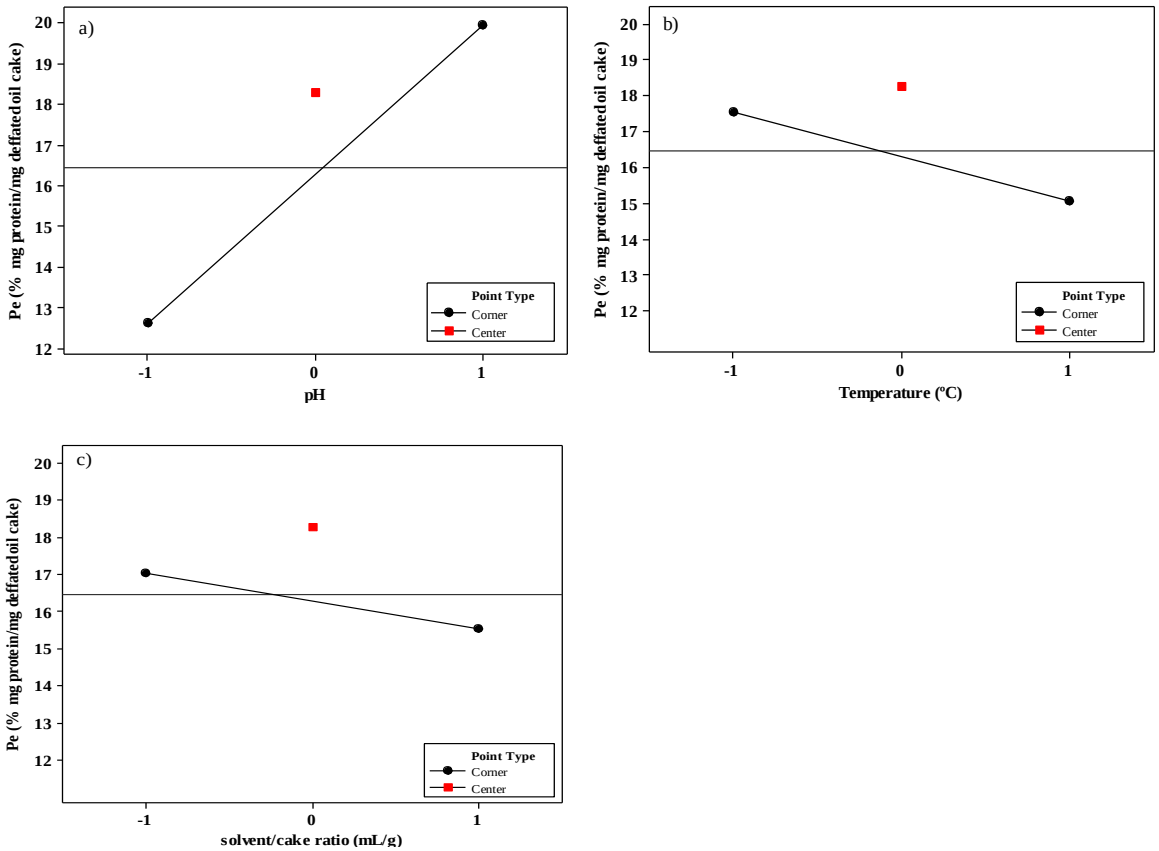

Figure 3.7. Plots for main effect of individual factors on protein extraction (P_e). a) pH b) temperature and c) solvent/cake ratio.

From Figure 3.8 could be confirmed that only the bifactorial interaction T * s/c ratio showed to be significant and maximal protein extraction could be obtained at the highest value of the s/c ratio (90 mL/g).

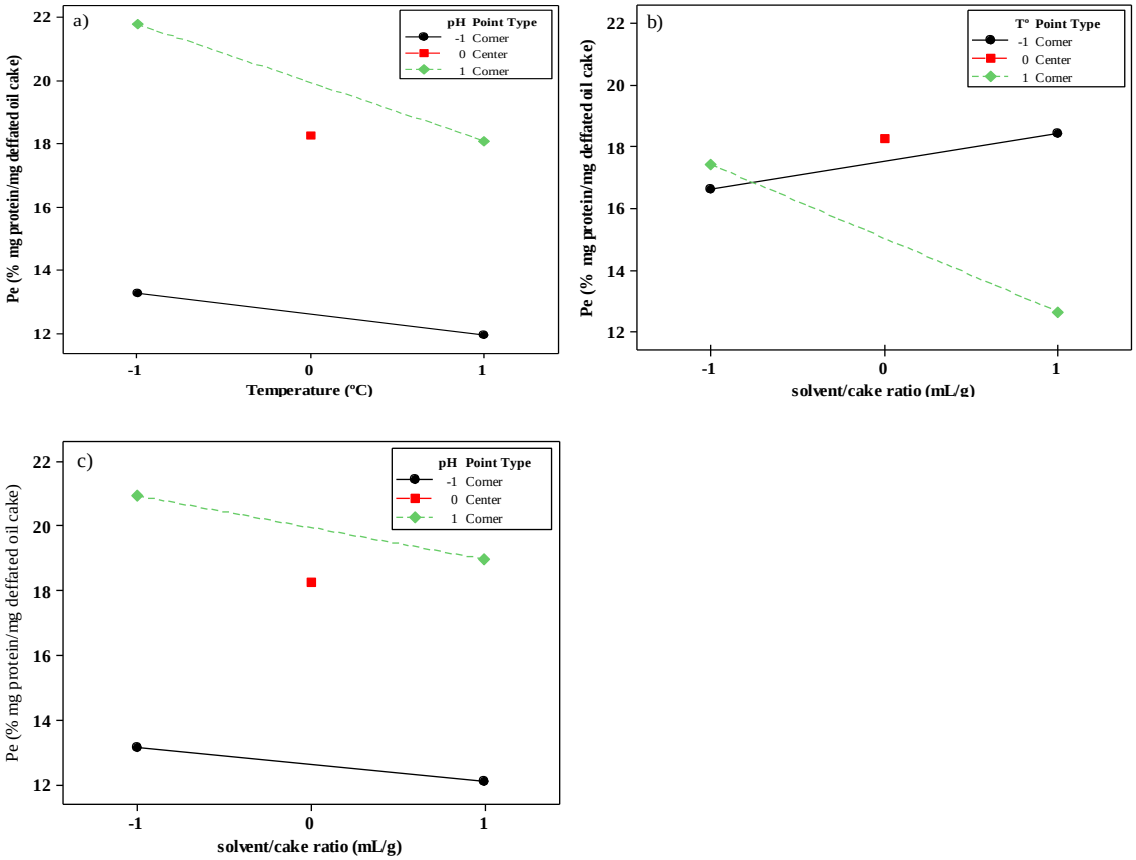

Figure 3.8. Plots for interaction effect of the factors on protein extraction (P_e). a) pH* temperature b) temperature and solvent/cake ratio c) pH*solvent/cake ratio.

Additionally, from the analysis of the results, was possible to obtain a model of prediction for the response protein extraction. This model relates the values of the protein extraction with the coded values of the factors and their interactions. The obtained model with only significant factors according to Table 3.3, corresponds to the Equation 3.2 presented below:

$$P_e = 16.284 + 3.659x_{pH} - 1.253x_T - 0.747x_{s/c} - 1.647x_T x_{s/c} \quad \text{(Eq.3.2)}$$

R^2= 0.854 ; R^2_{Adj}= 0.834

The value of the coefficient of multiple determinations R^2 of this model is close to 1, indicating that the regression model fits the experimental data well. Beside, the adjusted coefficient of multiple determination R^2_{adj} demonstrated that only significant terms have been included in the model, as their value is close to R^2. In addition the difference in between the theoretical and experimental values can be observed from Table 3.2. Accordind this results can see that the difference is very small, confirming the predictive ability of the equation. Subsequently, the analysis of variance (ANOVA) was used to evaluate the adequacy of the fit (see Table 3.4).

Table 3.4. ANOVA applied to the analysis of the effects for the response protein extraction the factorial design.

Source of variation	Degrees of freedom	Sum of square.	Mean square	*F*-value	*p*-value
Regression	4	583.40	145.85	43.74	0.000
Residual	30	100.04	3.33		
Lack of fit	4	24.64	6.16	2.12	0.106
Pure error	26	75.40	2.90		
Total	34	683.45			

Regarding the test for the lack of fit, it did not result to be significant (*p*-value = 0.106 > α = 0.05), it can be stated that the model given by Eq. (3.2) fits the experimental data well and that it can be used to predict P_e at any value of the factors, within the range studied.

Finally, the combination of the factors where P_e was maximal within the studied ranges was: pH 12, 90 mL/g solvent/cake ratio and temperature of 40 ºC and 30 min of extraction, according to the theoretical determination. Thus, in this work, through factorial design was possible to find the most influential factors on the protein extraction from defatted oil cake from *B. braunii*. Additionally, from the analysis of the results in this work was possible to obtain an empirical model capable of predicting the values of the protein extraction for a

range of values of the factors, where the factors and their interactions were included (Eq. 3.2). This model obtained had a suitable correlation, confirming the accuracy of the model (see ANOVA Table 3.4).

In relation to the significance of the pH effect, can be understood on the basis of the overall ion charge of the protein with pH. As pH increases to the alkaline region, the amino groups dissociate into $–NH_2$ and $–H^+$, and the overall protein charge becomes negative due to the presence of –COO groups and can consequently be hydrated and dissolved in water. Respect to the effect of the interaction of temperature and solvent/cake ratio, this interaction resulted to be the second factor most important on the response studied (see Figure 3.7) and the results of this interaction $x_T\, x_{s/c}$ showed a negative effect on the protein extraction yield. In this case the decrease in protein extraction (P_e) can be explained due to thermal degradation of the proteins as well as the temperature increases. Sathe *et al*. (1982) reported the formation of insoluble aggregates with sulphur-rich proteins in soybean flour when heated to 70 ºC and above. Aggregation, according to the authors, depends on several factors which among them is the heating medium. Since the extraction was carried out in aqueous medium, the decrease in the extraction of the protein might be due to reduced solubility of this protein aggregates at temperaturas above 40 ºC [142]. This explanation is valide for the effect of the temperature of protein extraction, being the third most influential factor in this study.

Finally, through the study of the protein extraction process by factorial design was possible to determine the effect of bi and tri-factorial interaction which by conventional planning of the experiments is not possible to observe. From this study the best conditions for the

protein extaction from defatted oil cake from *B. braunii* were determined and the protein concentrate was obtained after isoelectric precipitation.

3.3.3 Chemical composition of protein concentrate from defatted oil cake from green microalgae B. braunii

As the main characteristic of defatted oil cake from microalgae is their high protein content, the first step was determining the chemical composition of *B. braunii* biomass and defatted oil cake from *B. braunii*. The protein content for *B. braunii* biomass, defatted oil cake and protein concentrate is shown in Table 3.5. These values differ from those found by Dayanada *et al.* (2010) for *Botryococcus sp*., where the protein content in lyophilized biomass was 20%. This difference can be explained due to the proportion of constituents in the microalgae biomass varies with the strains, the race it belongs to and the physiological and cultural conditions [14, 124].

Table 3.5. Chemical and nutritional composition of *B. braunii* biomass, *B. braunii* oil cake and *B. braunii* protein concentrate.

Constituent (%)*	***B. braunii* biomass**	***B. braunii* defatted oil cake**	***B.braunii protein concentrate***
Protein	50.91 ± 1.23	48.10 ± 0.06	74.83 ± 2.89
Fat	17.43 ± 0.34	3.96 ± 0.04	3.18 ± 0.42
No nitrogen extract	14.30 ± 0.14	22.31 ± 0.05	15.37 ± 2.21
Fibre	6.83± 0.54	4.44 ± 0.01	1.98 ± 0.04
Ash	10.66 ± 0.26	21.16 ± 0.18	4.62 ± 0.01

*The constituents are expressed as g 100 g $^{-1}$ of dry matter**.**

However, the results found here are comparable with those reported for other freshwater microalgal species considered as possible sources of protein such as *Chlorella*

vulgaris (51-58%) [36, 38, 53], *Scenedesmus obliquus* (50-56%) [53], *Spirulina platensis* (46-63%) [39, 54]. Thus, the protein content of *B. braunii* oil cake (48.10%) allows considering it as feedstock for the production of protein products such as protein concentrate, isolates, hydrolysates and other non food applications.

According to the results of the factorial design for the protein extraction by alkaline method, the maximal protein extraction was of 25.3 % w/w. Under the conditions for the maximal protein extraction a protein concentrate was prepared after isoelectric precipitation, according to scheme in Figure 3.1, with a final protein content of 74% dry weight basis (determined by kjeldahl), as can be observed in Table 3.5. Comparing the values of the constituents for the samples analyzed from Table 3.5, the protein concentrate showed a reduction on fat, fibre, ash, and no nitrogen extract, respect to the defatted oil cake. As it was expected, an important rise of the protein content, about 55%, was observed in the protein concentrate (74%) in compare to defatted oil cake (48%). The difference observed in between each constituent for both samples analyzed, can be explained because of the solubility of the constituent. The protein extraction process consisted of alkaline extraction using water as solvent adjusted at alkaline pH. Subsequently a selective precipitation at isoelectric pH was carried to eliminate the non proteinaceus material, thus the final product is a protein concentrate with higher protein content in compare to the defatted oil cake from *B. braunii* and lower content of non-protein component. Additionally, from Table 3.5 a slight difference on protein content can be observed when comparing the biomass and defatted oil cake. However, this slight decrease could be explained because of some soluble proteins could be co-extracted in the process of lipids extraction, as has been described in similar works [143].

It is important to mention that the algae protein isolation method used in this study was a modification of the method used for the preparation of soy protein isolates. Normally, soy protein isolates are produced from hexane-defatted soy flakes and the protein content in the protein isolate is at least 90% on dry-basis. The lower protein content of the concentrate from defatted oil cake from *B. braunii* in compare to soybean, can be probably the result of the type of proteins present and their actual physiological function in the cell. Storage proteins that are present in protein bodies, such as those in soybeans, are usually easily extractable, while proteins that are associated with cell walls may be very difficult to extract, as can be the proteins present in *B. braunii*. However, the protein content of the concentrate elaborated from defatted oil cake from *B. braunni* (74%) is comparable to the protein content published in similar works based in microalgae as source of protein; being higher in compare to protein isolate elaborated from *Spirullina platensis* biomass with a final protein content of 68% [54], and higher than the protein content of the protein fraction prepared from *nannochloropsis spp.* biomass (40.5%) [98].

Later, amino acid composition of protein concentrate from *B. braunii* oil cake was determined and the results are presented in Table 3.6 in comparison to the *Chlorella vulgaris* hydrolysate and FAO/WHO/UNU (1985) amino acid requirement pattern. From these results it is possible to conclude that the protein concentrate from *B. braunii* oil cake contains adequate amounts of essential amino acids in relation to the FAO pattern, thus constitutes a suitable protein source for the elaboration of protein hydrolysate or as potential feed/food supplement. Additionally, the protein concentrate showed an essential amino acid ratio (essential/total amino acid %), of 41.65%, which proved its nitrogenous equilibrium according to the nutritional recommendations.

Table 3.6. Amino acid composition and chemical score of protein concentrate from defatted oil cake from *B. braunii* (g of amino acid/100 g protein).

Amino acid	*B. braunii* Protein concentrate	*C. vulgaris* hydrolysate	FAO pattern[a]
Aspartic acid	6.90	10.60	
Glutamic acid	7.85	14.30	
Serine	5.63	3.20	
Glycine	9.97	5.20	
Histidine	1.30	2.10	
Arginine	7.33	5.70	
Threonine	9.47	4.30	0.9
Alanine	11.26	11.20	
Proline	9.29	5.10	
Valine	5.87	8.00	1.3
Methionine + Cysteine	1.96	1.80	1.7
Fenilalanina + Tyrosine	9.89	7.50	2.4
Isoleucine	3.41	3.80	1.3
Leucine	6.29	9.20	1.9
Lysina	3.45	6.90	1.6
Essentials/total (%)	41.65	44.70	

a FAO/WHO/UNU (1985).

Although the essentials content of protein concentrate from *B. braunii* oil cake (41.65%) was lower than *Chlorella vulgaris* hydrolyzate (44.70%), amino acid profile of the protein concentrate showed higher contents of serine, glycine, threonine, arginine, proline and sulfur amino acids and aromatic amino acids. Finally, these results confirm that protein concentrate elaborated from defatted oil cake from *B. braunii* contain all essential amino acids and these are present in adequate amount in relation to the FAO pattern [144].

3.3.4 Mass-Balance calculations

The protein concentrate preparation from defatted oil cake from *B. braunii* was carried out according to the methodology described in section 3.2.4, under the alkaline conditions determined by factorial design. The preparation under alkaline extraction and later isoelectric precipitation, allowed a protein yield of 33% (w/w) respect to the defatted oil cake. In Figure 3.9 the mass balance is presented for the protein concentrate preparation from defatted oil cake from *B. braunii.*

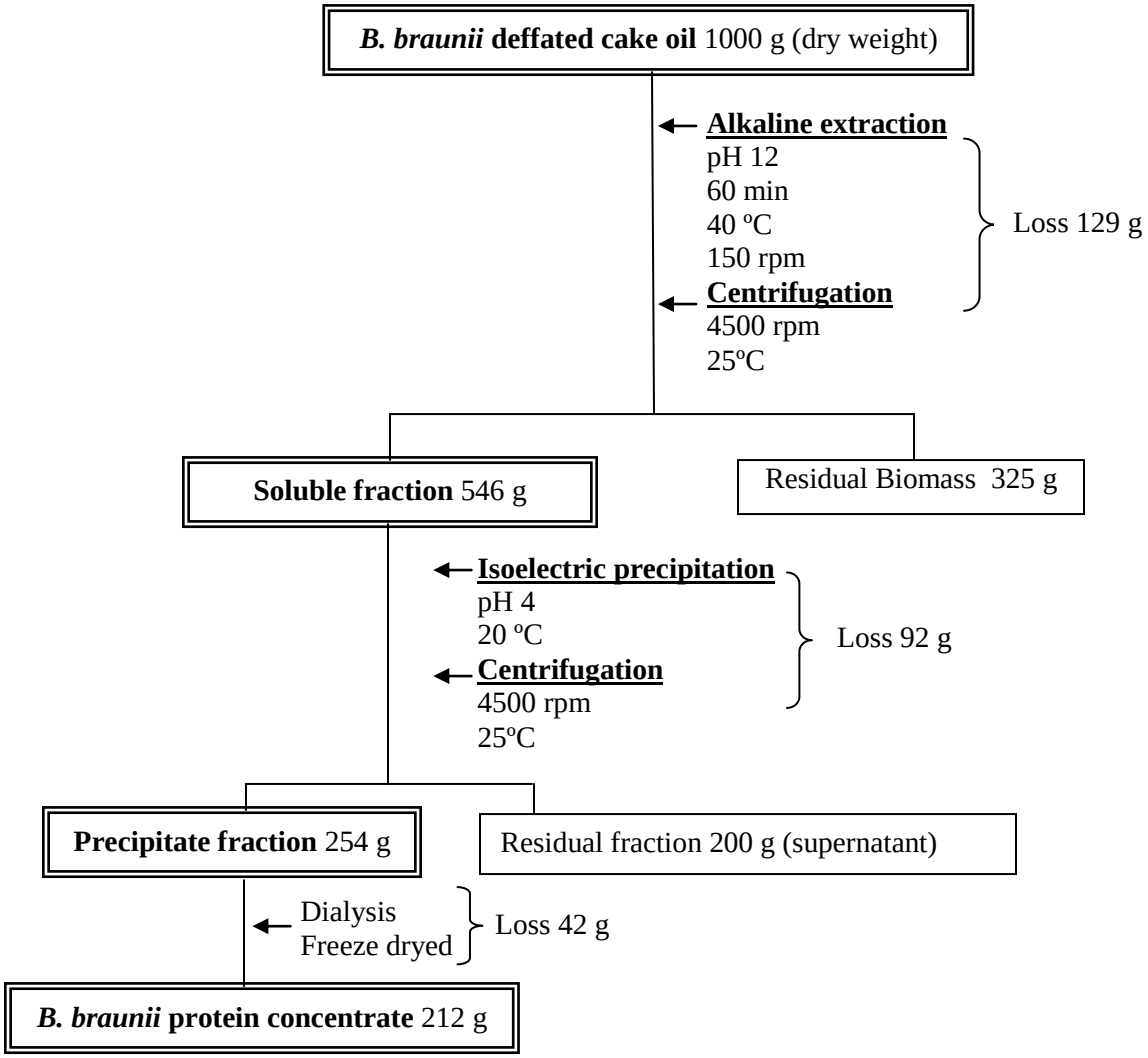

Figure 3.9. Mass balance calculations for the production of the protein concentrate from defatted oil cake from green microalgae *B. braunii*.

3.4 Conclusions

In this work the study of the operational factors, for maximizing the protein extraction from defatted oil cake from *B. braunii,* demonstrated that the protein extraction can be represented through a mathematical model as a function of pH, temperature and solvent/cake ratio and interaction of temperature and solven/cake ratio. At the same time the elaboration of a protein concentrate rich in protein (74%) was possible through the study of these operational factors involved in the extraction. In addition, a maximum protein extraction of 25.3% (w/w) was reached under the experimental conditions; pH 12, 30 min, 40ºC and s/c ratio of 90 mL/g. Moreover, the chemical composition of the protein concentrate from *B. braunii* oil cake, demonstrated to have interesting protein content (74%) and high quality in relationship to the content of the essencial amino acid comparable to the FAO protein reference. Finally, 33 % (w/w) of the proteins from defatted oil cake from *B. braunii* was extracted by means of the protein concentrate elaboration under the conditions determined by factorial design. These results confirmed the potential of this by-product of microalgae, from biodiesel production process, as a source of protein for protein concentrate elaboration for potential food aplications.

Chapter 4:

Electrospun Protein Concentrate Fibers from Microalgae Residual Biomass

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Electrospun protein concentrate fibers from microalgae residual biomass

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Abstract

Currently there is a growing interest in developing novel bioproducts and biomaterials derived from renewable sources that can reduce the dependence on fossil fuel feedstock. In this study a protein concentrate from microalgae *Botryococcus braunii* residual biomass (MPC) from a biorefinery process was used as a biopolymer to develop ultrafine fibers by electrospinning. Experiments were designed to study the effect of different formulations of MPC, poly(ethylene oxide) (PEO) and pH on morphology and diameter of fibers. The results indicated MPC fibers from acidic solutions prepared at pH 1 had smoother and smaller diameter than those fibers from alkaline solutions (pH 12). Moreover, under the conditions studied, it was conclude that pH and the concentration of MPC were the most significant factors in determining the diameter and morphology of the fibers obtained. Fourier transform infrared analysis showed there is a slight frequency shift for the secondary structure of MPC as induced by change in pH of the polymer solutions. Likely

this change in the protein structure improved the physical chain entanglement in the polymer blend. The results of this work revealed a potential to develop fibers from MPC from residual biomass by a promising technique that may find many end-use applications.

4.1 Introduction

Microalgae are photosynthetic microorganisms that use light energy and CO_2 to produce high-value compounds [97]. Recently, there has been an increasing interest in oil processing from microalgae for the production of biodiesel. In the literature, microalgae appear to be one of the most promising sources for the production of renewable biodiesel that is capable of meeting global demand for transport fuels [20, 28, 29, 51, 52, 54, 95, 126-128]. The extraction of oil from microalgae for biodiesel production results in considerable solid residue in the form of microalgae residual cake or residual biomass. In order to fully utilize algae feedstock, development of alternative uses for the residual biomass should be sought.

It is known that microalgae residual cake is rich in protein and carbohydrates, which are mainly being used as low-value animal feeds. To date, only a few studies have focused on exploring the utilization of this biomass residue, and to our knowledge, there is no report related to studying protein concentrate from *B. braunii*, especially on the conversion of protein concentrate into fibers.

Nowadays biopolymers are of great interest in various industries, giving rise to a broader utilization particularly in fields such as biomedical sciences, pharmaceuticals, cosmetics, and other related fields [145]. Electrospun fibers produced from biopolymers have been investigated extensively due to their biodegradability and biocompatibility. For example,

several proteins have been successfully converted into nanofibers using electrospinning, including silkworm silk, collagen, elastin, fibrinogen and soy protein. According has been reported, the resulting protein nanofibers could be useful as biological scaffolds for tissue engineering, hemostatic bandages or wound dressings, among others [130-136]. These protein fibers are also being explored for biosensors and in the medical and biomedical sectors [146]. Moreover has been described by Scheibel (2005) that protein fibers can also be employed as carrier molecules in therapeutic applications to induce oral tolerance to certain drugs [147].

Electrospinning is a process that uses an electrical field to control the formation and deposition of polymers [148]. In electrospinning, a polymer solution is stretched with an electrostatic force. Here, the polymer solution is charged positively or negatively with DC electrical potential, typically in the order of 10 to 20 kV. When the charge repulsion overcomes the surface tension of the solution, a polymer jet is ejected from the solution and directed toward a grounded collector, during which the solvent evaporates from the jet and a continuous solidified fiber is formed on the collector as a non-woven mat [139-142] . This technique produces fibers of diameter ranging from tens to hundreds nanometers [129, 138, 140, 143, 144].

Many fibrous proteins have been electrospun, such as collagen and gelatin, which can be electrospun readily as was above described. However, globular proteins must be unfolded to enable electrospinning [51, 149]. One of the common methods used to disrupt the quaternary structure is to use a combined alkaline and thermal treatment. At a pH well above the isoelectric point the protein unfolds and solubilizes, exposing hydrophobic and sulfhydryl groups. These groups may form hydrophobic and disulfide bonds, which have a

great impact on the stability of the protein network. With respect to the solvents used in electrospinning solutions, although organic solvents such as trifluoroethanol (TFE) and hexafluoro isopropanol (HFIP) have been used with good results in the electrospinning of proteins [145, 146], these solvents are toxic and not suitable for various applications. A biocompatible polymer poly(ethylene oxide) (PEO) is commonly used to overcome the difficulty of spinning aqueous polymer solutions. PEO is a low-cost, water-soluble and biocompatible polymer, which has already been used in biomedical applications, such as the development of wound dressings. The goal in this work was to study the electrospinnability of *B. Braunii* MPC, obtained from the microalgae residual biomass, as a biopolymer to produce fibers and to evaluate the effect of different formulations on the morphology and diameter of these fibers. In addition, by using the protein concentrate from microalgae residue, this project exploited low cost starting material to develop functional nano or microfibers with different fibers morphologies, thereby adding value to the microalgae residual biomass. The multifunctional and bioactive characteristics of the electrospun microalgae protein concentrate may also open up other niche applications, such as in biomedical areas.

4.2 Materials and methods

4.2.1 Materials

Microalgae oil cake from *Botryococcus braunii* was used as a feedstock for the preparation of protein concentrate. Microalgal protein concentrate was prepared according to Fig. 4.1 as we have previously reported [150]. The protein was extracted by dissolving defatted algal

powder in dilute alkali (pH 12), centrifuging at 4500 rpm at 25°C, followed by collecting the supernatant (supernatant A).

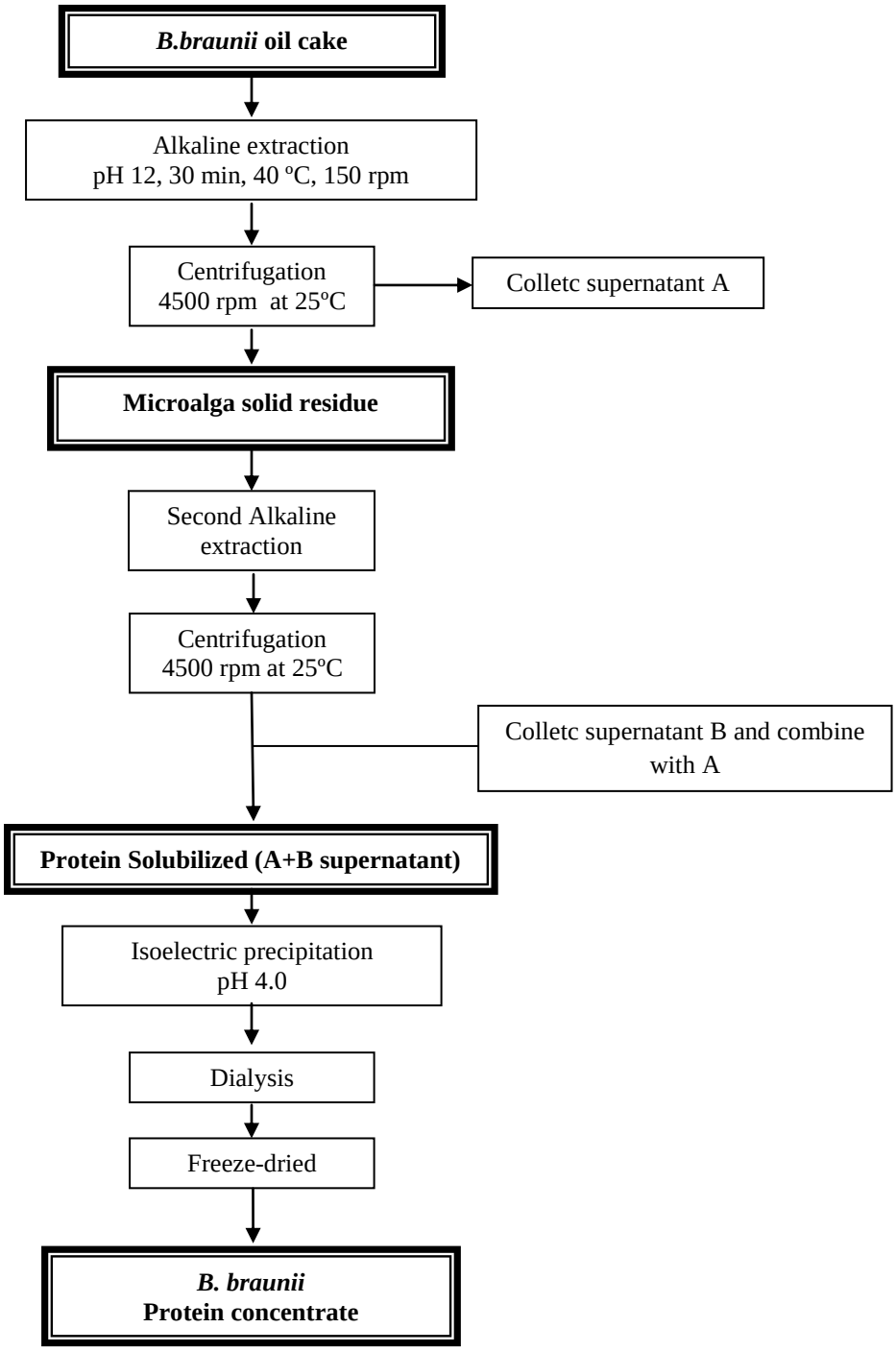

Figure 4.1. Schematic diagram of alkaline extraction and isoelectric precipitation process for preparation of protein concentrate from *B. braunii* residual biomass.

The pellet was collected and re-dissolved again in alkali followed by a second centrifuge treatment at 25 °C and 4500 rpm, from which supernatant B was collected. Both supernatants A and B were adjusted to pH 4 with 4 M HCl to precipitate the protein. The precipitate was recovered by centrifuge at 4500 rpm for 15 min at 25 °C. The pellet was dialyzed against distilled water for 3 days at 4 °C using a cellulose dialysis membrane (MWCO 6000–8000, Spectra/Pore, Spectrum Laboratories, Inc., CA, USA). Finally, the protein pellet was freeze-dried and stored until used. The nitrogen content of protein concentrate was determined according to the Kjeldahl method (a factor of 6.25 was used to convert nitrogen to protein) [134]. Protein concentrate was found to contain 71.7% protein. The chemical reagents such as hydrochloric acid and sodium hydroxide were purchased from Merck S.A. Chile. PEO (Mw ≈ 900,000), glacial acetic acid was purchased from Sigma Aldrich Company Canada (Oakville, ON).

4.2.2 Preparation of polymer solution for electrospinning

Preliminary experiments of three different combinations of MPC and PEO concentrations at pH 7 and pH 12, were conducted according to the methodology described by Vega-Lugo and Lim (2008) (Table 4.1). Concentrations of the solutions in all formulations were in w/w basis. Polymer solutions were prepared by adding PEO and MPC powders either in distilled water, aqueous sodium hydroxide (1%) solution, or glacial acetic acid to give neutral, alkaline, or acidic solutions, respectively. Polymer solutions were stirred for 12 h at room temperature to achieve complete dissolution of MPC. A two-level factorial experimental design was conducted to investigate the effect of MPC and PEO concentration and pH on the morphology of the fiber (Section 4.2.6). Electrical conductivity and pH of polymer

solutions were measured using a pH-conductivity meter (Accumet XL-20, Fisher Scientific, Ottawa, ON, Canada).

Table 4.1. Preliminary experiments for electrospinning *B. braunii* protein concentrate solutions.

	Solution composition (% w/w)		
Experiment	**PEO**	**MPC**	**pH**
1	2	2	7
2	2	2	12
3	0.8	10	12

4.2.3 Electrospinning of the polymer solutions

Electrospinning experiments were conducted at room temperature. Polymer solutions were placed into a 2.5 ml plastic syringe fitted with a 16-gauge blunt-end stainless steel needle spinneret. A positive electrode from a high voltage DC supply (Model PS/ER30P10.0-11 Glassman High voltage, High Bridge, NJ) was attached to the stainless steel needle. The syringe was loaded onto an infusion pump (Model KD 200, Kd Scientific, Holliston, MA) that delivered the polymer solution to the spinneret at a flow rate of 0.4 mL/h. A circular steel collector (26.5 cm diameter), positioned 25 cm away from the spinneret, was electrically grounded and covered with aluminum foil. Table 4.2 summarizes the process parameters used in the electrospinning of MPC.

Table 4.2. Electrospinning processing parameters.

Spinning parameters	**Working range**
Flow rate (mL/hr)	0.4
Temperature (ºC)	20
Collector distance (cm)	25
Operating voltage (kv)	15-20

4.2.4 Scanning Electron Microscopy (SEM)

The morphology of the fibers was analyzed using SEM (SEM S-570, Hitachi High Technologies Corp., Tokyo, Japan) at an accelerating voltage of 10 kV. Samples were coated with gold (20 nm) using a sputter coater (Model K550, Emitech, Ashford, Kent, England). The average diameter of the fibers was determined by measuring 50 different points from the SEM image, using image visualization software (Pro-Plus 5.1, Media Cybernetics Inc., Bethesda, MA).

4.2.5 Fourier Transform Infrared (FTIR) Spectroscopy analysis of MPC fibers

The fibers obtained were scanned using an FTIR spectrometer (IR Prestige-21; Shimadzu Corp., Tokyo, Japan) equipped with L-alanine doped triglycine sulfate (DLaTGS) detector. A MIRacle ATR accessory equipped with a diamond crystal (Pike Technologies, Madison, WI) was used for sampling. The background spectrum was collected using an empty ATR cell. The spectra were recorded between 4000 and 650 cm^{-1} with an 8 cm^{-1} spectral resolution. For each spectrum, 32 scans were co-added. The data were analyzed using IRSolution Software (Shimadzu Corp., Tokyo, Japan).

4.2.6 Design of experiments

A factorial design 2^3 was developed to evaluate the effect of the factors MPC concentration, PEO concentration and pH and their interactions on the responses fiber diameter and fiber morphology (Y_d and Y_m). Based in preliminary experiments the work levels for each factor were defined. Moreover, taking in account that the literature establishes that the extreme pH induces important effects on the conformation and

aggregation of the proteins, explaining the behavior of the proteins as a biopolymer, the range of pH studied included the extreme alkaline, acidic and neutral pH. The full design consisted of 16 experimental points and three replications in the center point (Table 4.3). Once the experiments were carried out, a data analysis was performed to find the values of the effect of the factors on Y_d and Y_m, which was calculated by Yates algorithm. The statistical significance was calculated using t distribution for α=0.05 and ab(n-1) degree of freedom. Subsecuently, a fit of the values of response could be obtained according to the statistical model of fixed effects (Montgomery, 2001). Data analysis was performed using Minitab 15 statistical software.

Table 4.3. Factors, levels of the factors and response values for the factorial design of the experiments.

Exp	%MPC	%PEO	pH	Conductivity	Mean fiber diameter (nm)	Morphology
	w/w	w/w		mS/cm		index
1	5	0.8	12	14.12	205	0.28
2	10	0.8	12	6.30	667	1
3	5	0.8	1	0.33	303	0.88
4	10	0.8	1	0.36	602	0.83
5	5	1.4	12	7.99	217	0.85
6	10	1.4	12	4.22	769	0.96
7	5	1.4	1	0.45	231	0.90
8	10	1.4	1	0.57	265	0.88
9	5	0.8	12	17.50	212	0.70
10	10	0.8	12	6.350	770	1
11	5	0.8	1	0.321	256	0.9
12	10	0.8	1	0.398	653	0.8
13	5	1.4	12	8.391	220	0.8
14	10	1.4	12	4.129	634	1
15	5	1.4	1	0.421	192	0.8
16	10	1.4	1	0.521	320	0.9
17	7.5	1.1	6.5	-	-	-
18	7.5	1.1	6.5	-	-	-
19	7.5	1.1	6.5	-	-	-

Diameter and morphology of the fibers are important determinants in many applications. In fact, as has been demonstrated by many studies found in the literature, for most applications, it is always desirable to have smooth, ultrafine nanofibres with uniform diameter to increase surface activity of the fiber. For this reason we considered them as responses in the experimental design. The average diameter of the fibers was determined by measuring from the SEM image as was described in section 4.2.4. and fiber morphology was measured as is described below.

Determination of morphology of the fibers

Fiber morphology is a qualitative characteristic of the fibers, thus it is necessary to define a procedure to quantify it as a numerical response, as follows and was previously described by Rodrigo et al. (2012) [151]. All fibers, agglomerates, beads, on the micrograph, for each experiment, were utilized to estimate the morphology index. Each micrograph was divided into 20 portions, as illustrated in Figure 4.2.

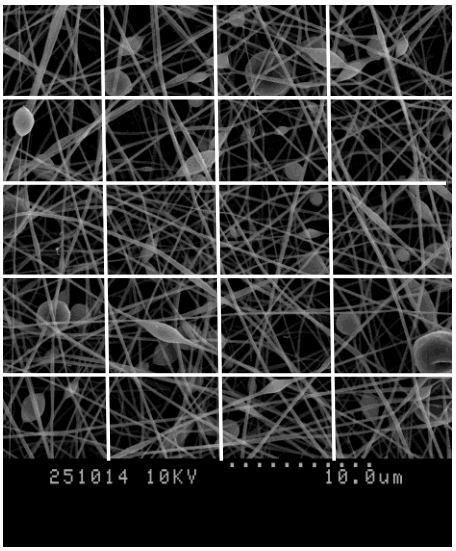

Figure 4.2. SEM micrographs of electrospun MPC/PEO fibers for morphologic index valuation.

Scores of beads and agglomerations were assigned for each micrograph portion. If at least one sphere, bead or agglomeration were present, the portion was assigned to one point (1). Otherwise, a zero point (0) was allocated. Finally, a compatibility matrix was performed to obtain final score for each experimental. To calculate the morphology index, Eq. (4.1) was used. With this procedure, fibers with smoother morphology had index values closer to unity.

$$Morhphology\ index = 1 - \frac{N^{o}_{beads} + N^{o}_{agglomerations} + N^{o}_{spheres}}{N^{o}_{features} * N^{o}_{portions}} \qquad \text{Eq. 4.1}$$

where N^{o}_{beads} is the number of portions with beads feature, $N^{o}_{agglomerations}$ is the number of portions on the micrograph with presence of agglomeration and $N^{o}_{spheres}$ correspond to the numbers of portions with spheres. $N^{o}_{features}$ is equal to 3 and $N^{o}_{portions}$ is 20.

4.3 Results and discussion

4.3.1 Preliminary experiments of electrospinning of B. braunii protein concentrate

MPC solutions without PEO could not be electrospun. Increasing the voltage caused the pendant droplet at the tip of the syringe to break away as droplets, rather than forming a polymer jet. These results might be due to the solution being electrically charged, but it did not acquire enough repulsion force to overcome its surface tension to form a fiber jet [135, 138, 149]. This difficulty with electrospinning of aqueous protein solution has been attributed to the polyelectrolyte characteristics of polypeptide macromolecules, which are

capable of bearing many charges accompanied by counterions that weaken the charge density required for spinning [152]. In preliminary experiments (Table 4.1), three experiments were performed to confirm the electrospinnability of MPC with the addition of PEO. These blend polymer solutions of MPC and PEO were spinnable, as depicted by the micrographs in Figure 4.3. As shown, at alkaline pH (Figures 4.3b, c) continuous fibers were successfully electrospun from both 2%MPC/2%PEO and 10%MPC/0.8% PEO formulations respectively. Moreover, higher concentration of MPC in the solution resulted in a greater fiber diameter. In contrast, the neutral MPC solutions only produced spherical particles, being difficult to electrospun this neutral aqueous polymer solution (Fig. 4.3a). The different morphology may be explained due to the pH of the solution when water was used as solvent without pH adjust. The pH of the protein concentrate is around pH 4, corresponding to the previous process developed to obtain the protein concentrate from microalga residual biomass. Thus, the pH of the polymer blend with water as solvent changed around 3.8 and may explain the morphology and difficulty to electrospun the aqueous solution, since isoelectric point of these proteins are close to pH 4, as was previously determined [150]. Under these conditions the protein molecules trended to aggregate more easily in the solution. The lack of chain entanglement may have prevented the formation of a stable polymer jet, which is an important requisite for the formation of fiber [153]. Similar behavior was reported by Dror et al. (2008) for BSA solutions at pH close to the isoelectric point [149].

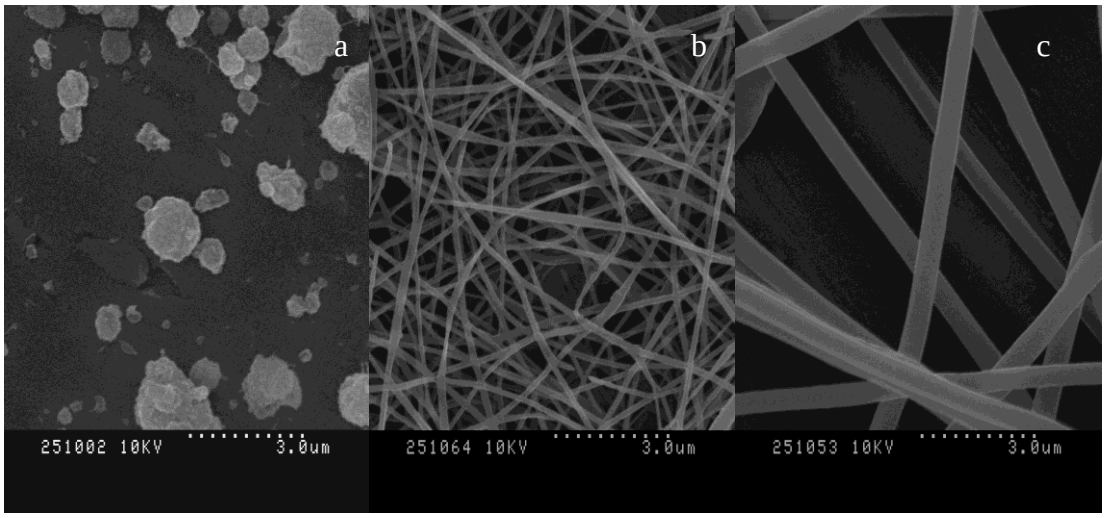

Figure 4.3. SEM micrographs of electrospun MPC/PEO fibers from different polymer blend concentrations for the preliminary experiments **a** 2% MPC, 2% PEO, pH 7; **b** 2% MPC, 2% PEO, pH 12; **c** 10% MPC, 0.8 % PEO, pH 12. Scale bars 3μm.

4.3.2 Results of factorial design: study of the morphology and diameter of the MPC/PEO fibers

MPC/PEO polymer solutions were successfully electrospun under alkaline and acidic conditions (see Figures 4.4 and 4.5). By means SEM analysis it could be observed that when pH was 12, at low and high protein concentration (5 and 10 % w/w), increase PEO content did not affect diameter of the fibers, while an increase in fiber diameter was observed with increasing MPC concentrations in the polymeric blend. As shown in Figure 4.4, increasing the MPC concentration from 5 to 10% (w/w) resulted in an increase in average fiber diameter from 209 ± 5 nm to 719 ± 73 nm (Figures 4.4a and 4.4c).

For morphology response, due to PEO increase from 0.8 % to 1.4%, a decrease in the occurrence of beads in the fibers was observed when the fibers were obtained from 5% MPC solution (Figures 4.4a and 4.4b). Instead, the PEO effect on morphology was not

observed when the MPC concentration was 10% in the polymer solution, being probably due to the effect of the high concentration of MPC was greater than PEO concentration effect in preventing the formation of beads (Figures 4.4c and 4.4d).

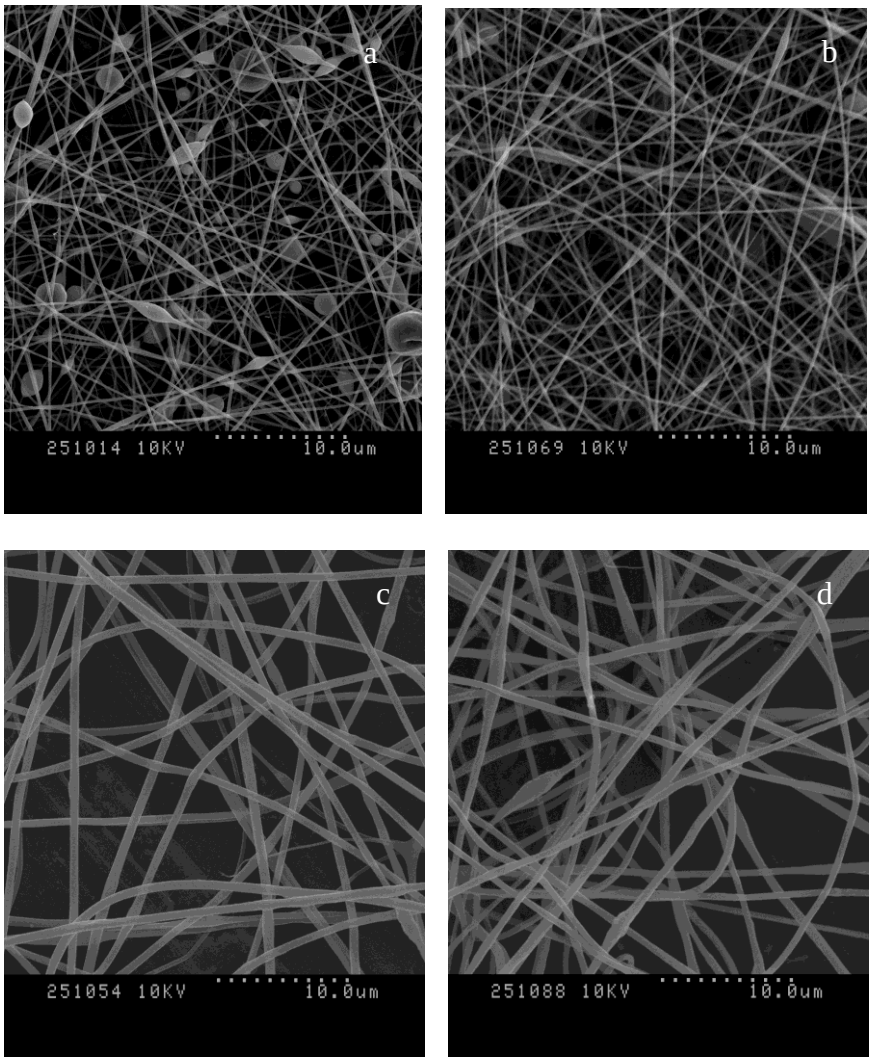

Figure 4.4. SEM micrographs of electrospun MPC/PEO fibers with varying concentrations of MPC and PEO at alkaline pH (12). **a** 5% MPC, 0.8% PEO; **b** 5% MPC, 1.4% PEO; **c** 10% MPC, 0.8% PEO; **d** 10 % MPC, 1.4% PEO. Scale bars 10 μm.

Micrographs of electrospun fibers obtained from acidic polymer solution are shown in Figure 4.5.

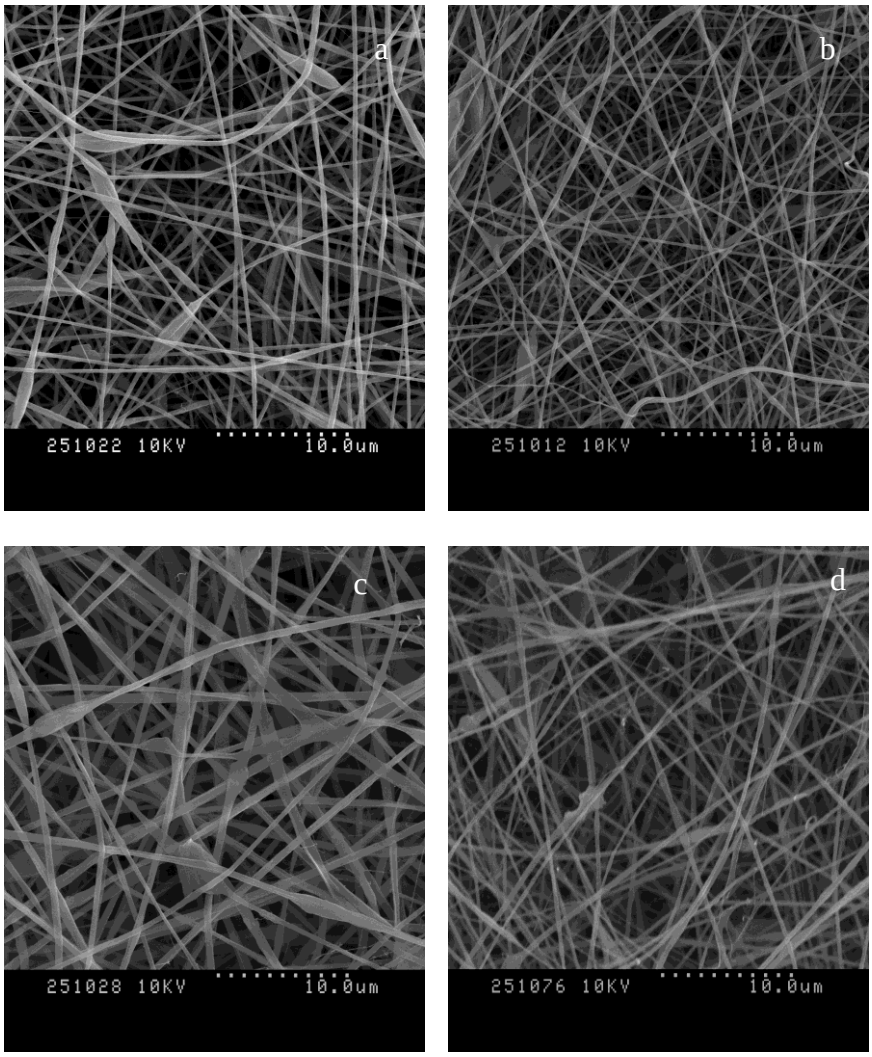

Figure 4.5. SEM image of electrospun MPC/PEO fibers with varying concentrations of MPC and PEO dissolved in acetic acid. **a** 5% MPC, 0.8% PEO; **b** 5% MPC, 1.4% PEO; **c** 10% MPC, 0.8% PEO; **d** 10 % MPC, 1.4% PEO. Scale bars 10 μm.

Overall, fibers exhibited smooth morphology with minimal occurrence of beads and/or agglomerates. Furthermore, these polymer solutions resulted in fiber of similar morphologies, regardless of MPC and PEO concentration. Similar to fibers electrospun from the alkaline solutions, the diameter of the fibers in acid polymer solutions produced trended to increase as the protein concentration increased, as shown Figures 4.5a and 4.5c the fiber diameter increased from 280 ± 3 nm to 628 ± 36 nm. However, the increase in the

concentration of PEO was not has an important effect on the diameter of the fibers at acidic pH, probably due to the effect of MPC concentration to had greater magnitude which can mask the effect of the PEO content.

The morphology of electrospun fibers is dictated by solution properties, especially viscosity, conductivity and surface tension. Process conditions such as solution flow rate, temperature, pressure, the tip-to-target distance and electrical field intensity have also been reported to affect the electrospinning behavior of a polymer solution [148]. In this study, electrical conductivity of the solutions was measured (Table 4.3). As shown, the addition of PEO leads to significant decrease in conductivity ($p< 0.05$). This is especially prevalent for experiments 1 and 5 under alkaline conditions, where increasing PEO concentration from 0.8 to 1.4 % w/w resulted in a decrease in conductivity from 14.12 to 7.99 mS/cm. Increasing MPC concentration from 5 to 10 % (w/w) (experiments 1 and 2) also led to a decrease in the conductivity from 14.12 to 6.30 mS/cm. The reduction in conductivity may be correlated with the increased fiber diameter. Similar effect was observed by [51] for the soy protein electrospun fibers. The lowered conductivity might have reduced the charge density on the polymer jet surface during spinning, reducing the repulsion force along the polymer jet, weakening the tendency of whipping instability, and thereby resulting in fibers of larger diameter.

In general, SEM results showed that all the formulations tested, with the exception of the central point, could be electrospun, yielding fibers of different morphology. In the central point 1.1% PEO/7.5% MPC at pH 6.5, the polymer solution could not be spun. For the central point electrospinnig not resulted in fibers formation, despite to vary processing parameters like flow rate and voltage. Formulation in the central point was prepared with

water as solvent and solution resulted to have a pH close to 3.8 without to reach the neutral pH. The explanation here could be corresponded with the preliminary experiments developed in similar conditions when water was used as solvent, being final pH of the polymer blend, close to the isoelectric point of the proteins of MPC. At this pH the molecules tend to aggregate and thus more easily may have disturbed the electrospinning process.

Overall, the results from the factorial experiments are consistent with the results reported by [154], who found that the blend system *Spirulina* biomass/PEO could be electrospun readily, achieving fibers with different *Spirulina* biomass contents. However, unlike our findings, Morais *et al*. used water as solvent and a relative high concentration of PEO 4 % (w/w) was added to enable the spinning of the biomass/PEO blend polymer solution. They reported average diameters for biomass/PEO fibers of about 110 ± 10 nm, which were comparable to pure PEO fibers. In our present study, we were able to obtain fibers of different diameters and morphologies from different MPC-PEO blends, with PEO concentrations as low as 0.8 % (w/w), with fiber diameters ranging from 200 to 700 nm. with variable MPC contents of up to 93 % (w/w).

4.3.3 Data analysis of the response morphology and diameter

Effect of the factors on morphology and diameter response

Responses of fiber morphology (Y_m) and fiber diameter (Y_d) are shown in Table 4.3. The diameter of the fibers varied from 192 to 770 nm for the different formulations tested in the experimental design. The fiber morphology index varied from 0.28 to 1, being valued as 1

the smoothest fibers observed. These results confirm the feasibility to use MPC from residual biomass as a biopolymer to develop fibers by electrospinning. At the same time was proven that fibers with different morpohology and diameters were obtained from different formulations, being promising results that may find applications in different scope.

The effect of the factor on the responses fiber diameter and fiber morphology has been evaluated according to the contour plots obtained from the data analysis of the factorial design. Figure 4.6 shows the contour plots of Y_m and Y_d as affected by PEO concentration, MPC concentration, and pH of the polymer solutions. As shown Figure 4.6a and 4.6b, diameter decreased when MPC concentration decreased. At acidic pH (Figure 4.6a) MPC concentration affect the diameter of the fiber, as can be observed from the Figure 4.6a, fiber diameter increase with the increase of MPC concentration. Similarly, at pH alkaline (Figure 4.6b) Y_d seemed to be influenced only by the MPC concentration could be observed larger fiber diameters with MPC increase.

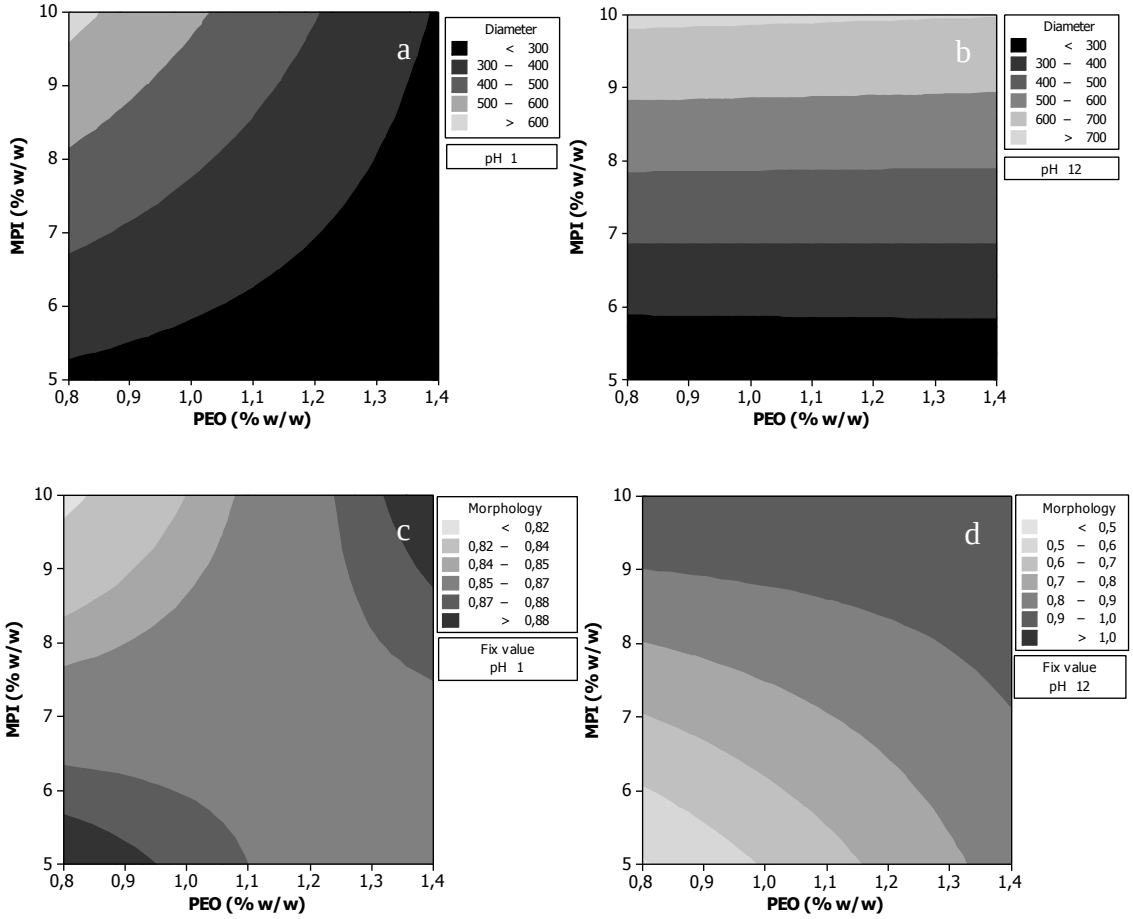

Figure 4.6. Contour plots of fiber diameter and fiber morphology. **a** MPC and PEO concentration effect on fiber diameters under acid conditions. **b** MPC and PEO concentration effect on fiber diameters at alkaline pH. **c** MPC and PEO concentration effect on fiber morphology under acid conditions. **d** MPC and PEO concentration effect on fiber morphology at alkaline pH.

The contour plots for Y_m are presented in Figure 4.6c and 4.6d in alkaline and acid media. From these plots a higher morphologic index was found at alkaline pH with higher concentrations of PEO and MPC in the polymer blend solutions (Figure 4.6d). However, at acid pH behavior of the morphology response was more stable, showing values between 0.825 and 0.885 (very close to unity). On the basis of these results, it can be concluded that at alkaline pH, lower concentrations of MPC led to a smaller fiber diameter, although more

presence of spheres or beads in morphology were found in comparison with fibers obtained under acid conditions. On the other hand, under acid conditions was possible to obtain fibers with a high fiber morphology index (about 0.85) with a smaller fiber diameter. This study has proven that the interaction between the different variables played a significant role over morphology and diameters of the fibers. On depending of the end application specific diameter and/or morphology will be required. More accurate studies will be necessary to find a particular application of theses fibers from MPC from *B. braunii* residual biomass and optimization will be required to reach a morphology and diameter specific. However, the nanofibers obtained here may have potential use in biomedical applications such as scaffolds for tissue engineering. As a sustainable feedstock, microalgae can be considered as an unlimited source of bioactive biocompounds. The "green" protein concentrate from algae could also address the inherent drawbacks associated with animal and/or synthetic polymers.

4.3.4 FTIR spectroscopy of electrospun fibers

In order to examine whether the secondary structure of MPC changed in the electrospinning process and to elucidate an interaction between MPC and PEO polymers, IR spectra of the pure MPC, pure PEO and electrospun MPC/PEO fibers were measured. Figure 4.7 shows the FTIR spectrum of pure PEO, which has a characteristic peak at around 1100 cm^{-1} that is related to the stretching of the C-O-C group present in the PEO molecule. The FTIR spectrum of the pure MPC and those of fibers containing PEO and MPC in alkaline and acid media are also shown in Figure 4.7. For pure MPC, two typical peaks around 1633 cm^{-1} and 1518 cm^{-1} were observed in the spectra that are corresponding to Amide I and II

groups [155]. This spectrum suggests there is a slight change for the frequencies at which the maximum occurred for amide I and II between two different formulations of the fibers analyzed. This fact suggests that a slight interaction could be happening between PEO and MPC, which is probably has relationship with a physical interaction due to an alterations in the secondary structures of MPC induced by pH changes, as previously have been described [153].

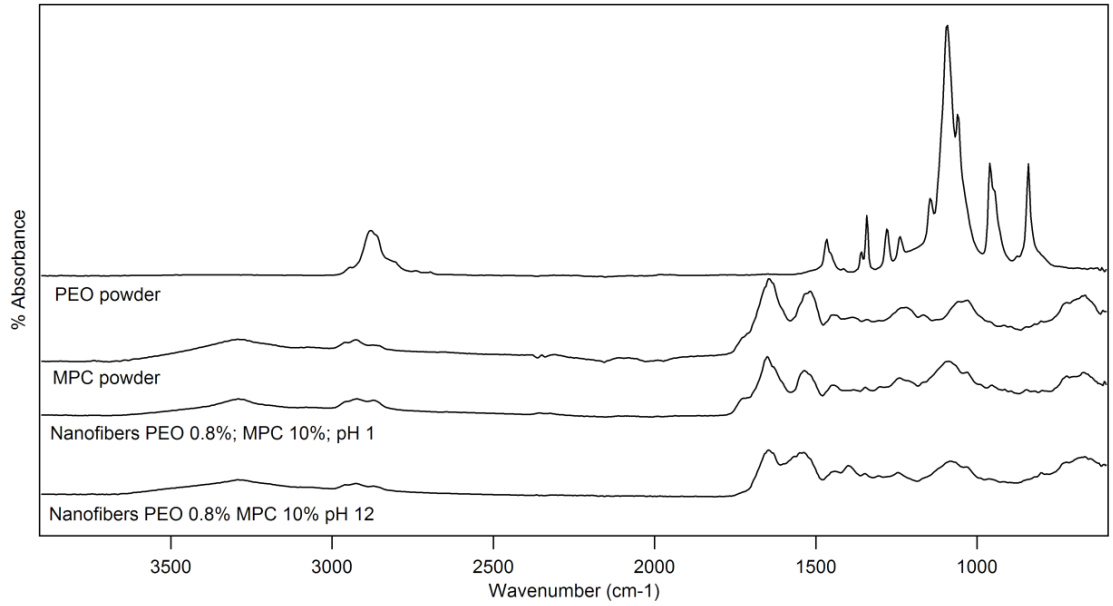

Figure 4.7. FTIR spectra for fibers obtained from electrospinning solutions with different components. The spectra correspond to pure PEO, pure MPC and fibers from precursor solutions of MPC 10% /PEO 0.8 % under acidic and alkaline conditions.

4.4 Conclusions

This study showed that it is possible to produce electrospun microalgal protein concentrate fibers with different morphologies by manipulating the pH, PEO and MPC concentration in the electrospinning solutions. The results show that PEO acted synergistically with MPC in reducing electric conductivity and improving the electrospinnability of the protein solution. Moreover, under the conditions studied, pH and the concentration of MPC were the most important factors in affecting the diameter and morphology of the fibers. Likewise, this work revealed that the PEO/MPC blend can be readily spun into fibers by electrospinning techniques, allowing the incorporation of MPC as high as 93% (w/w) in the fibers. FTIR analysis showed there was an evident of changes in the secondary structure of MPC induced by pH changes. It is likely that this change in the protein structure might have improved a physical chain entanglement in the polymer solution, which is important for the formation of fibers.

Future direction

The contribution of this investigation was to demonstrate that electrospun fibers could be prepared by electrospinning containing a high concentration of the protein concentrate from green microalgae *Botryococcus Braunii*. However, in the future is necessary to study other methods to enhance the entanglement of the protein without PEO aid. In addition, would be interesting that the antioxidant and antimicrobial properties of the protein concentrate were studied and/or to evaluate the incorporation and release of different antioxidants with focus on food packaging applications.

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Chapter 5:

Defatted oil cake from microalgae: a protein source for protein hydrolysate elaboration

Defatted oil cake from microalgae: a protein source for protein hydrolysate elaboration

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Abstract

The present work was carried out to examine the use of defatted oil cake from microalgae *Nannochloropsis gaditana* as feedstock to produce an enzymatic protein hydrolysate with bioactive properties. Firstly, a protein extract from *N. gaditana* oil cake (NgPE) was prepared and conditions for enzymatic hydrolysis were studied by response surface methodology (RSM). A screening with three different proteases, papain, proteinase k and trypsin, was carried out on protein extract to choose one of them based on the maximal degree of hydrolysis achieved. A central composite design (CCD) was used to study the influence of three independent operational factors, hydrolysis temperature (ºC), hydrolysis time (min) and enzyme/substrate (U/mg) ratio on the degree of hydrolysis (DH) and antioxidant activity (AA). An enzyme/substrate ratio of 4.15 U/mg, temperature of 48 ºC and a hydrolysis time of 93 min were the conditions found to reach the maximum degree of

hydrolysis (68%) using papain. The antioxidant activity (AA) of the NgPE hydrolysates was examined by the 1,1-diphenyl-2- picryl-hydrazyl (DPPH•) assay. The AA was also studied by RSM and polynomial model was presented. The conditions of the process for the lowest value of IC_{50} (33 mg/mL) were 48 ºC, 102 min and E/S ratio 5.69 U/mg. In addition, a chemical and physicochemical characterization of the protein extract and protein hydrolysate was carried out. Protein content and amino acid profile, demonstrated the potential of the protein extract and protein hydrolysate as source of proteins with amino acid profile comparable to the FAO reference protein. The SDS-PAGE profile confirmed the high degree of hydrolysis reached in the protein hydrolysate using papain. Moreover, results of the emulsifying and foaming capacity of the protein extract and protein hydrolysate showed that hydrolysis had a negative effect resulting in a drastic loss of these properties due to the enzymatic action ($P < 0.05$). Finally, *in vitro* antioxidant activity of both protein products, protein extract and protein hydrolysate, was determined by DPPH and ORAC method. The results demonstrated that the antioxidant activity of the protein hydrolysate was 10-fold stronger than the protein extract.

5.1 Introduction

Recently, microalgae appear to be one of the most promising sources for the production of renewable biodiesel that is capable of meeting global demand for transport fuels offering significant advantages over more conventional crops such as canola and soy bean [29, 30, 156-158]. The microalgae biomass composition is quite variable, commonly between 10% and 30% is oil; however this oil content is sometimes obtained under stressing conditions, such as long storage time or intense deprivation of nutrients. Besides lipids, other major

components in the microalgae biomass are carbohydrates, ash and proteins [53]. Thus, the extraction of oil from microalgae for biodiesel production results in considerable by-product called defatted oil cake from microalgae, which has high content in proteins and carbohydrates. Therefore, the protein recovery from this by-product represents an important opportunity to make the global process economically feasible as well as beneficial from environmental point of view.

Although microalgae have received increasing attention in last decade because of their potential for biofuel productions, some species have been used for food for thousands of years. In fact, the high protein content of various microalgal species has been one of the main reasons to consider them as a source of proteins for human consumption [33]. However, the possible applications of the proteins from defatted oil cake from microalgae, might be limited due to that the functional properties are affected by partial denaturation occurred during oil extraction, in addition to be practically indigestible to monogastric animals or humans [36]. Thus, to find a potential use for defatted oil cake from microalgae, modifications on extracted proteins have to be parts of the process for obtain protein products with potential for food applications. Hydrolysis of proteins from oilseeds and their oil cakes has experimented considerable development in recent years as strategy to recovery and adding value to these by-products from agro-industrial sector. Protein hydrolysates have been widely used in food technology [159] and studied for various biological activities [37, 40, 45, 160-162], but only few authors have published about enzymatic protein hydrolysates of microalga. [40-42] have studied the algae protein waste of the microalgae *Chlorella vulgaris*, which is by-product during production of algae essence from the microalgae in Taiwan. The main objective in mentionated work, was to

produce antioxidant and anticancer bioactive peptides by enzymatic hydrolysis of the proteins from protein waste, with potential to be used as dietary supplement for prevention of oxidative stress-related diseases, which was proved to be feasible. On the other hand Morris *et al.* (2008) have developed a protein hydolysate from *chlorella vulgaris* biomass. The findings of the authors indicate that *Chlorella* protein hydrolysate can be used for developing functional foods with immunopotentiating activity[36-38] .

In recent time, hydrolysates with antioxidant properties have attracted special attention as potential natural replacers for artificial antioxidants (BHA, BHT, TBHQ) for food/ feed industry, as well exogenous antioxidants in human nutrition [163].

The antioxidant ability of a protein hydrolysate is determined by its peptide composition. However, the peptide composition of a hydrolysate depends on proteolytic enzyme and the hydrolysis conditions. Thus, the study of the hydrolysis process, the influence of operational factors, such as enzyme to substrate ratio, hydrolysis time and temperature, and the interactive effects between hydrolysis parameters should also be regarded. Based on the previous background, this work was carried out to evaluate the alternative use of defatted oil cake from microalga *Nannochloropsis gaditana* (NgOC), as feedstock to prepare a bioactive protein hydrolysate. Hydrolysis of the protein extract prepared from defatted oil cake from microalgae (NgPE) was studied by RSM to maximize the hydrolysis degree and antioxidant activity. In addition, protein content, amino acid profile and molecular weight distribution (SDS-PAGE) were examined in NgPE and NgPE hydrolysate. Likewise, the emulsifying capacity, emulsifying stability and foaming properties were determined. Finally, the zeta potential and antioxidant activity by DPPH and ORAC assay of both proteins products were also studied.

5.2 Material and methods

5.2.1 Material

The microalgal biomass the marine microalgae Eustigmatophyceae *N. gaditana* Lubián CCMP 527 was provided by the Universidad de Antofagasta (Antofagasta, Chile). Defatted oil cake from microalgae *Nannochloropsis gaditana* (NgOC) is the by-product post oil extraction in biodiesel production from microalgae and it was used to develop this research. Hydrochloric acid and sodium hydroxide were purchased from Merck S.A. Chile. Dialysis Membrane MWCO 6000–8000, was purchased from Spectra/Pore, Spectrum Laboratories, Inc., CA, USA.

Trypsin and proteinase K were purchased from Merck S.A. Chile and papain (COROLASE® L10) from Dimerco S.A. Chile. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, Fluorescein and 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) were purchased from Sigma Aldrich (Milwaukee, WI, USA). 6-hydroxy-2,5,7,8-tetramethyl-2-carboxylic acid (Trolox) was purchased from Calbiochem, Germany.

5.2.2 Preparation of N.gaditana protein extract (NgPE)

NgPE was prepared in similar conditions as previously was reported by [164]. Briefly, the microalga was subjected to cell disruption by high pressure homogenizer at 20 psi (SFP, SPCH-10). Then the protein was solubilized at pH 12. Subsequently, an isoelectric precipitation at pH 4 was performed on the soluble protein and the supernatant was removed by centrifugation at 4000 rpm for 15 min. The precipitate obtained was dialyzed with MWCO dialysis bags (6-8 kDa, 32mm) for 72 h to remove interfering salts. Finally,

the NgPE was lyophilized and defatted with hexane considering a cake/solvent ratio of 1:4 g/mL for 5 h.

5.2.3 Enzymatic hydrolysis of NgPE from microalgae oil cake

Initially, the influence of the nature of proteolytic enzymes on the hydrolysis of NgPE was studied using the following proteases: papain, proteinase K and trypsin. The specific activities and optimum pH for these proteases are shown in Table 5.1, whose enzymatic activity was previously determined as described below. Briefly, one proteolytic unit, UHb, was defined by enzyme activity, which under standard conditions (37ºC, defined pH) catalyze the release of trichloroacetic acid soluble bovine hemoglobin compounds, equivalent to 1 µmol tyrosine per minute. After allowing the proteinases to act on denatured hemoglobin, the increase in hydrolysis products soluble in trichloroacetic acid is photometrically measured (280 nm).

The hydrolysis of the protein extract from defatted oil cake from *N. gaditana* was carried out for 4 h at 2.36 U/mg NgPE at temperature and pH considered as optimum for each enzyme. The hydrolysis reaction was stopped by heat treatment at 85 ºC for 15 min. The obtained slurry was centrifuged at 4000 rpm for 15 min. Degree of hydrolysis and antioxidant activity were measured in the supernatants as described below in section 5.2.5.

Table 5.1. Specific activity (UHb/mg of protein) and optimum pH of protease use in the enzymatic hydrolysis of NgPE from *Nanochloropsis gaditana.*

Proteolytic enzymes	Specific activitiy (UHb/mg of protein)	Optimum pH
Trypsin	462.87	7.5
Papain	852.50	7.5
Proteinase K	568.40	7.5

Once the screening of proteases was ended, the optimization of hydrolysis of NgPE was performed with the enzyme that showed higher degree of hydrolysis. The hydrolysis process was carried out as follow, NgPE was suspended in phosphate buffer solution (pH 7.5), to a final substrate concentration according to the experimental points in the design of experiments (section 5.2.4). NgPE solutions were stirred for 12 h at 25ºC to reach greater solubilization of proteins and homogenization of NgPE suspension. The mixtures were incubated at the appropriate temperature and the hydrolysis was started by the addition of the enzyme solution up to required concentration, according to the experimental plan (Table 5.3). The hydrolysis reaction was stopped by heat treatment at 85 ºC for 15 min. The solutions with inactivated enzyme were centrifuged at 4000 rpm for 15 min and the supernatant (protein hydrolysate) was freeze dried and used for further analysis.

5.2.4 Experimental design

The influence of the independent operational factors enzyme/substrate ratio ($x_{E/S}$), time (x_t) and temperature (x_T) on the degree of hydrolysis (Y_{DH}), was determined preliminary to establish the work levels of the design of experiments. The factors were tested at different experimental levels. While the levels of one factor were varied, the remaining factors were maintained fix at one level. The experimental values of the DH were plotted to establish the experimentation range. Factors and work levels, at which they were employed in the RSM, are presented in Table 5.3.

Optimization of the hydrolysis conditions were carried out by employing the response surface methodology (RSM) with a central composite design (CCD). Three independent operational factors (temperature, time and enzyme to substrate level) were employed at five

equidistant levels (-1.68, -1, 0, +1 and +1.68). Degree of hydrolysis (DH; %) and antioxidant activity (AA; mg/mL) were determined as the responses Y_{DH} and Y_{AA} respectively. Table 5.3 shows the design matrix of experiments, where run order, coded value and real value are presented. CCD consisted of 14 experimental points replicated, including 8 factorial points, 6 axial, and 12 centre points (Table 5.3), the experiments were carried out in a random order. Statistical analysis was performed with Minitab 15 software. In addition the correlation between the degree of hydrolysis and the antioxidant activity determined for either point of the design was calculated by Pearson's correlation coefficient (r), which measures the degree of association between two variables, and *p* was calculated with t-tests using Minitab 15 software.

Table 5.2. Factors and their coded and actual levels used in RSM studies for optimizing hydrolysis conditions of NgPE using papain.

		Coded levels				
Factors		-1.68	-1	0	+1	+1.68
$x_{E/S}$ U/mg NgPE	:	0.10	1.58	3.74	5.91	7.38
x_t min	:	39.54	60	90	120	140.45
x_T (ºC)	:	34.88	40	47.5	55	60.11

Table 5.3. Design matrix: Run order, coded and real values of the factors for CCD.

Run order	$x_{E/S}$ *(U/mg NgPE)*		x_t *(min)*		x_T *(ºC)*		y_{DH}	y_{AA}
	Coded value	*Real value*	*Coded value*	*Real value*	*Coded value*	*Real value*	*DH %*	*AA IC_{50}*
1	-1	1.58	-1	60	-1	40	49	40.13
2	-1	1.58	1	120	-1	40	47	44.23
3	1	5.91	-1	60	-1	40	46	46.28
4	1	5.91	1	120	-1	40	53	39.56
5	-1	1.58	-1	60	1	55	55	37.28
6	-1	1.58	1	120	1	55	52	39.28
7	1	5.91	-1	60	1	55	48	42.34
8	1	5.91	1	120	1	55	52	39.34
9	0	3.74	-1.68	39.54	0	47.5	52	40.89
10	0	3.74	1.68	140.45	0	47.5	55	38.78
11	-1.68	0.10	0	90	0	47.5	62	37.79
12	1.68	7.38	0	90	0	47.5	64	36.17
13	0	3.74	0	90	-1.68	34.88	42	48.78
14	0	3.74	0	90	1.68	60.11	48	46.98
15	0	3.74	0	90	0	47.5	69	34.98
16	0	3.74	0	90	0	47.5	66	35.51
17	0	3.74	0	90	0	47.5	68	34.78
18	0	3.74	0	90	0	47.5	67	37.43
19	0	3.74	0	90	0	47.5	68	33.76
20	0	3.74	0	90	0	47.5	67	36.87
21	-1	1.58	-1	60	-1	40	48	43.67
22	-1	1.58	1	120	-1	40	53	41.23
23	1	5.91	-1	60	-1	40	50	42.52
24	1	5.91	1	120	-1	40	54	38.76
25	-1	1.58	-1	60	1	55	48	42.76
26	-1	1.58	1	120	1	55	51	40.79
27	1	5.91	-1	60	1	55	55	37.56
28	1	5.91	1	120	1	55	52	40.73
29	0	3.74	-1.68	39.54	0	47.5	51	40.56
30	0	3.74	1.68	140.45	0	47.5	55	38.07
31	-1.68	0.10	0	90	0	47.5	61	37.07
32	1.68	7.38	0	90	0	47.5	65	35.04
33	0	3.74	0	90	-1.68	34.88	43	46.05
34	0	3.74	0	90	1.68	60.11	50	38.67
35	0	3.74	0	90	0	47.5	64	35.76
36	0	3.74	0	90	0	47.5	63	37.12
37	0	3.74	0	90	0	47.5	65	36.76
38	0	3.74	0	90	0	47.5	66	34.98
39	0	3.74	0	90	0	47.5	68	33.86
40	0	3.74	0	90	0	47.5	67	37.46

5.2.5 Determination of the degree of hydrolysis (DH)

The degree of hydrolysis of NgPE was determined according to the method reported by [165]. To a 2 mL aliquot of hydrolysates an equal volume of 20% w/v trichloroacetic acid (TCA) was added. The mixture was incubated for 30 min at 4◦C. Thereafter, the mixture was centrifuged at 4000 rpm for 15 min. The obtained TCA-soluble protein fraction and the hydrolysate mixture without addition of TCA were both analyzed to determine the protein content by method of Lowry et al. (1951) using bovine serum albumin as the standard protein. The DH was calculated as the ratio of TCA-soluble protein to total protein in the hydrolysate mixture, expressed as a percentage [165].

$$\%\,\mathrm{DH} = \frac{10\%\ \text{TCA-soluble protein fraction in the sample}}{\text{Total soluble protein in the sample}} \times 100 \qquad \text{(Eq.5.1)}$$

5.2.6 Protein content and amino acid profile determination

The protein determination of NgPE and NgPE hydrolysate was carried out by two methods. The nitrogen content was measured according to[134] and hence the protein content was calculated using a conversion factor of N*6.25. The soluble protein was determined using the method described by [132].

The amino acid profile was determined using high-performance liquid chromatography (HPLC) with an AccQ-Tag column and coupled to a UV detector (626-LC System) at 254 nm. The samples were hydrolyzed by acid hydrolysis for 18 h at 115 °C with 6M HCl containing 0.1% phenol in the absence of O_2. The samples were filtered and then derivatized using buffer and reagent (Kit AccQ-Fluor). Finally, concentrations of amino

acids were calculated with respect to the curve under the area obtained for the internal standard (2.5 mM α-aminobutyric acid). For elution conditions, the sample injection volume was 5 µL, and the eluents used were AccQ-Tag, acetonitrile and Milli-Q water.

5.2.7 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution for microalgal biomass, NgPE and NgPE hydrolysate was determined by SDS-PAGE, according was described by [166]. The NgOC was pulverized with liquid nitrogen and the samples NgPE and NgPE hydrolysate were incubated at -20°C for 1 h with a precipitation solution (7% TCA, 0.7% β-mercaptoethanol in acetone), and then centrifuged at 7000 rpm for 15 min. A washing solution (0.7% β-mercaptoethanol in acetone) was added to remove the microalgal pigments. The pellet was re-suspended in denaturant buffer (30 µL/mg pellets) (2M urea and 2M thiourea, 2% CHAPS, 2% DTT and 0.2% IPG buffer) which was analyzed by SDS-PAGE electrophoresis using 15% (w/v) polyacrylamide gel. The re-suspended solution loaded on to the acrylamide gel was run in a Mini Protean II unit (Bio-Rad Laboratories) at 100 mA. Protein bands were stained with Coomassie Blue (Bio-Rad, Coomassie Blue R-250) and the molecular mass of the proteins was determined using a molecular mass marker (10 to 250 kDa, Bio-Rad).

5.2.8 Emulsification index and emulsion stability

The emulsification index of NgPE and NgPE hydrolysate was determined according to the methodology described by Burgos-Díaz *et al.* (2011). Samples were mixed in a vortex for 3 min with an equal volume (2.5 mL) in the aqueous phase, which consisted of 2.5 mL of protein fraction at a concentration of 2% (w/v). The tubes were mixed in a vortex and left to stand for 1 h. After this time (considered the starting time, 0 h), the relative emulsion volume (E_{24}, %) and emulsion stability (ES, %) were calculated at 24 h intervals, from Equations 5.2 and 5.3.

$$Emulsification\ capacity\ (\%) = \frac{emulsion\ of\ \ portion\ (H)}{portion\ volume\ total\ (H_0)} \times 100 \qquad \text{(Eq. 5.2)}$$

$$Emulsification\ stability\ (\%) = \frac{\%\ emulsification\ capacity\ at\ 24\,hrs}{\%\ emulsification\ capacity\ at\ 0\ hrs} \times 100 \qquad \text{(Eq. 5.3)}$$

Where H is the height of the emulsion layer and H_0 is the height of the total solution.

5.2.9 Foaming Properties

Foaming capacity and stability were determined by the method described by Betschart *et al.* (1979) using equations 5.4 and 5.5, respectively. The sample was prepared at a concentration of 1:20 g/mL in distilled water (25°C) and the pH was adjusted at 7 with

0.1N NaOH. After homogenizing (homogenizer Benchtop, model 400DS) for 2 min at 10000 rpm, the volume was measured at different times (0, 5, 10, 30, 60 and 90 min) [167].

$$Foaming\ capacity\ (\%) = \frac{volume\ after\ homogenizing}{volume\ before\ homogenizing} \times 100 \qquad \text{(Eq. 5.4)}$$

$$Foaming\ stability\ (\%) = \frac{foam\ volume\ (elapsed\ time)}{initial\ \ foam\ volume} \times 100 \qquad \text{(Eq. 5.5)}$$

5.2.10 Determination of ζ- potential

The zeta potential was measured as was described by [168], with slight modifications. This measurement was used to study the effect of pH, protein concentration and ionic strength on the stability of NgPE and NgPE hydrolysate using a Malvern Zetasizer Nano ZS series HT instrumental. Solutions of NgPE and NgPE hydrolysate were prepared at 1 % (w/v) to evaluate the pH effect. The 12 aliquots at different values of pH (1.0 - 12.0), were adjusted with 1 M HCl or 1 M NaOH. Likewise, zeta potential was measured for different protein concentration of NgPE and NgPE hydrolysate (0-2% w/v). Later, a solution of 1% NgPE and NgPE hydrolysate at pH 7 was prepared to evaluate the effect of different ionic strength (0-500 mM NaCl), measuring zeta potential for each NaCl concentration.

5.2.11 Measurement of antioxidant activity: DPPH Method

The antioxidant activity of the NgPE and NgPE hydrolysates was determined by using the stable 1,1-diphenyl-1-picrylhydrazyl (DPPH) radical [169]. Solutions of each experimental point were prepared in fosfate buffer pH (7.5) to give a final concentration in the range of 5-400 mg/L. An aliquot of 1000 μL of each solution was mixed with 800 μL of 400 μM DPPH and 2200 μL of ethanol. After 30 min of incubation in the darkness, the absorbance was measured at 520 nm. The radical scavenging activity was obtained from interpolation into a calibration curve performed with Trolox solutions as standards (0-250 μg of Trolox/mL). The antioxidant activity was expressed as IC_{50}, which is defined as the concentration of the tested sample required for the inhibition of DPPH radical by 50%.

5.2.12 Measurement of antioxidant activity: ORAC Method

The antioxidant activity of NgPE and NgPE hydrolysate was measured by oxygen radical absorbance capacity (ORAC) analysis, using fluo-rescein as the flurorescent probe according to Ou et al. (2001) procedure [170]. The method was modified to adapt it to a 96-well fluorescent microplate reader (Biotek, Synergy HT). This method measures the ability of antioxidant compounds in tested materials to inhibit the decline of fluorescence which is induced by the peroxyl radical generator 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH). The reaction was carried out in 75 mM phosphate buffer (pH 7.4). Sample (100 μl) and fluorescein (100 μl; 0.082 mM final concentration) solutions were placed in the well of the microplate (black 96-well plates). AAPH solution (100 μl; 0.15 M final concentration) was rapidly added using a multichannel pipette. The plate was immediately

placed in the plate reader (Biotek, Synergy HT), and the fluorescence was recorded every minute for 150 min at 37 °C. Excitation and emission filters were 485-P and 520-P, respectively. The plate was automatically agitated prior to each reading. All reaction mixtures were prepared in triplicate and at least three independent runs were performed for each sample. A blank using phosphate buffer instead of the antioxidant solution and calibration solutions using Trolox (0-8 mM final concentration) as the antioxidant was also performed in the same run. The area under the fluorescence delay curve (AUC) was calculated using the KC4 v.3.4 software, and finally the ORAC value was expressed as the concentration of the tested sample required for the inhibition of DPPH radical by 50%, defined as IC_{50} value.

5.3 Results and discussion

5.3.1 Enzymatic hydrolysis of NgPE using various commercial proteases

In relation to the enzymatic hydrolysis, the specificity of the proteolytic enzyme used and the conditions of processing (temperature, time, enzyme-substrate level etc.) are crucial for the success of the process. In this work, three proteolytic enzymes such as papain, proteinase K and trypsin were used to evaluate their effectiveness on hydrolysis of NgPE. The results showed the degree of hydrolysis achieved for each of the proteases tested (Fig.5.1a); the highest degree of hydrolysis of NgPE was reached with papain (67%), followed by proteinase K (56%) and trypsin (47%).

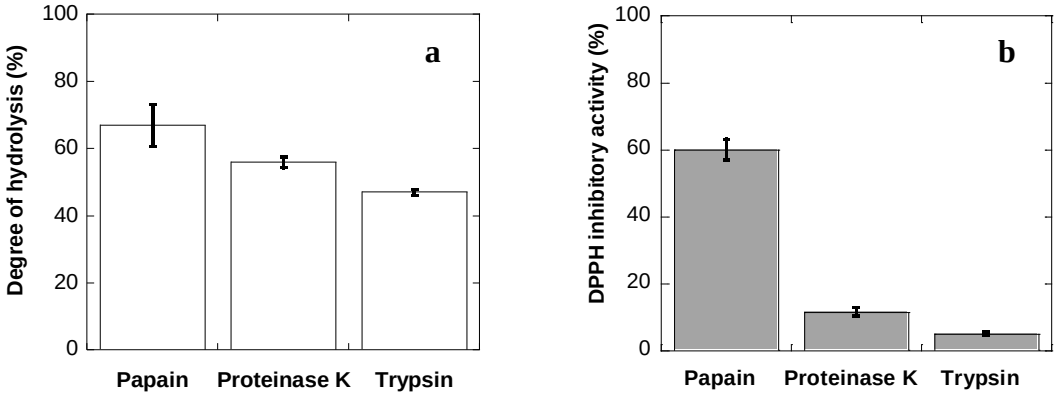

Figure 5.1. a Degree of hydrolysis of *N.gaditana* protein extract with different proteases. **b** antioxidant activity of NgPE hydrolysate from different proteases. A protein extract suspension of 25 mL was employed with 2.36 U/mg of E/S ratio, at optimum temperature for each enzyme for 4 h, accompanied by continuous stirring at 200 rpm.

The degree of hydrolysis of NgPE indicated that papain was the most efficient protease to hydrolyse the proteins of NgPE up to peptides or amino acids. This may be attributed to the presence of endo- and exo-peptidase activities in papain commercial employed in this study, which results in higher proteolytic activities when compared to the other enzymes that contain predominantly endo-peptidase activities. Our results are consistent with previous reports that have shown papain to produce protein hydrolysates with greater DH than similar hydrolysates produced with other different proteases. [36] reported comparable results on *Chlorella vulgaris* biomass, revealing that papain was the enzyme which reached the highest degree of hydrolysis of the microalga biomass. On the other hand DPPH inhibitory activity of NgPE hydrolysate obtained with papain, proteinase K and trypsin was measured and results are shown in Figure 5.1b. Similarly to the results observed for hydrolysis degree, the hydrolysate obtained with papain showed the highest value of DPPH inhibition (60%). In view of the highest degree of hydrolysis and the DPPH inhibitory activity was reached with papain, this enzyme was selected for further studies. The

utilization of papain, alone or combined with other proteases, has been previously reported in protocols for food protein hydrolysis such as microalgae biomass [36], shark [171] and salmon [172], among others.

5.3.2 Optimization of hydrolysis: degree of hydrolysis

The hydrolysis process of NgPE was studied by design of experiments. The influence of the independent factors enzyme/substrate ratio ($x_{E/S}$), hydrolysis time (x_t) and hydrolysis temperature (x_T) on the degree of hydrolysis of NgPE by papain was determined using CCD design as mentioned in the previous section. The values of hydrolysis degree for each experimental points are presented in Table 5.3. According to the results the degree of hydrolysis showed an experimental variation between 42 to 68%. The maximum DH% was observed close to the central point. Following from the ANOVA table (Table 5.4), among the independent factors studied, temperature and time were the significant operational factors ($p<0.05$). The quadratic term of time, temperature and enzyme/substrate ratio resulted equally significant. However the enzyme/substrate ratio and interactions between the different factors did not significantly influenced ($p>0.05$) the response degree of hydrolysis.Through the data analysis of RSM, was possible to obtain a model of prediction. This model relates the values of DH response with the coded values of the independent variables and their interactions. The obtained model corresponds to equation 5.6 presented below:

$$y_{DH} = 66.5784 + 0.9802\,x_t + 0.6257\,x_{E/S} + 1.2764\,x_T - 5.1972\,x_t^{\,2} - 1.7501\,x_{E/S}^{\,2} - 7.8489\,x_T^{\,2} + 0.5625\,x_t . x_{E/S} - 0.8125\,x_t . x_T - 0.3125\,x_{E/S} . x_T$$

(Eq.5.6)

Table 5.4. Analysis of variance (ANOVA) for the hydrolysis process of NgPE.

Source	df	SS	MS	*F*-value	*p*-value
Regression	9	2451.97	272.44	49.23	0.000
x_t	1	26.24	26.24	4.74	0.0374
$x_{E/S}$	1	10.69	10.69	1.93	0.1747
x_T	1	44.50	44.50	8.04	0.0081
x_t^2	1	778.53	778.53	140.69	0.0001
$x_{E/S}^2$	1	88.28	88.28	15.95	0.0004
x_T^2	1	1775.60	1775.60	320.88	0.0001
$x_t * x_{E/S}$	1	5.06	5.06	0.91	0.3465
$x_t * x_T$	1	10.56	10.56	1.91	0.1773
$x_T * x_t$	1	1.56	1.56	0.28	0.5991
Residual	30	166.01	5.534		
Pure error	25	115.50	4.620		
Total	39	2617.97			
R^2		0.9366			
R^2_{adj}		0.9176			
Lack of fit					0.0800

The model showed a good fit with the experimental data, with an R^2 value of 0.9366 (Table 5.4). In addition, regarding the test for lack of fit, it did not result to be significant (p-value= 0.08, $\alpha > 0.05$), it can be stated that the model given by Eq.(5.6) fits the experimental data well and it can be used to predict DH at any value of the factors, within the range studied. Response surface plots for DH of NgPE (Fig. 5.2) were obtained to determine the conditions of the hydrolysis to which the degree of hydrolysis is maximal. Figure 5.2 shows the surface plots for the factors studied and their interactions on the DH response. Figure 5.2a shows DH as a function of time and E/S ratio, revealing that the DH increase with the time until that an optimum point is reached, after that, the DH is reduced considerably. The same tendency can be observe for the temperature from Figure 5.2c, being temperature and time the most significant variables according the ANOVA analysis (Table 5.3). In a similar manner, DH to reach an optimum at E/S ratio around 3.74 U/mg NgPE, conrresponding to central point, as can be seen from Figures 5.2a and 5.2b.

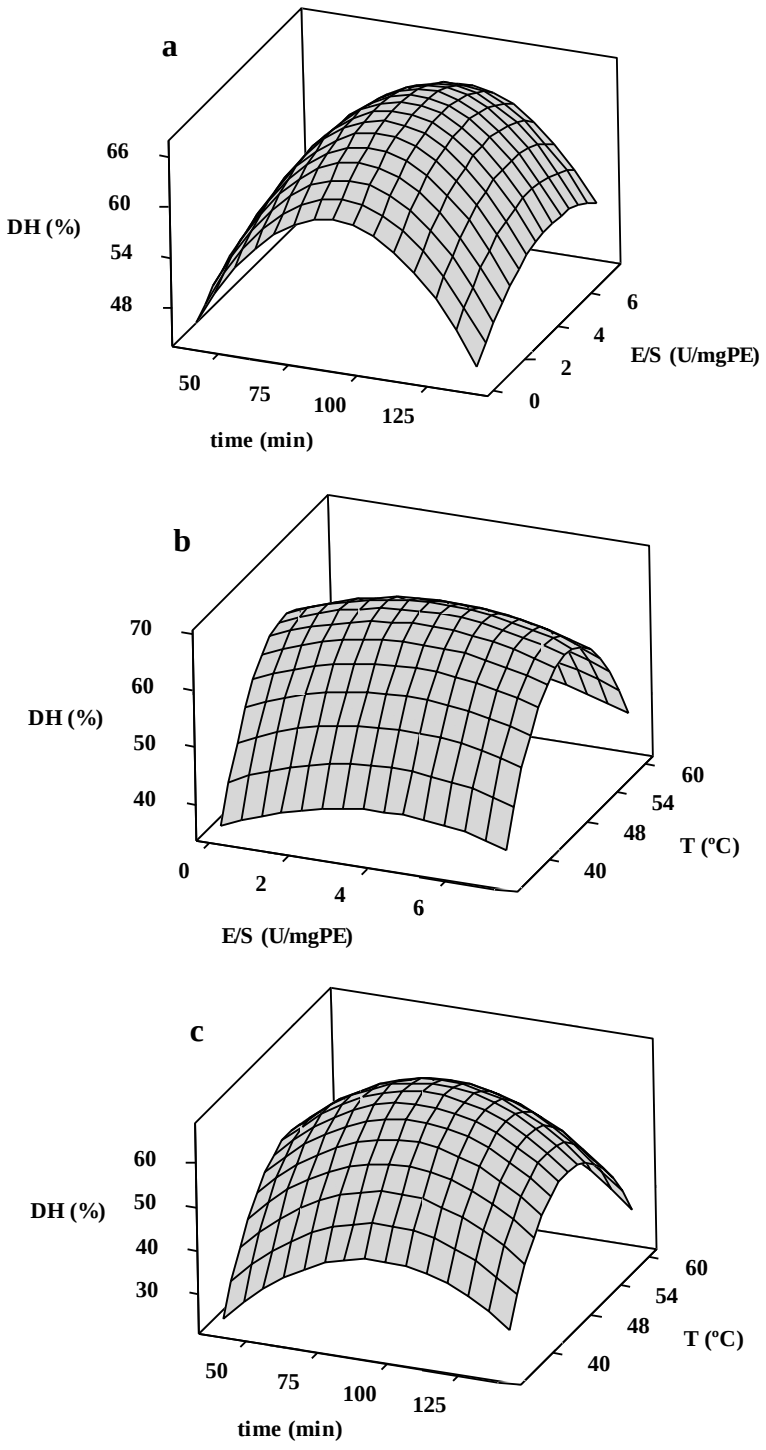

Figure 5.2. Response surface plot for the influence of the independent variables on DH of the NgPE **a** DH as a function of E/S ratio and time **b** DH as a function of E/S ratio and temperature, and **c** DH as a function of time and temperature. In figures a b y c the factor not present in the plot is at the center of their levels.

Respect to the hydrolysis time, as is shown in Figures 5.2 a y c, the hydrolysis of the NgPE was characterized by a high degree of hydrolysis for the first 90 min. The first 90 min of the hydrolysis could have relationship to the hydrolysis of the soluble fraction of proteins and hydrolysis of denatured protein present in the protein extract (NgPE). After 90 min of hydrolysis, the degree of hydrolysis was subsequently decreased, and then the enzymatic reaction seems to be limited due to the low concentration of the enzyme employed. In fact there is not significant effect of the factor E/S ratio, probably the hydrolysis of the proteins in this case, was dominated by the chemical hydrolysis. Denatured proteins of *N. gaditana* could be present in the NgPE due to the protein extract was prepared by alkaline extraction from microalgae oil cake to eliminate non proteins components, which could allows a major exposition of the peptide bonds to the enzyme action. This fact could explain the high degree of hydrolysis for the NgPE. The shape of this progress curve for the hydrolysis of NgPE was similar to what has been reported for enzymatic hydrolysis of other different protein substrates [36, 173]. Respect to the hydrolysis temperature, from Figure 5.2b and 5.2c can be observed an increase for DH when the temperature increasing from 40 to a 50ºC. This increase of temperature to produce an activation of the enzyme employed and contribute to unfolded of peptide bond of the NgPE, allowing a major accessibility the active enzyme site to these peptide bond and thus results a higher degree of hydrolysis, as it has been previously discussed by [174]. At the same time from Figure 5.2c can be observed that at upper temperatures (60ºC) a lower degree of hydrolysis was observed, probably due to an enzyme inactivation occurred. However, this is observed at 100-120 min of hydrolysis, bu at 70-90 min of hydrolysis, DH is near to the optimal value. This fact

demonstrate that temperature in this process is limiting due to denaturation of the enzyme and hence to the loss of activity.

The maximum of DH was determined according to the model equation (Eq.5.6), which was derived to obtain the protein hydrolysate with the maximum DH. The combination of the operational factors for maximum DH of 68% were obtained: x_t = 0.0849, $x_{E/S}$ = 0.1868 and x_T = 0.08842 corresponding to the coded values for the time, enzyme/substrate ratio and temperature respectively. Then, the maximum value for DH was found with the combination of the real value of the factors, enzyme/substrate 4.149 UHb/mg NgPE, time of 92.54 minutes and temperature of 48.13ºC.

Various authors have estimated different conditions for hydrolysis of protein waste materials, for example for hydrolysis of palm kernel cake protein using trypsin, a DH up to 57% was observed at 40ºC, pH 8.5, 3.5 g/ 100 g of enzyme/substrate after 6 h of hydrolysis [161], the hydrolysis process of pumpkin oil cake was studied by Vastag et al. (2010) where the optimum conditions were found at 40ºC, 4.38 U/mg substrate and 85 min of processing with DH of 43%. Likewise, the optimization of enzymatic hydrolysis of visceral waste proteins of Catla was found at 50ºC 1.5% of enzyme/substrate level at 135 min to obtain the highest degree of hydrolysis close to 50% [175]. Hence, can be established that not only operating conditions of hydrolysis are different, also the degree of hydrolysis is variable, thus the study of enzymatic hydrolysis of the proteins depende on each substrate to obtain a final product with certain desired characteristics.

5.3.3 Optimization of hydrolysis: antioxidant activity

In this study, the antioxidant potency of NgPE hydrolysates was evaluated using DPPH• radical scavenging assay. The antioxidant activity response was determined and expressed as IC_{50} value (mg/mL). The IC_{50} values for each experimental point of the matrix design are presented in Table 5.3 and the variation observed was from 33.76 to 48.78 mg/mL. From ANOVA in Table 5.5 can be observed the influence of the independent variables studied on the Y_{AA}. Both, the linear and quadratic terms of temperature (x_T) were significant within a 95% confidence interval ($p < 0.05$). The quadratic term of time (x_t) was also significant ($p < 0.05$); however, the interaction terms of the independent variables were not found to be significant.

Table 5.5. Analysis of variance (ANOVA) for the antioxidant activity of hydrolysis of NgPE.

Source	dF	SS	MS	*F*-value	*p*-value
Regression	9	426.70	47.41	12.06	0.000
x_t	1	9.79	9.79	2.49	0.1250
$x_{E/S}$	1	2.59	2.59	0.66	0.4230
x_T	1	36.88	36.88	9.38	0.0046
x_t^2	1	57.88	57.88	14.72	0.0006
$x_{E/S}^2$	1	3.25	3.25	0.83	0.3703
x_T^2	1	328.77	328.77	83.63	0.0001
$x_t * x_{E/S}$	1	9.00	9.00	2.29	0.1407
$x_t * x_T$	1	5.09	5.09	1.29	0.2644
$x_T * x_t$	1	0.25	0.25	0.064	0.8026
Residual	30	166.01	3.93		
Pure error	25	115.50	2.43		
Total	39	2617.97	4.23		
R^2			0.7835		
R^2_{adj}			0.7185		

The antioxidant activity response can be described by the fitted model in coded variables given in Eq. 5.7. The model showed a fit with the experimental data with an R^2 value of

0.78. The following model could be adequately used to explain the data variations of antioxidant activity of NgPE hydrolysate:

$$y_{AA} = 35.7614 - 1.007\,x_t - 0.5184\,x_{E/S} - 1.9543\,x_T + 4.0076\,x_t^{\,2} + 0.9501\,x_{E/S}^{\,2} + 9.5526\,x_T^{\,2} - 2.1213\,x_t.\,x_{E/S} + 1.5945\,x_t.\,x_T + 0.3536\,x_{E/S}.\,x_T$$

(Eq.5.7)

From the analysis of the RSM, response surface plots for the antioxidant activity were obtained (Figure 5.3).

Surface plots of Figure 5.3 show that when the factors studied are close to the central point, the response value of AA trend toward the minor value of IC_{50}, between 33 and 38 mg TE/mL. These results are consistent with those observed in the surface plots of Figure 5.2 for the study of the influence of the independent factors on the DH, where the values of the response degree of hydrolysis is also maximal in the experiments close to the central point. Thus for both responses, degree of hydrolysis and antioxidant activity was observed the same tendency (Figure 5.2 and Figure 5.3)

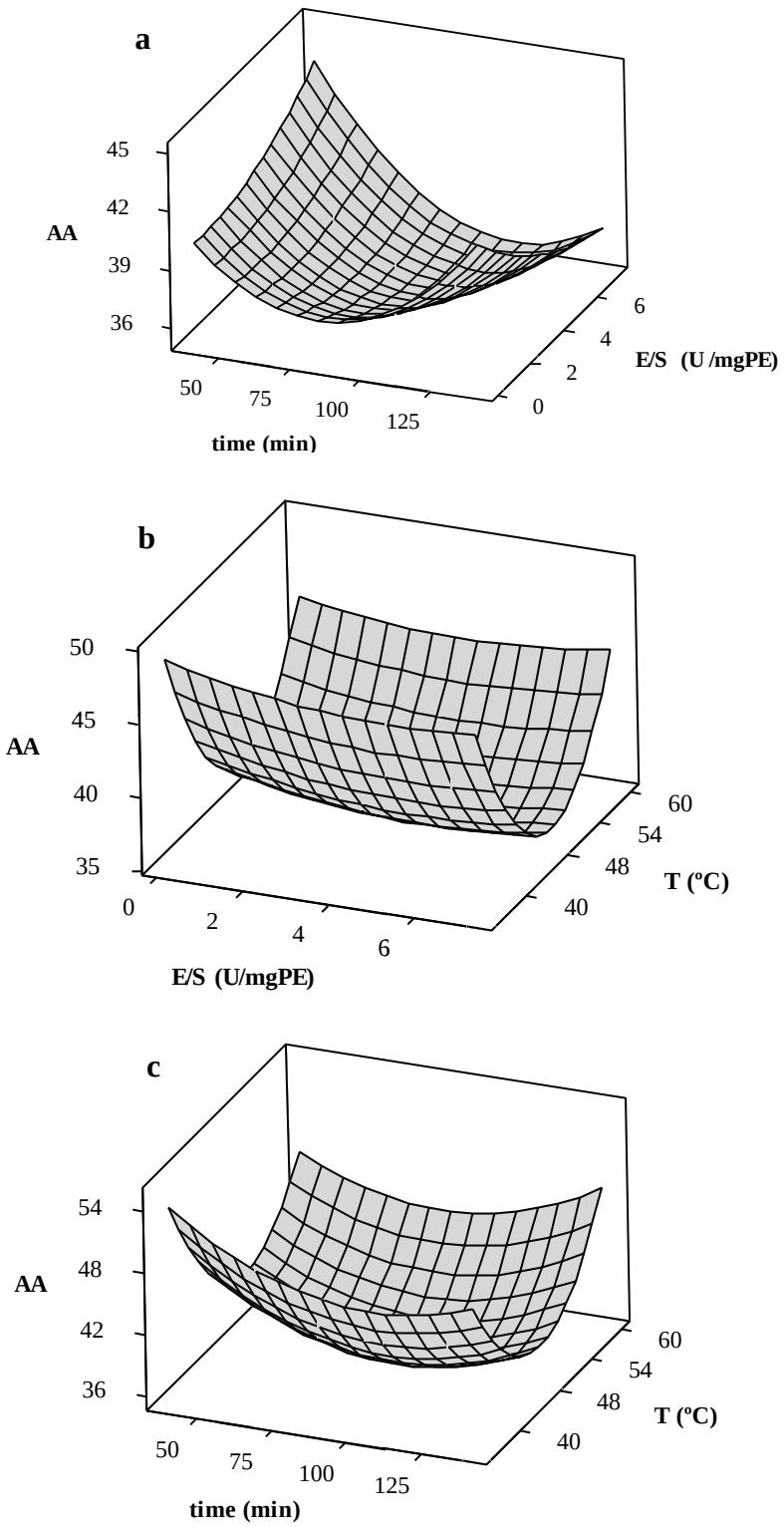

Figure 5.3. Response surface plot for the influence of the independent factors on antioxidant activity of the NgPE **a** DH as a function of E/S ratio and time **b** DH as a function of E/S ratio and temperature, and **c** DH as a function of time and temperature.

[176] reported similar results related to degree of hydrolysis in relation to the antioxidant activity. The results of this work demonstraded as the degree of hydrolysis of plasma protein increased DPPH radical scavenging activity significantly increased ($P < 0.05$), which presented 21.72%, 31.3%, 45.4% and76.8% scavenging activity corresponding to 0%, 6.2%, 12.7% and17.6% of DH, respectively. Therefore, higher DH values may be related to the production of smaller or shorter protein chains and thus greater antioxidant activity[176].

In this work RSM was an efficient method to maximize degree of hydrolysis under the conditions studied, and optimizing the antioxidant activity of the enzymatic hydrolysate. From the model equation for AA, the response variable (Y_{AA}) of NgPE as a function of the three independent factors(x_t, $x_{E/S}$ *and* x_T) and their interactions was derived to obtain the protein hydrolysate with the maximum antioxidant capacity. According to these results the hydrolysis conditions for obtaining the optimum AA using papain were – temperature of 48 ºC, time of 102 min, and an enzyme/substrate ratio of 5.69 U/mg. From this results, could be established that NgPE was effectively hydrolyzed by papain to produce protein hydrolysate of high degree of hydrolysis (68%) with a maximum antioxidant activity of IC_{50} = 33 mg /mL.

Following, Figure 5.4 shows the Pearson´s correlation between degree of hydrolysis and antioxidant activity. According to Sampaio (2002), *r* values greater than 0.7 with $p<0.05$ indicate a strong and significant association between the data. As can be observed from Figure 5.4 there is a high lineal correlation in between the responses studied being a significant correlation (r=-0.896, p=0.000). This negative correlation indicating that at highest degree of hydrolysis a maximal antioxidant activity is found.

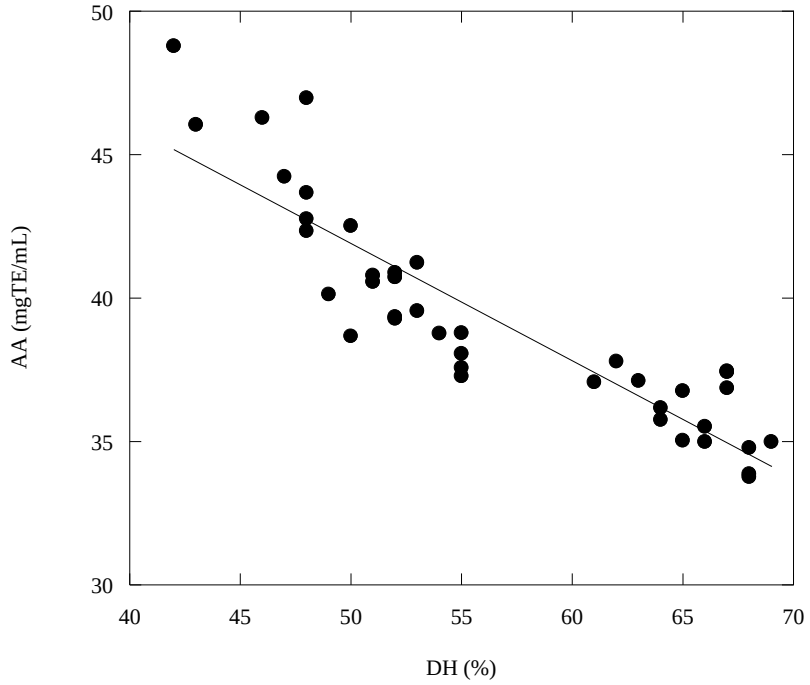

Figure 5.4. Pearson correlation between degree of hydrolysis and antioxidant activity.

Characterization of protein hydrolysate obtained under optimum experimental conditions for maximal antioxidant activity

5.3.4 Protein content and amino acid composition of the NgPE and NgPE hydrolysate

The protein content of the defatted oil cake from *N. gaditana*, protein extract and protein hydrolysate is displayed in Table 5.5. The protein hydrolysate was prepared under the optimal conditions to the maximal antioxidant activity, according to the results of the RSM, 48 ºC, 102 min and E/S ratio 5.69 U/mg. As can be observed from Table 5.6 the protein and soluble protein content from three different sources analyzed were statistically different ($p<0.05$). The protein extraction consisted of an akaline extraction, one of the most common methods to the protein extraction. Therefore, only solubilized protein was precipitated at isoelectric pH, which allowed obtaining a NgPE concentrated in protein

(63%). However, a decrease of the protein content in the enzymatic hydrolysate was observed in comparison with NgPE, showing a protein content of 33%.

On the other hand, the soluble protein in NgPE hydrolysate was twice the content present in NgPE and three times compared to the protein content of microalgae oil cake. This increase in the solubility of the proteins in the enzymatic hydrolysate, can be explained by breaking the molecular structure generated by the enzymatic action, being primarily the reduction in molecular weight and the increase in the number of polar groups.

Table 5.6. Protein content of the *N. gaditana* defatted oil cake, protein extract and NgPE hydrolysate

Protein content	***N.gaditana oil cake***	***N. gaditana protein extract***	***NgPE hydrolysate***
Protein*	25.34 ± 0.45^{a}	63.32 ± 0.06^{b}	33.68 ± 0.03^{c}
Soluble protein (mg/g)	143 ± 3.1^{a}	187.5 ± 8.7^{b}	406.1 ± 22.1^{c}

*The protein content determined by kjeldahl method is expressed as g 100 g $^{-1}$ of wet weight base

The nutritional value of food proteins depends on the type and amount of amino acids available for body functions. At the same time, the amino acid composition of protein hydrolysates is considered important related to antioxidant/antiradical capacity [177]. According to the literature, it has been reported that peptides with different compositions, structures and hydrophobicities will have different antioxidant properties [177]. Amino acid profile of NgPE and NgPE hydrolysate was determined and the results are presented in Table 5.7 in comparison with the FAO/WHO/UNU (1985) amino acid requirement pattern[144]. The results showed that there is no significant difference between NgPE and NgPE hydrolysate ($p < 0.05$). Based on this study, can be observed in Table 5.7 that aspartic acid and glutamic acid were the most major constituents among all the amino acids present in NgPE and NgPE hydrolysate, which coincides with the results published in the

literature for another species of microalgae [36, 129]. Likewise, for both analyzed samples, essentials amino acids are present in a sufficient quantity as required based on standard references. Among essentials amino acids, the exception is the low content of S-containing amino acids (Met and Cys) in the hydrolysate, which has been also observed in other species of microalgae [36]. In contrast, lysine is present in high concentration, surpassing the levels of the FAO reference protein. Hence, these protein fractions could be useful as supplement for other protein source with lysine deficit, for example cereals. From these results it is possible to conclude that the NgPE and NgPE hydrolysate contain adequate amounts of essential amino acids in relation to the FAO pattern, thus constituting a suitable protein source or as potential food or feed supplement. Additionally, the NgPE and NgPE hydrolysate showed an essential amino acid ratio (essential amino acid/total amino acid %), of 38.64 and 36.88% respectively, which proved its nitrogenous equilibrium according to the FAO nutritional recommendations [144].

Table 5.7. Amino acid composition of the protein extract and NgPE hydrolysate

Amino acid	*NgPE*	*NgPE Hydrolysate*	FAO pattern
Aspartic acid	10.47 ± 0.49[a]	12.84 ± 2.04[a]	
Glutamic acid	26.13 ± 1.28[a]	25.46 ± 1.56[a]	
Serine	4.03 ± 0.21[a]	4.33 ± 1.86[a]	
Glycine	7.90 ± 0.89[a]	8.00 ± 0.80[a]	
Asparagine	0.84 ± 0.11[a]	0.58 ± 0.31[a]	
Arginine	4.56 ± 0.05[a]	3.93 ± 0.31[a]	
Threonine	3.96 ± 0.43[a]	4.72 ± 0.39[a]	3.4
Alanine	4.81 ± 2.41[a]	5.87 ± 1.29[a]	
Proline	3.44 ± 0.59[a]	2.64 ± 1.14[a]	
Valine	4.73 ± 0.73[a]	3.70 ± 2.91[a]	3.5
Methionine + Cysteine	3.75 ± 0.64[a]	2.06 ± 0.59[a]	2.5
Fenilalanina + Tyrosine	8.77 ± 0.63[a]	9.37 ± 1.81[a]	2.4
Isoleucine	4.22 ± 0.26[a]	3.56 ± 0.73[a]	2.8
Leucine	7.81 ± 1.58[a]	7.14 ± 0.91[a]	6.6
Lysine	5.40 ± 0.56[a]	6.35 ± 1.14[a]	5.8
Essentials/total (%)	38.64	36.88	

5.3.5 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Figure 5.5a shows the SDS–PAGE of *N. gaditana* oil cake (lane Ng) and Figure 5.5b shows electrophoretic pattern of NgPE (lanes PE1-PE2) and NgPE hydrolysate (lanes H). In Figure 5.5a the electrophoretic pattern of *N. gaditana* oil cake showed the protein bands corresponding to the different molecular weight distribution between 10 and 100 kDa. Similar molecular size distribution was observed by Sheih et al. (2009) for *Chlorella vulgaris* protein waste, showing the hydrolysis pattern of non-hydrolyzed algae protein waste with molecular size distribution between 14-97 kDa. In Figure 5.5b (lanes PE_1-PE_2) it is evident that proteins in NgPE was unfolded and hydrolyzed due to alkaline condition used for its obtaining, thus molecular size distribution of this protein was mostly observed between 15 y 37 kDa. Later, most of the protein of NgPE were hydrolyzed to low-molecular weight peptides and or amino acids (Figure 5.5b, lane H), after hydrolysis of NgPE with papain virtually all proteins observed in Figure 5.5a have disappeared in Figure 5.5b (lane H) from the gel except peptides fractions <10 kDa (band lane H). Similar observations were reported by [42] in algae protein waste digested with pepsin enzyme, showing a electrophoretic pattern < 14 kDa [42]. Finally, the electrophoretic pattern of NgPE hydrolysate presented in Figure 5.5b indicates that the peptides fractions after enzymatic hydrolysis are less than 10 kDa, corroborating well the highest DH observed in this study (68%).

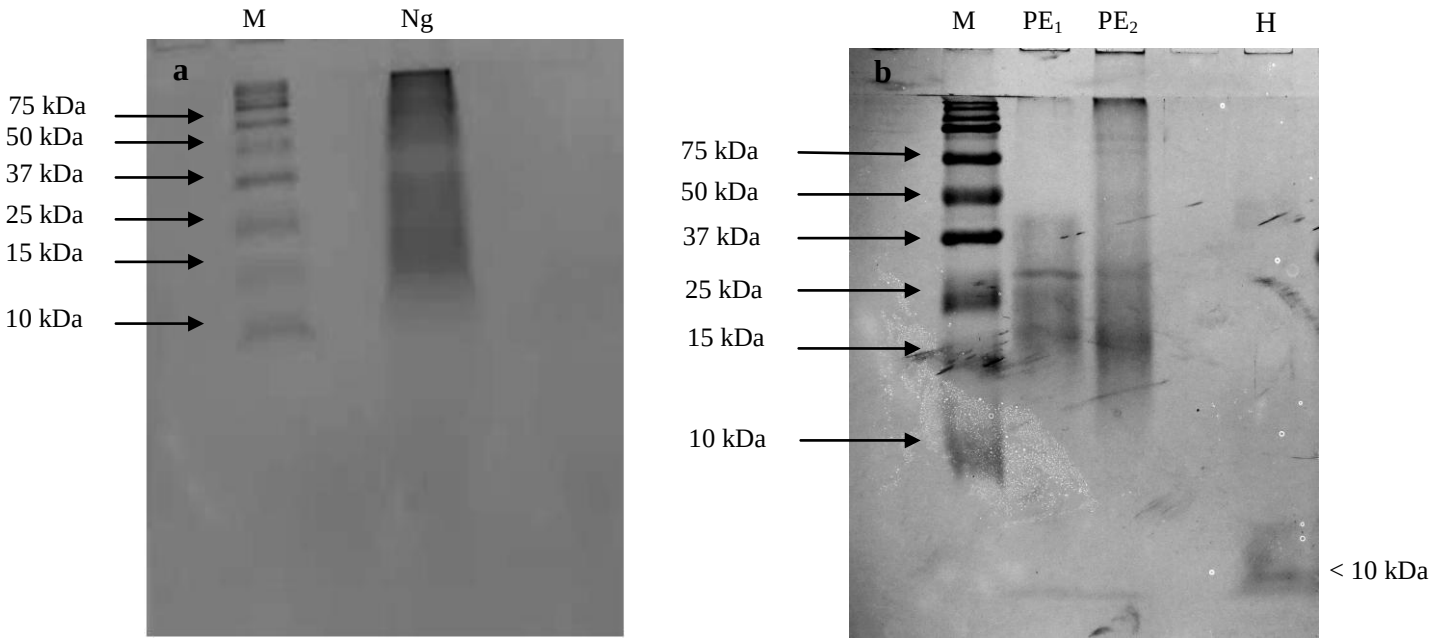

Figure 5.5. Electrophoretic pattern of NgPE hydrolysate. a) M – standard marker proteins; Ng – *N. gaditana* oil cake b) M – standard marker proteins; PE_1–PE_2 – Ng protein extract corresponding in duplicate; H–protein hydrolysate.

5.3.6 Technofunctional properties

Proteins play a very important role in the physical and organoleptic properties of many foods, where they influence the functional characteristics such as the formation of emulsions, gels, foam, or water or oil absorption [92].

Table 5.8 shows the results from the evaluation of the emulsification properties and foam formation of NgPE and NgPE hydrolysate. The composition of many food emulsions includes proteins as emulsifiers and polysaccharides as stabilizers, biopolymers that jointly control the consistency and texture in a food. The proteins or peptides help in the formation of emulsions, mainly by reducing the interface tension [178].

The results showed that the foaming capacity and emulsifying capacity of NgPE was maintained over 50%, as well as the stability of the emulsion at 24 h ($\%E_{24}$). According to

the literature, the protein fraction that show values over 50% of emulsifying capacity are considered biopolymers suitable for emulsifying applications. Thus, based in the results this work, can be established that the NgPE presents good emulsifying properties and emulsion stability to be considered for emulsifyin applications. Moreover, the emulsifying capacity of NgPE (63%) is comparable to the emulsifying capacity determined for some plant proteins like the protein isolate of lupin (74 %) [142], soy concentrate (70 %) and rapeseed concentrate (61.3 %) [179]. However, NgPE hydrolysate does not show any capacity to form emulsions. The hydrolysis reaction produces smaller peptides (with a DH greater than 10%) and therefore the generation of polar groups like NH_4 and COO^- is increased. These factors are translated into increased solubility and decreased oil absorption for forming emulsions [180]. [92] Proposed that the minimum size of peptides should be 20 kDa so the peptides can stabilize the oil-water interfaces in the case of emulsions, or air-water interfaces in the case of foam. Some studies carried out by [178] have shown that excessive enzymatic hydrolysis could be detrimental to their emulsifying properties. Different enzymatic hydrolysis effects on the functional properties of proteins have been reported, such as [181] who reported an increase in foam and stability of the wheat gluten emulsion using moderate enzymatic hydrolysis. On the other hand, [182] reported the reduction in emulsion stability of rapeseed seed protein fractions with a high degree of enzymatic hydrolysis. Therefore, the technological properties of a protein can be modified through of enzymatic hydrolysis, but the treatment must be defined on the basis of the desired product, because is proved that a high degree of hydrolysis has a negative effect on the most of the technological properties.

Table 5.8. Foaming and emulsifying capacity of NgPE and NgPE hydrolysate.

Properties	NgPE	NgPe hydrolysate
Foaming capacity (%)	92.3 ± 3.8	21. ± 0.6
Emulsifying capacity (%)	63.0 ± 3.0	2.8 ± 0.1
Foam stability (%)*	13.3 ± 1.7	6.8 ± 0.1
Stability of the emulsion (%)*	82.9 ± 0.4	42.5 ± 4.4

*The stability of the foaming and emulsifying capacity was evaluated after 24 h.

5.3.7 Zeta- Potential measurement

Zeta potential is a measure of the magnitude of the electric potential or charge on the interfacial surface of the suspend particles, is a useful parameters to predict the stability of protein-based preparations and how a protein will behave in solution. Developing protein-based products require an understanding of how they behave in solution since this denoted the ability to predict aggregation behavior and to determine appropriate conditions for storage, crystallisation, and other processing parameters. Thus, its measurement can be applied to improve the formulation of dispersions, emulsions and suspensions [183, 184].

The measurement of the ζ-potential for NgPE and NgPE Hydrolysate, under different pH levels, range of protein concentrations and range of ionic strength (0-500 mM NaCl) is showed in Figure 5.6. In Figure 5.6a can be observed the pH effect for both samples studied. The charge of the NgPE was negative at high pH values (5-12) and increased and became positive as the pH was decreased (1-4). The net charge of the proteins decreased with increasing pH passing through zero at pH 4.3, corresponding to the isoelectric point of NgPE proteins (pH 4–5). In contrast ζ –potential for NgPE hydrolysate approaches to zero as well as the pH moves from 6 to 1 and can be stablished that the protein solution present instability in this range of pH. At pH values between 7 and 12 the protein hydrolysate shows ζ –potential around -30 mV, indicating the anionic character of the NgPE

hydrolysate and higher stability in compare to acid pH. According has been described in the literature by Malhotra *et al.* (2004), the charge on a protein can be influenced from the sum of the charges on the individual amino acid residues [185]. In this case, the anionic character of the protein hydrolysate, may be due to the release of acidic amino acids, being the most important amino acids present the glutamic and aspartic acid as could be observed in Table 5.7. Other amino acids carry charge but are not present on an adequate mass basis to influence the total charge.

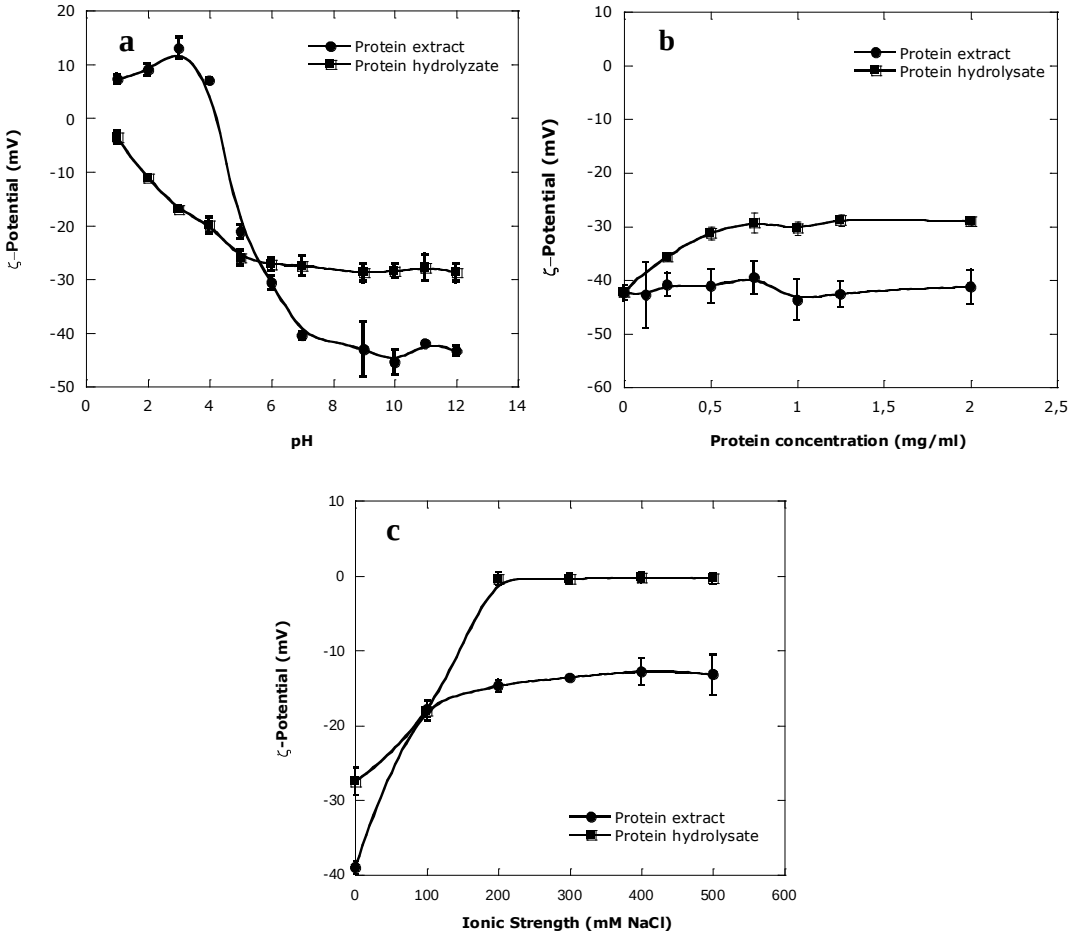

Figure 5.6. Variation of ζ –potential of protein extract (NgPE) and NgPE hydrolysate **a** ζ –potential of protein extract and protein hydrolysate from *N. gaditana* as a function of pH (solutions at 1% w/v) **b** ζ –potential of protein extract and protein hydrolysate from *N. gaditana* as a function of protein extract concentration (solutions at pH 7) **c** ζ –potential of protein extract and protein hydrolysate from *N. gaditana* as a function of salt concentration (solutions at pH 7, 1 %w/v)

On the other hand, Figure 5.6b shows that the ζ -potential was largely independent of the protein concentration, for both NgPE and NgPE hydrolysate, presenting a charge about -40 mV for NgPE and about -30 mV for NgPE hydrolysate, demonstrating these values its anionic character as well as an important stability of protein solution according has been described by Butler et al. (1996). Respect to the ζ -potential when the ionic strength was varied, from figure 5.6c can be observed the zeta potential measurements increased as the salt concentration was increased from 0-500 mM NaCl. This result demonstrated that the samples NgPE and NgPE hydrolyzate were neutralized due to the Na^+ presence. Thus the values of measured of zeta potentials for the proteins solutions became closer to zero as the salt concentration increased, which showed a larger instability for the protein hydrolysate in compare to the NgPE related to the NaCl concentration.

5.3.8 Antioxidant activity of protein hydrolysate

DPPH is a free radical generating compound and has been widely used to evaluate free radical scavenging ability of various antioxidant compounds [45]. In this work DPPH radical scavenging capacity assay was chosen to determine the IC_{50} of the protein extract and protein hydrolysate from *N.gadiatana* oil cake. Here, the antioxidant activity of NgPE and NgPE hydrolysate is shown in Figure 5.7a. For both samples an IC_{50} value was determined by DPPH scavening ability. However, 5-fold concentration of NgPE (160 mg/mL) was required to produce a radical scavenging equivalent to that of NgPE hydrolysate (31 mg/mL). In similar studies it has been demonstrated that a number of food-derived peptides or protein hydrolysates were capable of interacting and quenching DPPH radicals [45, 46, 48, 186, 187]. In particular Yoshie-Stark *et al.* (2004) determined the

median effective dose as $ED_{50.}$ They reported that hexane deoiled lupin, protein isolates, and their hydrolysates showed ED_{50} at the concentration of 118-238 mg/mL, whereas CO_2-deoiled lupin had ED_{50} at the concentration of 111-272 mg/mL [188].The ED_{50} values to the NgPE eached 160 mg/mL.

ORAC assay was further used to test the antioxidative capacity of the NgPE and NgPE hydrolysate. As shown in Figure 5.7b, the antioxidant capacity of the NgPE hydrolysate increased with respect to NgPE. In fact, the ORAC assay demonstrated that a concentration 10-fold of NgPE (0.54 mg/mL) was required to produce the same antioxidant effect as NgPE hydrolysate (0.05 mg/mL), confirming the antioxidant capacity of the protein hydrolysate obtained from NgPE enzymatic hydrolysis.

In this study changes in the antioxidant activity between NgPE and NgPE hydrolysate were observed by means of DPPH and ORAC method, and they can be explained in part by the differentiation observed in their electrophoretic patterns, in particular, by the distinctive protein band compositions for each sample (Figure 5.5b). In this case, disruption of the native protein structure by hydrolysis resulted in the release of protein fraction with molecular weight <10 kDa, thus the opening and exposure of active amino acid residues with antioxidant activity can react with oxidants as has been reported in similar works[176, 189]. However, some of their other characteristics, as amino acid composition, may also play an important role. It has been reported that aromatic amino acids, have the capability to convert radicals to stable molecules by donating electron, while keeping their own stability via resonance structure. Likewise, hydrophobic amino acids enhance the solubility of peptide in lipid which facilitates accessibility to hydrophobic radical species. On the other hand, sulfur amino acid group can act as radical scavenger, protecting tissue from

oxidative stress [177]. The amino acid profile presented in Table 5.7 shows the presence of these three groups of amino acid in NgPE hydrolysate and NgPE. Within the aromatic amino acids presents in the samples analyzed are Tyr and Phe; Cys and Met are present and correspond to the sulfur amino acids and the hydrophobics aminoacid in the samples are Gly, Al, Val, Leu, Pro Met Phe and Ile. However, it is important to keep in mind that the contribution of these amino acids has been related to their presence and position in the amino acid sequence and not necessarily to their concentration in the peptide fraction. Therefore, although there is no significant difference in the amino acid profile of the samples NgPE and NgPE hydrolysate, the amino acid sequence is different in between them due to hydrolysis effect and explains the difference found in the antioxidant activity.

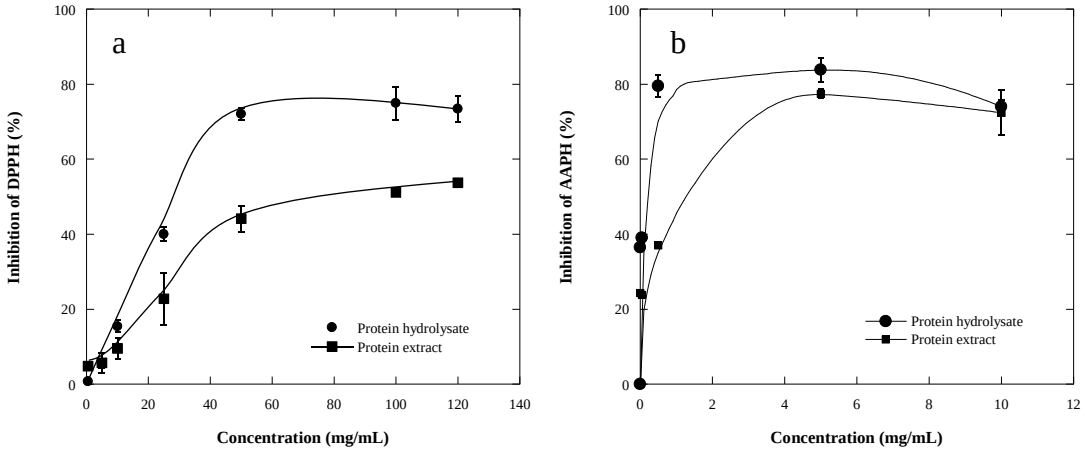

Figure 5.7. a Radical (DPPH•+) scavenging activity of *N. gaditana* protein extract and protein hydrolysate. The IC_{50} of each preparation was determined and expressed as mg/mL. **b** Antioxidant activity of protein extract and protein hydrolysate (ORAC assay). The ORAC result was expressed as IC_{50} value (mg/mL).

5.4 Conclusion

In this paper a protein extract and protein hydrolysate were obtained from defatted oil cake from *N. gaditana*. According to the results found in this study, both fractions showed interesting features that suggest promising applications. While the protein extract (NgPE) showed a higher protein content compared to the hydrolysate, both protein products had adequate amino acid balance comparable to FAO/WHO reference protein profile. Regarding the techno-functional properties, NgPE proved to have a remarkable emulsifying capacity and stability (> 50%) and significantly higher emulsifying properties than the protein hydrolysate. According to the optimization of enzymatic hydrolysis of NgPE, this substrate was effectively hydrolysed by papain reaching a high degree of hydrolysis (68%), which was confirmed in the SDS-PAGE electrophoretic assay, where peptides about molecular weight of 10 kDa were observed. This releasing of peptides of low molecular weight, could contribute to enhance of antioxidant activity of NgPE hydrolysate respect to NgPE as was observed by IC_{50} values determined by DPPH and ORAC assay. At the same time the stronger antioxidant capacity of the protein hydrolysate in compare to the extract may be attributed to the increased availability of hydrogen ions (protons and electrons) due to peptide cleavages by using papain, which could exert their antioxidant activity acting as acceptors or electron donors. In conclusion, the defatted oil cake from *N.gaditana* has potential to be used as feedstock to produce a protein extract and/or protein hydrolysate with functional and bioactive properties to be considered as natural or green ingredient which could be applied in food/feed industry. However, this study demonstrated that hydrolysis had enormously negative effects on the functional properties, mainly on

emulsifying capacity of the protein extract. Therefore, protein hydrolysate could be used as a potential antioxidant but may not be suitable as a functional food ingredient.

Finally, this work provides an alternative use as source proteins for elaboration of protein hydrolysate with antioxidant activity, which may find applications in food industry as functional ingredient.

Chapter 6:

General discussion and conclusions

6.1 General discussion

In the last decade an important number of studies about protein extraction from different alternative protein sources have been carried out with the idea of meeting a growing demand for this macronutrient because of the increase in world population. Some agriculture by-products have being studied as proteins sources such as, soybean meal, peanut meal, mucuna bean, rapeseed and sunflower meal. This need for proteins is an opportunity in the agriculture scope, especially in areas where biorefinery must be developed to make the production process economically feasible. However, the availability of proteins from agriculture by-products has not the potential to fulfill the worldwide protein demand. Under this context, microalgae oil cake represents a promising source of protein. At the same time the potential use of this by-product improve the economic feasibility of the biodiesel production from microalgae. Some uses have been described for defatted oil cake from microalgae such as ethanol and methane production, livestock feed, used as organic fertilizer due to its high N:P ratio, or simply burned for energy co-generation (electricity and heat). However, the opportunity to take advantage as protein source has not been developed until now.

In this thesis the opportunity to develop an alternative use for the defatted oil cake from microalgae is analyzed, through of the elaboration of electrospun proteins fibers formation by electrospinnig technique and enzymatic protein hydrolysate. In Chapter 3, the protein extraction process from defatted oil cake from microalgae was studied as a preliminary step in the potential use of this by-product. The main objective was maximized the protein extraction through the study of the influence and interactions of the operational factors such as pH, solvent/cake ratio, temperature and time of extraction. Results showed that the pH

was the most significant operational factor on protein extraction, followed by the interaction of the temperature-solvent/cake ratio, temperature and solvent/cake ratio. In addition, from the data analysis of the results was possible to obtain an empirical model capable of predicting the values of the protein extraction, where the factors and their interactions were included. According to our study a maximum protein extraction yield of 25.3 % was reached under the best conditions defined.

Soy protein isolate is the most common one used in foods. In general the soy protein isolate accounts for approximately 60% of recovery of the total protein present in the defatted white flakes and their protein content in the final product is generally > 90%. Respect to the protein content, our protein concentrate from *B. braunii* oil cake (74%), resulted lower in compare to the soy protein isolate. However, in soybean material the protein correspond to storage proteins that are present in protein bodies and are usually easily extractable. In contrast, proteins that are associated with cell walls or photosystems may be more difficult to extract, as is the microalgae case.

Previously to the protein extraction study, microalgae oil cake characterization was carried out to establish the starting point. This characterization demonstrated high protein content, therefore the protein concentrate obtained under optimal conditions, according to its protein content and amino acid profile, resulted to be suitable to be used in different applications, such as substrate in the protein hydrolysate production and as biopolymer in the fibers formation by electrospinning technique.

In chapter 4 of this work, the protein concentrate prepared from defatted oil cake from microalgae was used as biopolymer for the fibers formation by electrospinning technique. According to the results, the protein concentrate obtained from *B. braunii* cake showed to

be suitable feedstock for the production of nanofibers by electrospinning technique. Moreover, modifying the initial formulation of the protein solution by addition of the biodegradable polymer, allowed to obtain fibers with different diameters and morphologies, thus with potential to many end-applications.

In Chapter 5, we analyzed the idea for obtaining a protein hydrolysate from defatted oil cake from *N. gaditana* from biodiesel production process and to characterize this protein fraction. In the first part, results showed that a protein extract of high protein content and quality was obtained by means alkaline extraction from *N. gaditana* oil cake. Subsequently, an enzymatic protein hydrolysate was obtained using the protein extract as substrate. The enzymatic hydrolysate was optimized by RSM to reach a high degree of hydrolysis and to optimize the antioxidant capacity. In this section protein extract and protein hydrolysate were characterized by chemical, thecno-functional and physicochemical properties, and bioactive properties. From chemical characterization, was confirmed the high protein content and protein profile rich in essentials amino acid, comparable to FAO reference for both protein fractions analyzed. Moreover, from the results of the technofunctional properties, emulsifying activity, emulsifying stability and foaming properties of the NgPE were significantly higher than emulsifying and foaming properties of NgPE hydrolysate ($P < 0.05$). These results can be explained due to the high degree of hydrolysis of the NgPE, being related the peptide size with an increase of the generation of polar groups like NH_4 and COO^-. These factors are translated into increased solubility and thus decreased oil absorption for forming emulsions. Finally, antioxidant activity of both protein products using *in vitro* techniques was determined by DPPH and ORAC method. The results demonstrated that NgPE and NgPE hydrolysate have antioxidant activity, however the

antioxidant activity of the protein hydrolysate was 10-fold stronger than antioxidant activity of the protein extract.

6.2 General conclusions

The results obtained demonstrated that the protein extraction from *B. braunii* oil cake carried out by alkaline extraction is a function of independent factors studied (pH, temperature and solvent/cake ratio) as well as of interaction temperature and solvent/cake ratio. This implies that at the optimal combination of these factors (pH 12, 30 min, 40ºC and s/c ratio of 90), the maximum protein extraction was 23.5 % w/w. At the same time the elaboration of a protein concentrate (74%) was possible through the study of these operational factors. In addition, *B. braunii* oil cake and their protein concentrate have demonstrated to have interesting quality and content of protein, 48% and 74%, respectively. These results confirmed the potential of the by-product of microalgae from biodiesel production process as source of proteins.

Respect to the second objective of this thesis, this work showed that it is possible to produce electrospun microalgal protein concentrate fibers with different morphologies by manipulating the pH, PEO and MPC concentration in the electrospinning solutions. Therefore, this work revealed that the PEO/MPC blend can be readily spun into fibers by electrospinning techniques, allowing the incorporation of as high as 93% (w/w) of MPC in the fibers. Thus, in this case the protein from defatted oil cake from *B. braunii* effectively was used as biopolymer.

Through of the methodology employed for developing the last objective of this thesis was demonstrated is possible by means of simple processes at laboratory scale, to obtain a

protein hydrolysate with antioxidante activity from *N. gaditana* oil cake. Moreover in this study, protein extract and protein hydrolysate showed interesting features that suggest promising applications; while NgPE hydrolysate showed a higher soluble protein content compared to the protein extract, both fractions had adequate amino acid balance comparable to FAO/WHO/UN reference protein profile. In relation to the techno-functional properties, NgPE proved to have a remarkable emulsifying capacity and stability (> 50%) and significantly higher emulsifying properties than the protein hydrolysate.

According to the optimization of enzymatic hydrolysis of NgPE, this substrate was effectively hydrolysed by papain reaching a high degree of hydrolysis (68%), which was confirmed in the SDS-PAGE electrophoretic assay. This releasing of peptides of low molecular weight, could contribute to enhance of antioxidant activity of NgPE hydrolysate respect to NgPE about 10-fold as was determined by ORAC assay. In conclusion, *N.gaditana* oil cake has potential to be used as feedstock to produce a protein extract and/or protein hydrolysate with functional and bioactive properties to be considered as natural or green ingredient which could be studied in the future for applications on food or feed industry.

Finally, this work provides two alternative ways for potential applications for defatted oil cake from microalgae. On the one hand the protein is recovered to be used as a biopolymer in the fibers formation for potential applications in the nanomaterial scope with focus in food packaging; on the other hand the protein is extracted to the preparation of two products such as protein extract and protein hydrolysate with functional and bioactive properties with potential to be used in food/feed industry.

REFERENCES

1. Parker, M.S., T. Mock, and E.V. Armbrust, *Genomic Insights into Marine Microalgae*, in *Annual Review of Genetics*. 2008. p. 619-645.
2. Spolaore, P., et al., *Commercial applications of microalgae.* Journal of Bioscience and Bioengineering, 2006. **101**(2): p. 87–96.
3. Khan, S.A., et al., *Prospects of biodiesel production from microalgae in India.* Renewable and Sustainable Energy Reviews, 2009. **13**(9): p. 2361-2372.
4. Chen, C.Y., et al., *Strategies to Enhance Cell Growth and Achieve High-Level Oil Production of a Chlorella vulgaris Isolate.* Biotechnology Progress, 2010. **26**(3): p. 679-686.
5. Converti, A., et al., *Effect of temperature and nitrogen concentration on the growth and lipid content of Nannochloropsis oculata and Chlorella vulgaris for biodiesel production.* Chemical Engineering and Processing, 2009. **48**(6): p. 1146-1151.
6. Debska, D., et al., *Effects of Medium Composition on the Growth of Chlorella vulgaris During Photobioreactor Batch Cultivations.* Journal of Biobased Materials and Bioenergy, 2010. **4**(1): p. 68-72.
7. Lv, J.M., et al., *Enhanced lipid production of Chlorella vulgaris by adjustment of cultivation conditions.* Bioresource Technology, 2010. **101**(17): p. 6797-6804.
8. Sostaric, M., et al., *Studies on the Growth of Chlorella vulgaris in Culture Media with Different Carbon Sources.* Chemical and Biochemical Engineering Quarterly, 2009. **23**(4): p. 471-477.
9. Widjaja, A., C.C. Chien, and Y.H. Ju, *Study of increasing lipid production from fresh water microalgae Chlorella vulgaris.* Journal of the Taiwan Institute of Chemical Engineers, 2009. **40**(1): p. 13-20.
10. Yoo, C., et al., *Selection of microalgae for lipid production under high levels carbon dioxide.* Bioresource Technology, 2010. **101**: p. S71-S74.
11. Zhang, W., et al., *Biodiesel production with microbial oil of high oleic acid content from a microalga Chlorella vulgaris.* Abstracts of Papers of the American Chemical Society, 2008. **235**.

12. Damiani, M.C., et al., *Lipid analysis in Haematococcus pluvialis to assess its potential use as a biodiesel feedstock.* Bioresource Technology, 2010. **101**(11): p. 3801-3807.

13. Dayananda, C., et al., *Isolation and characterization of hydrocarbon producing green alga Botryococcus braunii from Indian freshwater bodies.* Electronic Journal of Biotechnology, 2007. **10**(1): p. 78-91.

14. Dayananda, C., et al., *Autotrophic cultivation of Botryococcus braunii for the production of hydrocarbons and exopolysaccharides in various media.* Biomass & Bioenergy, 2007. **31**(1): p. 87-93.

15. Meng, X., et al., *Biodiesel production from oleaginous microorganisms.* Renewable Energy, 2009. **34**(1): p. 1-5.

16. Metzger, P., M.N. Rager, and C. Largeau, *Polyacetals based on polymethylsqualene diols, precursors of algaenan in Botryococcus braunii race B.* Organic Geochemistry, 2007. **38**(4): p. 566-581.

17. Metzger, P., et al., *Lycopanerols I-L, four new tetraterpenoid ethers from Botryococcus braunii.* Journal of Natural Products, 2003. **66**(6): p. 772-778.

18. Rao, A.R., et al., *Effect of salinity on growth of green alga Botryococcus braunii and its constituents.* Bioresource Technology, 2007. **98**: p. 560–564.

19. Tran, H.L., S.J. Hong, and C.G. Lee, *Evaluation of extraction methods for recovery of fatty acids from Botryococcus braunii LB 572 and Synechocystis sp PCC 6803.* Biotechnology and Bioprocess Engineering, 2009. **14**(2): p. 187-192.

20. Tran, H.L., et al., *Statistical Optimization of Culture Media for Growth an Lipid Production of Botryococcus braunii LB572.* Biotechnology and Bioprocess Engineering, 2010. **15**: p. 277-284.

21. Tsukahara, K. and S. Sawayama, *Liquid fuel production using microalgae.* Journal of the Japan Petroleum Institute, 2005. **48**(5): p. 251-259.

22. Yamaguchi, K., et al., *Lipid Composition of a Green Alga, Botryococcus braunii.* Agricultural and Biological Chemistry, 1987. **51**: p. 493-498.

23. Zhila, N.O., G.S. Kalacheva, and T.G. Volova, *Influence of nitrogen deficiency on biochemical composition of the green alga Botryococcus.* Journal of Applied Phycology, 2005. **17**(4): p. 309-315.

24. Lee, J.Y., et al., *Comparison of several methods for effective lipid extraction from microalgae.* Bioresource Technology, 2010. **101** p. S75–S77.

25. Alu'datt, M.H., et al., *Optimisation, Characterisation and Quantification of Phenolic Compounds in Olive Cake.* Food Chemistry, 2010. **123**: p. 117-122.

26. Li, X., H.Y. Hu, and J. Yang, *Lipid accumulation and nutrient removal properties of a newly isolated freshwater microalga, Scenedesmus sp LX1, growing in secondary effluent.* New Biotechnology, 2010. **27**(1): p. 59-63.

27. Miao, X. and Q. Wu, *Biodiesel production from heterotrophic microalgal oil.* Bioresource Technology, 2006. **97**(6): p. 841-846.

28. Ren, M., K. Ogden, and B. Lian, *Effect of culture conditions on the growth rate and lipid production of microalgae Nannochloropsis gaditana.* Journal of Renewable and Sustainable Energy, 2013. **5**(6).

29. Deng, X., Y. Li, and X. Fei, *Microalgae: A promising feedstock for biodiesel.* African Journal of Microbiology Research, 2009. **3**(13): p. 1008-1014.

30. Verma, N.M., et al., *Prospective of biodiesel production utilizing microalgae as the cell factories: A comprehensive discussion* African Journal of Biotechnology, 2010. **9**: p. 1402-1411.

31. Sánchez, A., et al., *Shear stress tolerance and biochemical characterization of Phaeodactylum tricornutum in quasi steady-state continuous culture in outdoor photobioreactors.* Biochemical Engineering Journal, 2003. **16**: p. 287-297.

32. Wang, B., et al., *CO2 bio-mitigation using microalgae.* Applied Microbiology and Biotechnology 2008. **79**: p. 707–718.

33. Becker, E.W., *Microalgae — Biotechnology and Microbiology*. 1994, Cambridge: Cambridge University Press.

34. Barrow, C. and F. Shahidi, *Marine nutraceuticals and functional foods*, ed. F. Shahidi. 2008: Taylor & Francis Group.

35. Carolina, S., et al., *Characterization of proteins from Spirulina platensis microalga using capillary electrophoresis-ion trap-mass spectrometry and capillary electrophoresis-time of flight-mass spectrometry.* ELECTROPHORESIS, 2005. **26**(13): p. 2674-2683.

36. Morris, H.J., et al., *Utilisation of Chlorella vulgaris cell biomass for the production of enzymatic protein hydrolysates* Bioresource Technology 2008. **99** p. 7723-7729.

37. Morris, H.J., et al., *Immunostimulant activity of an enzymatic protein hydrolysate from green microalga Chlorella vulgaris on undernourished mice.* Enzyme and Microbial Technology, 2007. **40**(3): p. 456-460.

38. Morris, H.J., et al., *Composicion bioquímica y evaluación de la calidad proteica de la biomasa autotrófica de chorella vulgaris.* Revista Cubana de Alimentación y Nutrición, 1999. **13**(2): p. 123-128.

39. Nirmala, C., V. Prakash, and L.V. Venkataraman, *Physico-chemical and functional properties of proteins from spray dried algae (Spirulina platensis).* Food / Nahrung, 1992. **36**(6): p. 569-577.

40. Sheih, I.C., T.J. Fang, and T.-K. Wu, *Isolation and characterisation of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste.* Food Chemistry, 2009. **115**(1): p. 279-284.

41. Sheih, I.C., et al., *Anticancer and Antioxidant Activities of the Peptide Fraction from Algae Protein Waste.* Journal of Agricultural and Food Chemistry, 2010. **58**(2): p. 1202-1207.

42. Sheih, I.C., T.-K. Wu, and T.J. Fang, *Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems.* Bioresource Technology, 2009. **100**(13): p. 3419-3425.

43. Kuehler, C.A. and C.M. Stine, *Effect of enzymatic hydrolysis on some functional properties of whey protein.* Journal of Food Science, 1974. **39**(2): p. 379-382.

44. Ng, K.L., et al., *Optimization of enzymatic hydrolysis of palm kernel cake protein (PKCP) for producing hydrolysates with antiradical capacity.* Industrial Crops and Products, 2013. **43**(0): p. 725-731.

45. Vastag, Z., et al., *Hydrolysis of pumpkin oil cake protein isolate and free radical scavenging activity of hydrolysates: Influence of temperature, enzyme/substrate ratio and time.* Food and Bioproducts Processing, 2010. **In Press, Corrected Proof**.

46. Yoshie-Stark, Y., Y. Wada, and A. Wäsche, *Chemical composition, functional properties, and bioactivities of rapeseed protein isolates.* Food Chemistry, 2008. **107**(1): p. 32-39.

47. Yoshie-Stark, Y. and A. Wäsche, *In vitro binding of bile acids by lupin protein isolates and their hydrolysates.* Food Chemistry, 2004. **88**(2): p. 179-184.

48. Zhu, K., H. Zhou, and H. Qian, *Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase.* Process Biochemistry, 2006. **41**(6): p. 1296-1302.

49. Thawien, W., *Protein-Based Edible Films: Characteristics and Improvement of Properties*. 2012.

50. Lin, L., et al., *Alimentary 'green' proteins as electrospun scaffolds for skin regenerative engineering*. Journal of Tissue Engineering and Regenerative Medicine, 2013. **7**(12): p. 994-1008.

51. Vega-Lugo, A.-C. and L.-T. Lim, *Electrospinning of Soy Protein Isolate Nanofibers.* Journal of Biobased Materials and Bioenergy, 2008. **2**(3): p. 223-230.

52. Vega-Lugo, A.-C. and L.-T. Lim, *Controlled release of allyl isothiocyanate using soy protein and poly(lactic acid) electrospun fibers.* Food Research International, 2009. **42**(8): p. 933-940.

53. Becker, E.W., *Micro-algae as a source of protein* Biotechnology Advances 2007. **25**: p. 207–210.

54. Chronakis, I.S., *Gelation of edible blue-green algae protein isolate (Spirulina platensis strain Pacifica): Thermal transitions, rheological properties, and molecular forces involved.* Journal of Agricultural and Food Chemistry, 2001. **49**(2): p. 888-898.

55. Devi, M.A. and L.V. Venkataraman, *Functional Properties of Protein Products of Mass Cultivated Blue-Green Alga Spirulina Platensia.* Journal of Food Science, 1984. **49**(1): p. 24-27.

56. Ramadan, M.F., M.Z. Sitohy, and J.T. Moersel, *Solvent and enzyme-aided aqueous extraction of goldenberry (Physalis peruviana L.) pomace oil: impact of processing on composition and quality of oil and meal.* European Food Research and Technology, 2008. **226**: p. 1445-1458.

57. Bautista, J., J. Parrado, and A. Machado, *Composition and fractionation of sunflower meal: use of the lignocellulosic fraction as substrate in solid-state fermentation.* Biological Wastes, 1990. **32**: p. 225–33.

58. Pandey, A. and C.R. Soccol, *Economic utilization of crop residues for value addition – a futuristic approach.* Journal of Scientific and Industrial Research, 2000. **59**: p. 12-22.

59. Kao, T.H., et al., *Anti-inflammatory Effects of Isoflavone Powder Produced from Soybean Cake.* Journal of Agricultural and Food Chemistry, 2007. **55**(26): p. 11068-11079.

60. Kao, T.-H. and B.-H. Chen, *Functional Components in Soybean Cake and Their Effects on Antioxidant Activity.* Journal of Agricultural and Food Chemistry, 2006. **54**(20): p. 7544-7555.

61. Suja, K.P., et al., *Antioxidant efficacy of sesame cake extract in vegetable oil protection.* Food Chemistry, 2004. **84**(3): p. 393-400.

62. Ramachandran, S., et al., *Oil cakes and their biotechnological applications - A review.* Bioresource Technology, 2007. **98**(10): p. 2000-2009.

63. Felizardo, P., et al., *Production of biodiesel from waste frying oils.* Waste Management, 2006. **26**(5): p. 487-494.

64. Kulkarni, M.G. and A.K. Dalai, *Waste Cooking OilAn Economical Source for Biodiesel: A Review.* Industrial & Engineering Chemistry Research, 2006. **45**(9): p. 2901-2913.

65. Barnwal, B.K. and M.P. Sharma, *Prospects of biodiesel production from vegetable oils in India.* Renewable and Sustainable Energy Reviews, 2005. **9**(4): p. 363-378.

66. Sivaramakrishnan, S. and D. Gangadharan, *Edible oil cake*. 2009, Coleraine: Biotechnology for Agro-industrial Residues Utilisation: Utilization of Agro-residues.

67. Pastuszewska, B., et al., *The protein value of differently processed rapeseed solvent meal and cake assessed by in vitro methods and in tests with rats.* Animal Feed Science and Technology, 2003. **106**(1-4): p. 175-188.

68. Glencross, B., W. Hawkins, and J. Curnow, *Nutritional assessment of Australian canola meals. I. Evaluation of canola oil extraction method and meal processing conditions on the digestible value of canola meals fed to the red seabream (Pagrus auratus, Paulin).* Aquaculture Research, 2004. **35**: p. 15 - 24.

69. Anil Kumar, G.K., et al., *Mustard cake as a source of dietary protein for growing lambs.* Small Ruminant Research, 2002. **44**(1): p. 47-51.

70. Omar, J.M.A., *Effects of feeding different levels of sesame oil cake on performance and digestibility of Awassi lambs.* Small Ruminant Research, 2002. **46**(2-3): p. 187-190.

71. Chen, H.-M., et al., *Antioxidant Activity of Designed Peptides Based on the Antioxidative Peptide Isolated from Digests of a Soybean Protein.* Journal of Agricultural and Food Chemistry, 1996. **44**(9): p. 2619-2623.

72. Schiffman, J.D. and C.L. Schauer, *A Review: Electrospinning of Biopolymer Nanofibers and their Applications.* Polymer Reviews, 2008. **48**(2): p. 317-352.

73. Huang, Z.-M., et al., *A review on polymer nanofibers by electrospinning and their applications in nanocomposites.* Composites Science and Technology, 2003. **63**(15): p. 2223-2253.

74. Subhadra, B.G., *Sustainability of algal biofuel production using integrated renewable energy park (IREP) and algal biorefinery approach.* Energy Policy, 2010. **38**(10): p. 5892-5901.

75. Zhu, Y., et al., *Development of hydrothermal liquefaction and upgrading technologies for lipid-extracted algae conversion to liquid fuels.* Algal Research, 2013. **2**(4): p. 455-464.

76. Yang, Z., et al., *Hydrogen and methane production from lipid-extracted microalgal biomass residues.* International Journal of Hydrogen Energy, 2011. **36**(5): p. 3465-3470.

77. Geun Goo, B., et al., *Characterization of a renewable extracellular polysaccharide from defatted microalgae Dunaliella tertiolecta.* Bioresource Technology, 2013. **129**(0): p. 343-350.

78. Morris, H.J., et al., *Protein hydrolysates from the alga Chlorella vulgaris 87/1 with potentialities in immunonutrition*, in *Biotecnología Aplicada*. 2009: Cuba.

79. Guil-Guerrero, J.L., et al., *Functional properties of the biomass of three microalgal species.* Journal of Food Engineering 2004. **65** p. 511–517.

80. Barbarino, E. and S.O. Lourenço, *An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae.* Journal of Applied Phycology, 2005. **17**: p. 447-460.

81. Meijer, E.A. and R.H. Wijffels, *Development of a fast, reproducible and effective method for the extraction and quantification of proteins of micro-algae.* Biotechnology Techniques, 1998. **12**(5): p. 353-358.

82. Hao, C. and Z. Mao. *Application of response surface methodology for optimization of protein extraction from Chlorella sorokiniana*. in *ASABE Annual meeting* 2010. Pittsburgh, Pennsylvania.

83. Rausch, T., *The estimation of micro-algal protein content and its meaning to the evaluation of algal biomass I. Comparison of methods for extracting protein.* Hydrobiologia, 1981. **78**: p. 237-251.

84. Coustets, M., et al., *Optimization of protein electroextraction from microalgae by a flow process.* Bioelectrochemistry, 2015. **103**(0): p. 74-81.

85. Eboibi, B.E., et al., *Influence of process conditions on pretreatment of microalgae for protein extraction and production of biocrude during hydrothermal liquefaction of pretreated Tetraselmis sp.* RSC Advances, 2015. **5**(26): p. 20193-20207.

86. Safi, C., et al., *Release of hydro-soluble microalgal proteins using mechanical and chemical treatments.* Algal Research, 2014. **3**(0): p. 55-60.

87. Safi, C., et al., *Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods.* Algal Research, 2014. **3**(0): p. 61-65.

88. Ju, Z.Y., D.-F. Deng, and W. Dominy, *A defatted microalgae (Haematococcus pluvialis) meal as a protein ingredient to partially replace fishmeal in diets of Pacific white shrimp (Litopenaeus vannamei, Boone, 1931).* Aquaculture, 2012. **354–355**(0): p. 50-55.

89. S.L., L.-I., T. L.N., and S. A., *Ruminant Nutrition Symposium: The utility of lipid extracted algae as a protein source in forage or starch-based ruminant diets.* Journal of Animal Science, 2014. **92**(4): p. 1331-1342.

90. Gavrilescu, M. and Y. Chisti, *Biotechnology—a sustainable alternative for chemical industry.* Biotechnology Advances, 2005. **23**(7–8): p. 471-499.

91. Sawayama, S., et al., *CO_2 fixation and oil production through microalga.* Energy Conversion and Management, 1995. **36**(6-9): p. 729-731.

92. Wijffels, R.H. and M.J. Barbosa, *An Outlook on Microalgal Biofuels.* Science, 2010. **329**(5993): p. 796-799.

93. Wicke, B., et al., *Exploring land use changes and the role of palm oil production in Indonesia and Malaysia.* Land Use Policy, 2011. **28**(1): p. 193-206.

94. Lima, M., M. Skutsch, and G.d.M. Costa, *Deforestation and the Social Impacts of Soy for Biodiesel: Perspectives of Farmers in the South Brazilian Amazon.* Ecology and Society, 2011. **16**(4).

95. Timilsina, G.R., et al., *The impacts of biofuels targets on land-use change and food supply: A global CGE assessment.* Agricultural Economics, 2012. **43**(3): p. 315-332.

96. Mata, T.M., A.A. Martins, and N.S. Caetano, *Microalgae for biodiesel production and other applications: A review.* Renewable and Sustainable Energy Reviews, 2010. **14** p. 217–232.

97. Borowitzka, M.A., *Comercial production of microalgae: ponds, tanks, tubes and fermentors.* Journal of Biotechnology, 1999. **70**(313-321).

98. Gerde, J.A., et al., *Optimizing protein isolation from defatted and non-defatted Nannochloropsis microalgae biomass.* Algal Research, 2013. **2**(2): p. 145-153.

99. Benemann, J., *Microalgae aquaculture feeds.* Journal of Applied Phycology, 1992. **4**(3): p. 233-245.

100. Brown, M.R. and S.W. Jeffrey, *Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. Amino acids, sugars and pigments.* Journal of Experimental Marine Biology and Ecology, 1992. **161**(1): p. 91-113.

101. Brown, M.R., et al., *Nutritional properties of microalgae for mariculture.* Aquaculture, 1997. **151**(1-4): p. 315-331.

102. da Silva, R. and J. Barbosa, *Seaweed meal as a protein source for the white shrimp Litopenaeus vannamei.* Journal of Applied Phycology, 2009. **21**(2): p. 193-197.

103. Belarbi, E.H., E. Molina, and Y. Chisti, *A process for high yield and scaleable recovery of high purity eicosapentaenoic acid esters from microalgae and fish oil.* Enzyme and Microbial Technology, 2000. **26**(7): p. 516-529.

104. Chauton, M.S., et al., *A techno-economic analysis of industrial production of marine microalgae as a source of EPA and DHA-rich raw material for aquafeed: Research challenges and possibilities.* Aquaculture, 2015. **436**(0): p. 95-103.

105. Durmaz, Y., et al., *The effect of low temperature on fatty acid composition and tocopherols of the red microalga, Porphyridium cruentum.* Journal of Applied Phycology, 2007. **19**(3): p. 223-227.

106. Cohen, Z., S. Didi, and Y.M. Heimer, *Overproduction of {gamma}-Linolenic and Eicosapentaenoic Acids by Algae.* Plant Physiol., 1992. **98**(2): p. 569-572.

107. Geresh, S., et al., *Characterization of the extracellular polysaccharide of Porphyridium sp.: molecular weight determination and rheological properties.* Carbohydrate Polymers, 2002. **50**(2): p. 183-189.

108. Sun, Y., et al., *The isolation and antioxidant activity of polysaccharides from the marine microalgae Isochrysis galbana.* Carbohydrate Polymers, 2014. **113**(0): p. 22-31.

109. Bermejo Román, R., et al., *Recovery of pure B-phycoerythrin from the microalga Porphyridium cruentum.* Journal of Biotechnology, 2002. **93**(1): p. 73-85.

110. Gouveia, L., et al., *Functional food oil coloured by pigments extracted from microalgae with supercritical CO2.* Food Chemistry, 2007. **101**(2): p. 717-723.

111. Mendes, R.L., et al., *Supercritical CO2 extraction of carotenoids and other lipids from Chlorella vulgaris.* Food Chemistry, 1995. **53**(1): p. 99-103.

112. Nobre, B., et al., *Supercritical carbon dioxide extraction of astaxanthin and other carotenoids from the microalga Haematococcus pluvialis.* Europe Food Research and Technology, 2006. **223**: p. 787–790.

113. Abe, K., H. Hattori, and M. Hirano, *Accumulation and antioxidant activity of secondary carotenoids in the aerial microalga Coelastrella striolata var. multistriata.* Food Chemistry, 2007. **100**(2): p. 656-661.

114. Cerón, M.C., et al., *Antioxidant activity of Haematococcus pluvialis cells grown in continuous culture as a function of their carotenoid and fatty acid content.* Applied Microbiology and Biotechnology, 2007. **74**: p. 1112-1119.

115. Mendiola, J.A., et al., *Screening of functional compounds in supercritical fluid extracts from Spirulina platensis.* Food Chemistry, 2007. **102**(4): p. 1357-1367.

116. Herrero, M., et al., *Dunaliella salina Microalga Pressurized Liquid Extracts as Potential Antimicrobials.* Journal of Food Protection, 2006. **69**: p. 2471-2477.

117. Santoyo, S., et al., *Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from Haematococcus pluvialis microalgae.* LWT - Food Science and Technology, 2009. **42**(7): p. 1213-1218.

118. Meyer, M.A. and A. Weiss, *Life cycle costs for the optimized production of hydrogen and biogas from microalgae.* Energy, 2014. **78**(0): p. 84-93.

119. Mussgnug, J.H., et al., *Microalgae as substrates for fermentative biogas production in a combined biorefinery concept.* Journal of Biotechnology, 2010. **150**(1): p. 51-56.

120. Ramos-Suárez, J.L. and N. Carreras, *Use of microalgae residues for biogas production.* Chemical Engineering Journal, 2014. **242**(0): p. 86-95.

121. Riaño, B., B. Molinuevo, and M.C. García-González, *Treatment of fish processing wastewater with microalgae-containing microbiota.* Bioresource Technology, 2011. **102**(23): p. 10829-10833.

122. Ruiz-Martinez, A., et al., *Microalgae cultivation in wastewater: Nutrient removal from anaerobic membrane bioreactor effluent.* Bioresource Technology, 2012. **126**(0): p. 247-253.

123. Banerjee, A., et al., *Botryococcus braunii: a renewable source of hydrocarbons and other chemicals.* Critical Reviews in Biotechnology, 2002. **22**(3): p. 245–279.

124. Dayananda, C., et al., *Isolation, characterization and outdoor cultivation of green microalgae Botryococcus sp.* Scientific Research and Essays, 2010. **5**(17): p. 2497-2505.

125. Kanda, H., et al., *Wet extraction of hydrocarbons from Botryococcus braunii by dimethyl ether as compared with dry extraction by hexane.* Fuel, 2013. **105**(0): p. 535-539.

126. Saga, K., et al., *Comparative evaluation of wet and dry processes for recovering hydrocarbon from Botryococcus Braunii.* Applied Energy, 2015. **141**(0): p. 90-95.

127. Zhang, F., et al., *Application of memberane dispersion for enhanced lipid milking from Botryococcus braunii FACHB 357.* Journal of Biotechnology, 2013. **165**(1): p. 22-29.

128. Andrade, R.D., et al., *Obtaining of flour to leave of the cultivation of Chlorella vulgaris and their protein analysis* Temas Agrarios, 2007. **12**(1): p. 50 - 57.

129. McArdle, R.N., et al., *Protein and Amino Acid Content of Selected Microalgae Species.* Lebensmittel-Wissenschaft und-Technologie, 1994. **27**(3): p. 249-252.

130. Vioque, J., et al., *Obtención y aplicaciones de concentrados y aislados protéicos* Grasas y Aceites, 2001. **52**(2): p. 127-131.

131. Boye, J., F. Zare, and A. Pletch, *Pulse proteins: Processing, characterization, functional properties and applications in food and feed.* Food Research International, 2010. **43**(2): p. 414-431.

132. Lowry, O.H., *Protein measurement with the Folin-Phenol reagents.* J. Biol. Chem., 1951. **193**: p. 265-275.

133. Montgomery, D.C., ed. *Design and Analysis of Experiments*. 2001, Wiley: New York.

134. A.O.A.C., ed. *Official methods of analysis*. 16th ed. 1995, (Ed.). Arlington, VA: Association of Analytical Chemists. 1094 p.

135. Deak, N.A., et al., *Soy protein products, processing, and utilization*, in *In Soybeans: Chemistry, Production, Processing, and Utilization, AOCS Press, Urbana, IL.*, P.J.W. Johnson, R. Galloway (Eds.), Editor. 2008: L.A. p. 661–724.

136. Adebowale, Y.A., *A study of the control variables during the preparation of protein isolate from Mucuna Ben (Mucuna Pruriens).* Electronic Journal of Environmental, Agricultural and Food Chemistry, 2008. **7**(9): p. 3223-3238.

137. Kain, R.J., et al., *Study on the effect of control variables on the extraction of penaut protein isolate from penaut meal (Arachis hypogaea L.).* American Journal of Food Technology, 2009. **4**(1): p. 47-55.

138. Allard, B. and E. Casadevall, *Carbohydrate composition and characterization of sugars from the green microalga Botryococcus Braunii.* Phytochemistry, 1990. **29**(6): p. 1875-1878.

139. Claire, B., et al., *Variability of cell wall structure and hydrocarbon type in different strains of "Botryococcus braunii".* Journal of Phycology, 1984. **20**(3): p. 377-389.

140. Blokker, P., et al., *Chemical fingerprinting of algaenans using RuO4 degradation.* Organic Geochemistry, 2006. **37**(8): p. 871-881.

141. Nurdiyana, H. and M.K. Siti Mazlina, *Optimization of protein extraction from fish waste using response surface methodology.* Journal of Applied Sciences, 2009. **9**(17): p. 3121-3125.

142. Sathe, S.K., S.S. Deshpande, and D.K. Salunkhe, *Functional Properties of Lupin Seed (Lupinus mutabilis) Proteins and Protein Concentrates.* Journal of Food Science, 1982. **47**(2): p. 491-497.

143. Yao, L., J. Gerde, and T. Wang, *Oil extraction from microalga Nannochloropsis sp. with isopropyl alcohol.* Journal of the American Oil Chemists' Society, 2012. **89**(12): p. 2279-2287.

144. FAO/WHO/UNU, *Energy and protein requirements*, in *Technical Report Series. Report of the Joint FAO/WHO/UNU Expert Consultation*. 1985, FAO, WHO and the United Nations University: Switzerland.

145. Kriegel, C., et al., *Fabrication, Functionalization, and Application of Electrospun Biopolymer Nanofibers.* Critical Reviews in Food Science and Nutrition, 2008. **48**(8): p. 775-797.

146. Kowalczyk, T., et al., *Electrospinning of Bovine Serum Albumin. Optimization and the Use for Production of Biosensors.* Biomacromolecules, 2008. **9**(7): p. 2087-2090.

147. Scheibel, T., *Protein fibers as performance proteins: new technologies and applications.* Current Opinion in Biotechnology 2005, 2005. **16**: p. 427-433.

148. Ramakrishna, S., et al., eds. *An Introduction to Electrospinning and Nanofibers*. 2005, World Scientific publishing: Singapore.

149. Dror, Y., et al., *Nanofibers Made of Globular Proteins.* Biomacromolecules, 2008. **9**(10): p. 2749-2754.

150. Verdugo, M., B. Cancino, and M. Rubilar. *Caracterización de un Aislado proteico de Torta Microalgal de Botryococcus braunii* in *XVIII Congreso Nacional de Ciencia y Tecnología de los Alimentos Ciencia, Tecnología e Ingeniería de los Alimentos: su rol en el bienestar y el buen vivir (Sumak Kawsay) II Congreso Latinoamericano IV del Caribe sobre Inocuidad y Calidad en Alimentos*. 2011. Valdivia, Chile.

151. Rodrigo, R., C.A. Toro, and J. Cuellar, *Influence of the geometric factors of the experimental device used in suspension polymerization on the properties of poly(styrene-co-divinylbenzene) microparticles.* Journal of Applied Polymer Science, 2012. **124**(2): p. 1431-1446.

152. Huang, Z.-M., et al., *Electrospinning and mechanical characterization of gelatin nanofibers.* Polymer, 2004. **45**(15): p. 5361-5368.

153. Vega-Lugo, A.-C. and L.-T. Lim, *Effects of poly(ethylene oxide) and pH on the electrospinning of whey protein isolate.* Journal of Polymer Science Part B: Polymer Physics, 2012. **50**(16): p. 1188-1197.

154. Morais, M.G., et al., *Preparation of nanofibers containing the microalga Spirulina (Arthrospira).* Bioresource Technology, 2010. **101**: p. 2872–2876.

155. Pavia, D.L., G.M. Lampman, and G.S. Kritz, eds. *Introduction to Spectroscopy*. Saunders Golden Sunburst Series. 1996: USA.

156. Brennan, L. and P. Owende, *Biofuels from microalgae—A review of technologies for production, processing, and extractions of biofuels and co-products.* Renewable and Sustainable Energy Reviews, 2010. **14**: p. 557– 577.

157. Chisti, Y., *Biodiesel from microalgae.* Biotechnology Advances, 2007. **25**(3): p. 294-306.

158. Sharif, A.B.M., et al., *Biodiesel Fuel Production from Algae as Renewable Energy.* American Journal of Biochemistry and Biotechnology, 2008 **4**(3): p. 250-254.

159. Clemente, A., *Enzymatic protein hydrolysates in human nutrition.* Trends in Food Science &Technology, 2000. **11**(7): p. 254-262.

160. Mendis, E., N. Rajapakse, and S.-K. Kim, *Antioxidant Properties of a Radical-Scavenging Peptide Purified from Enzymatically Prepared Fish Skin Gelatin Hydrolysate.* Journal of Agricultural and Food Chemistry, 2004. **53**(3): p. 581-587.

161. Cheng, Y., Y.L. Xiong, and J. Chen, *Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions.* Food Chemistry, 2010. **120**(1): p. 101-108.

162. Vastag, Z., et al., *Antioxidant and angiotensin-I converting enzyme inhibitory activity in the water-soluble protein extract from Petrovac Sausage (Petrovská Kolbása).* Food Control, 2010. **21**(9): p. 1298-1302.

163. Wang, J.-s., et al., *Antioxidant properties of papain hydrolysates of wheat gluten in different oxidation systems.* Food Chemistry, 2007. **101**(4): p. 1658-1663.

164. Medina, C., et al., *Protein fractions with techno-functional properties fromNannochloropsisgaditana microalgal biomass.* Journal of Biobased Material and Bioenergy, 2015. **In Press, Corrected Proof**.

165. Hoyle, N.T. and J.H. Merritt, *Quality of Fish Protein Hydrolysates from Herring (Clupea harengus).* Journal of Food Science, 1994. **59**(1): p. 76-79.

166. Bollag, D., M. Rozicky, and S. Edelstein, eds. *Protein Methods* 1996: New York. USA. 405.

167. Betschart, A.A., R.Y. Fong, and M.M. Hanamoto, *Safflower Protein isolates: Functional Properties in Simple Systems and Breads* Journal of Food Science, 1979. **44**(4): p. 1022-1035.

168. Cheng, Y., Y.L. Xiong, and J. Chen, *Antioxidant and emulsifying properties of potato protein hydrolysate in soybean oil-in-water emulsions.* Food Chemistry, 2010. **120**: p. 101–108.

169. Hatano, T., et al., *Two New Flavonoids and Other Constituents in Licorice Root : Their Relative Astringency and Radical Scavenging Effects.* Chemical & Pharmaceutical Bulletin, 1988. **36**(6): p. 2090-2097.

170. Ou, B.X., M. Hampsch-Woodill, and R.L. Prior, *Development and Validation of an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as the Fluorescent Probe* Journal of Agricultural and Food Chemistry, 2001. **49**(10): p. 4619-4626.

171. Wang, B., et al., *Preparation and evaluation of antioxidant peptides from ethanol-soluble proteins hydrolysate of Sphyrna lewini muscle.* Peptides, 2012. **36**(2): p. 240-250.

172. Enari, H., et al., *Identification of angiotensin I-converting enzyme inhibitory peptides derived from salmon muscle and their antihypertensive effect.* Fisheries Science, 2008. **74**(4): p. 911-920.

173. Song, L., et al., *Antioxidant Activities of Hydrolysates of Arca Subcrenata Prepared with Three Proteases.* Marine Drugs, 2008. **6**(4): p. 607-619.

174. Van der Plancken, I., et al., *Kinetic Study on the Changes in the Susceptibility of Egg White Proteins to Enzymatic Hydrolysis Induced by Heat and High Hydrostatic Pressure Pretreatment.* Journal of Agricultural and Food Chemistry, 2004. **52**(18): p. 5621-5626.

175. Bhaskar, N., et al., *Optimization of enzymatic hydrolysis of visceral waste proteins of Catla (Catla catla) for preparing protein hydrolysate using a commercial protease.* Bioresource Technology, 2008. **99**(2): p. 335-343.

176. Liu, Q., et al., *Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis.* Food Chemistry, 2010. **118**(2): p. 403-410.

177. Sarmadi, B.H. and A. Ismail, *Antioxidative peptides from food proteins: A review.* Peptides, 2010. **31**(10): p. 1949-1956.

178. Chen, L., et al., *Modifications of soy protein isolates using combined extrusion pre-treatment and controlled enzymatic hydrolysis for improved emulsifying properties.* Food Hydrocolloids, 2011. **25**(5): p. 887-897.

179. Pedroche, J., et al., *Brassica carinata protein isolates: chemical composition, protein characterization and improvement of functional properties by protein hydrolysis.* Food Chemistry, 2004. **88**(3): p. 337-346.

180. Benítez, R., A. Ibarz, and J. Pagan, *Protein hydrolysates: processes and applications.* Acta Bioquímica Clínica Latinoamericana, 2008. **42** (2): p. 227-236.

181. Kong, X., H. Zhou, and H. Qian, *Enzymatic preparation and functional properties of wheat gluten hydrolysates.* Food Chemistry, 2007. **101**(2): p. 615-620.

182. Chabanon, G., et al., *Hydrolysis of rapeseed protein isolates: Kinetics, characterization and functional properties of hydrolysates.* Process Biochemistry, 2007. **42**(10): p. 1419-1428.

183. Koch, S., et al., *Protein detection with a novel ISFET-based zeta potential analyzer.* Biosensors & Bioelectronics, 1999. **14**(4): p. 413–421.

184. Sze, A., et al., *Zeta-potential measurement using the Smoluchowski equation and the slope of the current–time relationship in electroosmotic flow.* Journal of Colloid and Interface Science, 2003. **261**: p. 402–410.

185. Malhotra, A. and J.N. Coupland, *The effect of surfactants on the solubility, zeta potential, and viscosity of soy protein isolates.* Food Hydrocolloids, 2004. **18**: p. 101–108.

186. Jiménez-Ruiz, E.I., et al., *Partial Characterization of Ultrafiltrated Soy Protein Hydrolysates with Antioxidant and Free Radical Scavenging Activities.* Journal of Food Science, 2013. **78**(8): p. C1152-C1158.

187. Seung-Hong, L., et al., *Potential Antioxidant Activities of Enzymatic Digests from Fresh Water Microalgae, Pediastrum duplex and Dactylococcopsis fascicularis.* ALGAE, 2009. **24**(3): p. 169-177.

188. Yoshie-Stark, Y., et al., *Functional Properties, Lipoxygenase Activity, and Health Aspects of Lupinus albus Protein Isolates.* Journal of Agricultural and Food Chemistry, 2004. **52**(25): p. 7681-7689.

189. Kong, B. and Y.L. Xiong, *Antioxidant activity of zein hydrolysates in a liposome system and the possible mode of action.* Journal of Agricultural and Food Chemistry, 2006. **54**(16): p. 6059-6068.