UNIVERSIDAD DE LA FRONTERA

Facultad de Ingeniería y Ciencias

Doctorado en Ciencias de Recursos Naturales

"GREEN SYNTHESIS OF COPPER NANOPARTICLES MEDIATED BY *Macrocystis pyrifera*"

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

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TEMUCO-CHILE

2020

"Green synthesis of copper nanoparticles mediated by *Macrocystis pyrifera*"

Esta tesis fue realizada bajo la supervisión de la Dra. Olga Rubilar Araneda, perteneciente al Departamento de Ciencias Químicas, Facultad de Ingeniería y Ciencias de la Universidad de La Frontera y es presentada para su revisión por los miembros de la comisión examinadora.

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To all those who have ever felt *different.*

For those who inspired me.

Acknowledgements

I would like to thank my parents, brother and sister, for being supportive at different stages of my life, each in their own way. I am also grateful to other family members and friends for their kind words throughout this process.

To my life partner, Benjamín, who has offered unwavering support and encouragement, for every laugh when research fails or succeeds. To our son, Máximo, for his kisses and little hands giving me love in its purest form like I never felt before.

I also want to express my gratitude to the members of the evaluation committee, Dr. Michael Seeger, Dra. Mariella Rivas, Dr. Cledir Santos, and Dr. Leonardo Bardehle, for their useful feedback in each Thesis Advance. A very special mention goes out to my Thesis advisors, Dra. Olga Rubilar and Dr. Gustavo Ciudad for their understanding and constant support during my doctoral journey.

I would like to thank the Environmental Nanobiotechnology Center (UFRO, Chile) for being welcoming and for organizing a lovely baby shower when my little one was about to come to this world. I also thank Noelia for her contention, love, unconditional help and for organizing an amazing second baby shower with all members from the Centre of Waste Management and Bioenergy. Thank you all!

In New Zealand, from the School of Engineering and Advanced Technology, Massey University, I thank Dr. Benoit Guieysse and Dr. Richard Haverkamp for their help and guidance during the first steps of my thesis project. A special mention to Ann Marie Jackson, for being such a lovely person and for her support during my internship at Massey. I also want to acknowledge Roland Schaap, Maxence Plouviez, Ray Mohan and

Daa Kitiya for sharing their experience with me and making my stay very pleasant and full of good memories.

In Canada, from the Nanotechnology Research Group, University of Alberta, I'm grateful to Dr. Mike Serpe and Will Carvalho, who kindly hosted a young researcher looking for equipment and advice. The same country gave me the chance to meet Dr. Tzu-Chiao Chao, from the Institute of Environmental Change and Society at the University of Regina, I landed his lab looking for an expert eye, and I found experience, someone who challenge me in order to improve, and a long-lasting bond. I also want to thank Juvilyn Jabonete for her friendship, kindness, and awesome food.

A final acknowledgment goes to the Agencia Nacional de Investigación y Desarrollo (ANID) for their financial support throughout the Doctoral Scholarship 21141234, FONDECYT project 1191089, FONDECYT project 1192389, and ANID/FONDAP/15130015. In addition, thanks to Dirección de Investigación from Universidad de La Frontera for funding my participation at international congresses.

Summary and thesis outline

The green synthesis of metal nanoparticles by algae is an advantageous alternative to chemical or physical protocols since it involves clean, non-toxic and ecofriendly procedures, avoiding chemical agents or co-products associated with environmental toxicity. Moreover, it is a cost-effective method in comparison to the use of other organisms since the existent biorefinery concept can be supported by new value-added products. In this sense, the use of brown algae extracts for the green synthesis of silver and gold nanoparticles has been reported by several authors; however, the green synthesis of copper nanoparticles (Cu-NPs) has been barely explored, and the possible biomolecules involved in the nucleation process and consequent synthesis are yet-to-be described. Therefore, this work aims to evaluate the formation of copper nanoparticles using a biomolecule from a *Macrocystis pyrifera* aqueous extract.

In Chapter I, we presented a general introduction of this Doctoral Thesis, indicating the hypothesis and goals of this study. Also, we present a comprehensive update about the considerable advances that have been achieved aimed to improve the understanding behind the synthesis mechanisms for silver and gold nanoparticles, and the challenges when copper is used as a precursor.

In Chapter II, we evaluated the influence of reaction parameters in the synthesis of Cu-NPs using *Macrocystis pyrifera* free-biomass non-boiled (FBNB) extract. For this purpose, response surface methodology (RSM) based on a central composite design (CCD) was used to evaluate the following independent variables for nanoparticle formation in the extract: X_1: $CuSO_4$ concentration; X_2: pH; and X_3: temperature. Their effects were assessed on synthesized CuO-NPs average size distribution, zeta potential, and polydispersity index (PDI) by dynamic light scattering (DLS). Shape, size, and

elemental mapping at the microstructural level were measured by scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS). Results from CCD showed that predicted optimal reaction conditions for Cu-NPs formation using *M. pyrifera* extract were 2.2 mM $CuSO_4$ concentration, pH8, and incubation at 25,5°C, obtaining an average size distribution, Z potential and PDI of 121 nm, -23.5 mV and 0.3, respectively. This work demonstrated that *M. pyrifera* extract is a feasible medium for the synthesis of Cu-NPs and that the control of the reaction parameters can determine the nanoparticle characteristics.

In Chapter III, we analyzed a green synthesis method to obtain CuO-NPs using HPLC-SEC separated protein fractions from an aqueous extract of *M. pyrifera* as a reductant and capping agent. The characterization of CuO-NPs was evaluated by Dynamic Light Scattering (DLS), Z-potential, Fourier Transform Infrared (FTIR), Transmission Electron Microscope (TEM) equipped with Energy Dispersive X-ray Spectroscopy (EDS) detector. Low Molecular Weight (LMW) and High Molecular Weight (HMW) protein fractions were able to synthesize spherical CuO-NPs. TEM images showed CuO-NPs ranged from 2 to 50 nm in diameter. FTIR measurements showed functional groups from proteins having a pivotal role in the reduction and stabilization of the nanoparticles. Our study suggests that using water-soluble proteins from *M. pyrifera* represents a consistent, straightforward green method to synthesize homogenous nano-scale size CuO-NPs that exhibits high-stability. This work may also provide a suitable tool to synthesize other nanoparticles that have different application areas.

Finally, Chapter IV corresponds to general discussion, concluding remarks and future directions.

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CHAPTER I

General Introduction

1.1 General Introduction

Nanomaterials are defined as materials with at least one external dimension in size range from approximately 1-100 nanometers (nm). Inside this group, nanoparticles are solid particles with all three external dimensions at the nanoscale [1–3]. The broad interest in the manufacturing of nanoscale materials is primarily because of the wide range of applications that they have due to their interesting properties. Macroscopic properties such as electrical conductivity, colour, mechanical strength or melting point, among others, can vary dramatically with respect to the same material on a nanometric scale [4], which can drastically modify physic-chemical properties compared to the bulk material. Their actions can be explained depending on their chemical composition, size and shape. Due to the large surface area to volume ratio, they find a wide range of applications in industries [5–7]. In recent years, nanomaterials have received particular attention for their positive impact in improving many sectors of the economy, including consumer products, energy, transportation, cosmetics, pharmaceuticals, antimicrobial agents and agriculture. Presently, the synthesis of inorganic nanoparticles has been demonstrated by many physical and chemical means [8]. Chemical methods have low productivity, are non-eco-friendly, capital intensive and toxic. Therefore, biological synthesis either extracellularly or intracellularly from higher plants or microbes have gained the upper hand [2], [9–11]. At present, the importance of biological synthesis is being emphasized globally, but the research in the field of biosynthesis has focused mainly on silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs), followed by zinc oxide nanoparticles (ZnO NPs), and finally, copper/copper oxide nanoparticles (Cu/CuO-NPs).

Copper has been widely used and recognized as a safe inorganic antibacterial agent and is also of great interest due to its optical, catalytic, mechanical, and electrical properties. Besides, it is a good alternative material to noble metals like gold and silver, since copper

is highly conductive and much more economical [12, 13]. Copper-based nanoparticles are of great interest because of their low cost, availability and demonstrated properties, showing high potential for biotechnological uses, with possible applications in heat transfer systems as super-strong materials, sensors [14], antimicrobial [15, 16], bactericidal [17], as agents used to coat hospital equipment [18], and also as catalysts [19], where nanoparticles have shown a higher efficiency than particles [20–22]. Various physical and chemical methods have been extensively used to synthesize Cu-NPs, and their properties can be controlled depending on the synthesis method. The most important ones for the synthesis of Cu-NPs are chemical methods such as chemical reduction, electrochemical techniques, photochemical reduction and thermal decomposition [23].

The available literature on the synthesis of Cu-NPs reported that it is susceptible to oxidation [24–26]. Therefore, the most successful chemical synthesis of metallic Cu-NPs is either carried out in organic or aqueous phase synthesis to avoid potential oxidation of copper [27]. The biosynthesis of pure metallic Cu-NPs in aqueous phase is still an open challenge for bionanotechnologists. During the last decade, algae mediated synthesis of noble metal nanoparticles has been explored by some authors [28, 29]. The use of biological species and biochemical constituents from algae, such as proteins [30–33], fucoidans [34, 35], diterpenoids [16, 36], as potential responsible agents for metal-ions reduction and nanoparticle stabilization from different metal salts has been reported. It is important to mention that the incorporation of this technology into the current algal biorefinery system represents an attractive opportunity since the existing ready-to-use industry of algae biofuels, biomaterials, and high-value co-products can be favoured by adding new algal bioproducts. Nevertheless, studies about the biosynthesis of Cu-NPs have been examined barely, stopping in most of the cases at a phenomenological level, but without proposing experiments to evidence the biomolecules responsible for the

reduction of copper from solution. The possible role of diterpenoids as reducing and capping agent for the biosynthesis of CuO-NPs [16] was reported as the only approach for macroalgae-mediated synthesis. In this context, a follow-up study should be carried out to explore other compounds for their eventual capability for the macroalgae-mediated synthesis of Cu-NPs, to open up the development of a new possible bioproduct for a decaying algae-based energy industry [37]. The marine algae *Macrocystis pyrifera* is a multicellular brown alga belonging to the class Phaeophyceae and order Laminariales. This seaweed can grow from depths of over 25 m to reach and spread along the surface in a dense canopy. During upwelling, *M. pyrifera* can grow at a phenomenal rate, up to 50 cm a day [38]. In Chile, this abundant alga is produced for national internal production and for exportation with a price that in 2013 reached 1.735 UDS/ton, being a volume of 81.7 tons commercialized same year [39]. In terms of its biochemical composition, *M. pyrifera* biomass contain 75% carbohydrates, 13% proteins, 11% ash, and 0.7% lipids [40]. Ortiz J. and collaborators [41] explored the composition of 17 amino acids in *M. pyrifera*, showing an important contribution of water-soluble amino acids such as glutamic acid, aspartic acid, arginine and cysteine. It has been described that biomolecules with carboxyl, hydroxyl, and amine functional groups have the potential for metal-ion reduction and for capping in the green synthesis of nanoparticles, in replacement of chemical or physical methods [31], [42–46]. In this context, *M. pyrifera* biomass may be considered a potential candidate for the green synthesis of Cu-NPs. Moreover, the existent biorefinery concept can be supported by new value-added products.

Therefore, the general goal of this thesis will be to evaluate the biosynthesis of copper nanoparticles by *Macrocystis pyrifera* macroalgae. The results obtained in this study will contribute to describe an approach to the possible biomolecule involved in synthesis and

operation parameters that are affecting the biosynthesis of Cu-NPs from this macroalgae specie.

1.1.1 Biosynthesis of nanoparticles using algae

Amongst different living organisms studied as potential candidates for the synthesis of metallic nanoparticles, algal biomass is presented as a novel, easy-to-handle, and environmentally acceptable "green procedure" that has been examined barely. Currently, the algae industry is moving forward. Scientists are discovering novel application possibilities, and the entire spectrum of energy and non-energy products that can be obtained using algal biomass as a raw material can effectively boost the development of a biorefinery system. According to the Algae Biomass Organization's 2015 Industry Survey (http://www.algaebiomass.org/resource-center/references/industry-statistics/), approximately 11.3 % of the companies and industries that produce algal products are linked to material and services including, manufactured goods, plastics, chemicals and wastewater treatment.

However, to make sense of the algal biorefinery concept, there is an urgent need to establish a proper connection between the various input and output streams of the products, as well as the synergism between different industries, since effective biorefinery using algae can only be constructed through its integration with other industries. The emphasis on algal lipids is not new because of the high interest in biodiesel from lipids. In addition, biomethane production from defatted algal biomass through anaerobic digestion, generation of ethanol, from species that are high in carbohydrates/polysaccharides and thin cellulose walls, and the extraction of chlorophyll and carotenoid pigments for their application in different industries, such as cosmetics or

pharmaceutical products are also of great importance. These are all examples of the growing interest in utilizing algal biomass in an optimal way and not just to produce biofuels but also valorizing co-products in the process [37].

Marine ecosystems are very important for the overall health of both marine and terrestrial environments, and the use of natural sources like marine biological resources is essential for nanotechnology. During the last years, some authors have reported the use of biological species and biochemical constituents from algae such as proteins [30–33], fucoidans [34, 35] and diterpenoids [16, 36], as potential responsible agents for the reduction of metal ions and nanoparticle stabilization from different metal salts. Nevertheless, the bioreduction mechanism and steps that lead to the formation of nuclei, which subsequently grow through further reduction of metal ions, has not been sufficiently described. Seaweeds such as green *Caulerpa peltata*, red *Hypnea* valentiae, and brown *Sargassum myriocystum* were used for the synthesis of zinc oxide nanoparticles [35]. Preliminary screening of physic-chemical parameters such as metal concentration, seaweed extract concentration, temperature, pH, and reaction time revealed that one seaweed, *S. myriocystum* was able to synthesize zinc oxide nanoparticles. It was confirmed through the initial colour change of the reaction mixture and UV-visible spectrophotometer. The extracellular biosynthesized clear zinc oxide nanoparticles of 36 nm size were put through characterization techniques, such as DLS, AFM, SEM-EDX, TEM, XRD, and FTIR. The biosynthesized ZnO nanoparticles were more effective antibacterial agents against gram-positive than the gram-negative bacteria. Based on the FTIR results, fucoidan water-soluble pigments present in *S. myriocystum* leaf extract were responsible for the reduction and stabilization of zinc oxide nanoparticles. This approach was quite stable, and no visible changes were observed, even after six months. These soluble elements could have acted as both reducing and stabilizing

agents, preventing the aggregation of nanoparticles in solution and extracellular biological synthesis of zinc oxide nanoparticles.

On the other hand, few studies refer to the biosynthesis of Cu-NPs using algae (Table 1). Abboud et al. [16] explored the synthesis using brown macroalgae *Bifurcaria bifurcata*, and the possible hypothesis of the formation mechanism states that water-soluble diterpenoids present in the algal extract perform dual functions of reduction and stabilization of CuO-NPs. The similitude of the spectral features between the copper nanoparticles and the features from the algal extract suggested that the compound present on the surface of the CuO-NPs has a very similar chemical composition. Water-soluble diterpenoids are also mentioned for the reduction of other metals. Rajathi et al. [36] demonstrated that gold nanoparticles were synthesized by the brown macroalgae *Stoechospermum marginatum*, and the FT-IR spectrum of the seaweed was analyzed before and after bioreduction in order to identify the potential biomolecules in *S. marginatum* responsible for the reduction and efficient stabilization of bio-reduced gold nanoparticles. As a result, the appearance of new peaks at 1550 cm^{-1} confirmed that reduction was carried out by hydroxyl groups present in the diterpenoids of the brown alga.

Even when these results represent evidence of the feasibility of one macroalgal specie to have a natural and effective reductant agent capable of reducing copper to successfully synthesize Cu-NPs, a deep characterization of a specific biomolecule needs to be studied. In addition, to validate the feasibility of algae-mediated synthesis of Cu-NPs, a different macroalgal specie need to be proved. Finally, these answers could contribute to obtaining better control of the process itself and its nanoproducts, to consequently open a new opportunity to scale up this technology.

Table 1. Algae-mediated synthesis of metallic nanoparticles

Algal specie	Precursor	Method	Size	Shape	Application	Reference
Padina pavonica	$AgNO_3$	Thallus extract	10-72nm (e)[a]	Spherical	Microbiocidal (against cotton phytopatogens)	Sahayaraj et al., [47]
Stoechospermum marginatum	$HAuCl_4$	Algal powder < 0.5 mm	18.7-93.7nm (e)[a]	Spherical, triangular, and hexagonal	Antibacterial	Rajathi et al., [36]
Bifurcaria bifurcate	$CuSO_4$	Algal extract	5-45mn (e)[a]	Spherical and elongated (small minority)	Antibacterial	Abboud et al., [16]
Sargassum myriocystum	$HAuCl_4x3H_2O$	Algal extract	10-23nm (e)[a]	Spherical, Triangular	-	Dhas et al., [48]
Sargassum plagiophyllum	$AgNO_3$	Algal extract	18-42nm (e)[a]	Spherical	Antibacterial	Dhas et al., [49]
Sargassum wightii	$HAuCl_4^-$	Algal powder	8-12mn (e)[a]	Thin plannar and spherical	-	Singaravelu et al., [50]
Fucus vesiculosus	$HAuCl_4$	Algal powder	- (e)[a]	Spherical	Biosorption	Mata et al., [31]
Cladosiphon okamuranus & Kjellmaniella crassifolia	$HAuCl_4x3H_2O$	Algal fucoidan extract	8-10nm (e)[a]	Spherical	-	Soisuwan et al., [34]

Sargassum wightii	$AgNO_3$	Algal extract	8-27nm (e)[a]	Spherical	Antibacterial	Govindaraju et al., [51]
Chlorella vulgaris	$HAuCl_4^-$ (among others)	Live cells	40-60nm (i)[a]	Spheroidal or polyhedral	-	Luangpipat et al., [52]
Spirulina platensis	$HAuCl_4x3H_2O$	Protein extract	2-8nm (e)[a]	Spherical	Antibacterial	Suganya et al., [33]
Spirulina platensis	$AgNO_3$	Protein extract	6nm (e)[a]	Spherical	Antibacterial	Suganya et al., [32]
Gelidellia acerosa	$AgNO_3$	Algal extract	22um (e)[a]	Spherical	Antifungal	Vivek, et al., [53]
Ulva fasciata	$AgNO_3$	Algal extract	28-41um (e)[a]	Spherical	Antibacterial	Rajesh, et al., [54]
Nannochloropsis oculata & *Chlorella vulgaris*	$AgNO_3$	Live cells	< 15nm (e)[a]	-	-	Mohseniazar et al., [55]
Ulva flexousa	$AgNO_3$	Algal extract	2-32nm (e)[a]	Spherical	-	Zohreh et al., [56]
Rhizoclonium fontinale & *Ulva intestinalis*	$HAuCl_4 xH_2O$	Entire algal biomass	~ 42.39nm (i)[a]	Spherical		Parial et al., [28]
Chlorococcum humícola	$AgNO_3$	Extract and live cells	2-16nm (i) (e)[a]	Spherical	-	Jena et al., [57]
Rhizoclonium fontinales	$HAuCl_4 xH_2O$	Live cells	5-88nm (i)[a]	Spherical, triangles, hexagons, rod-shaped	-	Parial et al., [58]

Chlamydomonas reinhardtii	$AgNO_3$	Extract and live cells	5-35nm (i) 5-15(e)[a]	Spherical	-	Barwal et al., [59]
Turbinaria conoides	$HAuCl_4$	Dead algal biomass	20-80nm (e)[a]	Spherical	Biosorption	Vijayaraghavan et al., [42]
Chlorella vulgaris	$HAuCl_4$, $AgNO_3$	Extract from lyophilized cells	0.8-2nm, <20nm (e)[a]	Triangular, truncated triangular, hexagonal	-	Xie et al., [30, 44]
Tetraselmis kochinensis	$HAuCl_4$	Live cells	5-35nm (i)[a]	Spherical	-	Senapati et al., [60]
Chaetomorpha linum	$AgNO_3$	Algal extract	3-44nm (e)[a]	Coalesced nano-clusters	-	Kannan et al., [29]

– not reported

[a] (i)—intracellular; (e)—extracellular

1.1.2 Production processes and characterization of nanoparticles synthesized from algae

The use of macro- and microalgae species in the synthesis process of nanoparticles has drawn more interest because they can provide protocols involving few steps that are also free from toxicants; furthermore, natural capping agents are readily supplied by algal compounds [61].

The main differences between the intracellular and extracellular process to synthesize noble metal nanoparticles using algae is that in the first pathway, the live cells from

harvested macro- or microalgae are directly incubated with the metal ion solution, and physical or chemical methods are required for cell disruption to recover the nanoparticles from inside the cell, or in other cases, to release the particles that could be retained in the cell wall due to electrostatic interactions [28, 57, 59, 60]. Unfortunately, these last steps that are needed once the nanoparticles are formed can implicate a negative effect on the surface area and physic-chemical properties of the resulting nanoproducts. On the other hand, a pre-treatment of the algal biomass is carried out when the extracellular biosynthesis of metal nanoparticles is performed using algae extracts. First, the algal biomass is dried and milled, or sometimes just previously lyophilized. Then, the options are to obtain the algal extract with or without boiling the biomass. Some authors have reported that boiling the biomass to obtain an extract is a disadvantage since the non-controlled release of biomolecules has rendered the analysis of the synthesis response a daunting task. This may be explained since the identification and follow-up of the whole biological processes of the responsible agents for the nucleation and growth of the metal nanoparticles is not yet throughout characterized and defined [62]. Once the extract is ready to use (filtered), it can be placed in contact with the metal-ion solution and incubated from 24 to 72 hrs. Free nanoparticles can be recovered by centrifugation and stored for further analysis. The general process of algae-mediated synthesis of metal nanoparticles is summarized in Figure 1.

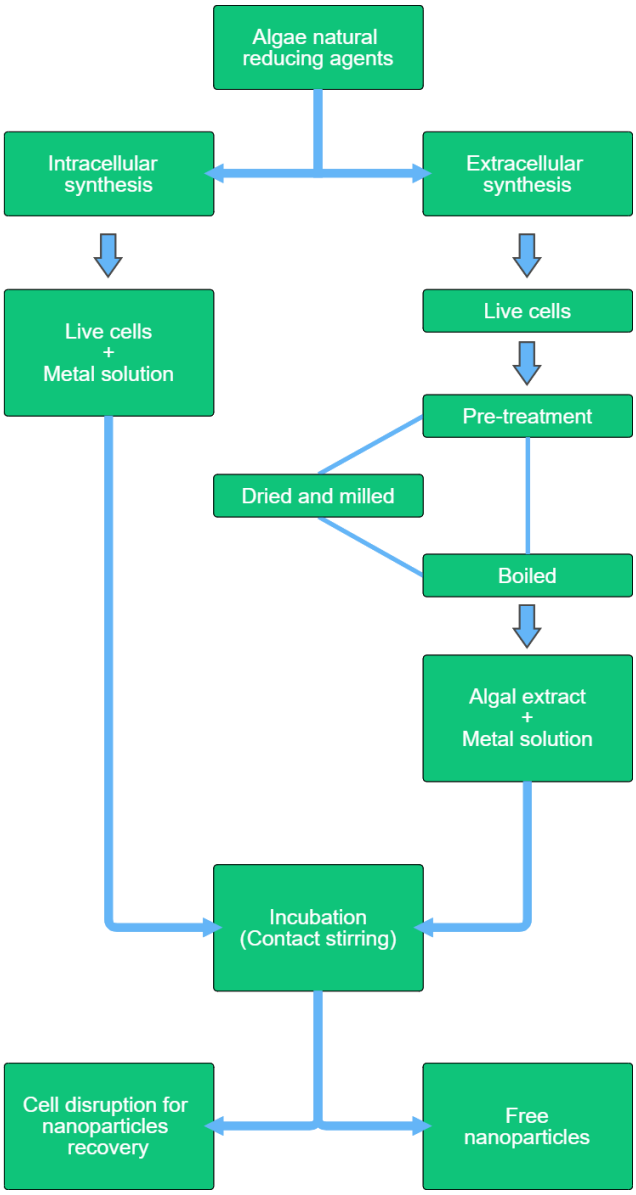

Figure 1. Synthesis process diagram of the algae-mediated synthesis of metal nanoparticles.

The characterization of the nanoparticles is as relevant as the synthesis process itself since the physic-chemical properties and morphology is directly related to the possible applications for each nanoproduct [58]. The different techniques used are helpful to resolve diverse parameters such as particle size, shape, crystallinity, fractal dimensions, pore size and surface area. Nowadays, this characterization is carried out using a range of diverse techniques, such as scanning and transmission electron microscopy (SEM, TEM),

Fourier transform infrared spectroscopy (FTIR), X-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM), powder X-ray diffractometry (XRD), and UV–Vis spectroscopy. The last technique mentioned is common to confirm sample formation by exhibiting the plasmon resonance, and light wavelengths in the 200-800 nm are generally used for characterizing various metal nanoparticles in size range of 200-100 nm. Spectrophotometric absorption measurements in the wavelength ranges of 400-450 nm, 510-560 nm, and 572-582 nm [63] are used to characterize silver, gold, and copper nanoparticles, respectively. However, the frequency and width of the surface plasmon absorption depend on the size and shape of the metal nanoparticles as well as on the dielectric constant of the metal itself and the surrounding medium [64]. For instance, non-spherical gold nanoparticles exhibit multiple absorption bands, correlated with their multiple axes, in comparison to the single one for isodiametric particles [28]. In the case of gold nanorods, it shows two surface plasmon oscillations, namely transverse and longitudinal bands, which correspond to electron oscillations perpendicular and parallel on the rod length direction, respectively [65]. Dynamic light scattering (DLS) is used to characterize surface change and size distribution of the particles suspended on a liquid.

Morphology and particle size are possibly determined by TEM, SEM and AFM. The TEM technique has a 1000-fold higher resolution compared to SEM [66]. The improvement of AFM over conventional microscopes such as SEM and TEM is that AFM technique measures 3D images, so that particle height and volume can be intended. Moreover, XRD is used for the determination of crystalline structures in nanoparticles since X-rays can penetrate nanomaterial, and the resulting diffraction pattern can be compared to standards to obtain structural information. FT-IR spectroscopy is useful for characterizing surface chemistry, so organic functional groups such as carbonyls or hydroxyls that could be attached to the surface of nanoparticles are detected by using this technique.

1.1.3 Applications of synthesized NPs from algae

The widespread applications of nanoparticles such as pharmaceuticals, cosmetics, food, energy, agriculture, surface coating, polymers, etc., are well known [7, 67, 68]. Specifically, nanoparticles from gold and other noble metals such as copper have attracted much attention because of their potential applications in microelectronics [69], sensors [70], catalysis [71], non-linear optical materials [72], etc. However, few of them were found in the current scientific literature, which involves nanoproducts from algae mediated synthesis (Table 1).

The rapid development of multidrug resistance (MDR) in different microorganisms is currently motivating the research of cost-effective therapeutics, which could act as improved antibacterial agents to effectively control such resistance. In this sense, copper and silver are known to have a wide range of antibacterial properties against different microorganisms like *Fusarium oxysporum* and *Xanthomonas campestris* (both cotton pathogens) using AgNPs biosynthesized by *Padina pavonica* [47]; *Escherichia coli* using AgClNPs from the marine alga *Sargassum plagiophyllum* [49]; *Staphylococcus aureus*, *Bacillus rhizoids*, *Escherichia coli* and *Pseudomonas aeruginosa* using AgNPs from *Sargassum wightii* Grevilli [51]; pathogens isolated from HIV patients such as *P. aeruginosa*, *E. coli* and *S. sciuri* using AuNPs synthesized from *Spirulina platensis* [32]; AgNPs against *Xanthomonas campestris* using an extract from the marine algae *Ulva fasciata*; *Enterobacter aerogenes* and *Staphylococcus aureus* when CuO-NPs biosynthesized by *Bifurcaria bifurcata* were used as antimicrobial by using disc diffusion tests with *E. aerogenes* and *S. aureus* [16]. Evidently, the small size and high surface to volume ratio of these kinds of nanoparticles, with modified structure rather than macromolecules and their own biocompatibility, makes them suitable for pharmaceutical applications, being able to overcome the performance of conventional antibiotics in

several cases. Abboud et al. [16] mentioned that the antimicrobial activity of CuO-NPs is due to copper ions released may bind with DNA molecules and lead to a disorder of the helical structure by cross-linking within and between the nucleic acid strands. Copper ions inside bacterial cells also disrupt biochemical pathways; however, the exact mechanism behind it is unknown.

A challenge in nanotechnology is to tailor the optical, electronic and electrical properties of nanoparticles by controlling their size and shape. For biological synthesis, this represents a pending issue for further research since optimization regarding the bioreactant concentration, interaction time with the metal solution, pH, mixing ratio, and reaction kinetics are parameters that need to be studied in the algae-mediated synthesis of noble metal nanoparticles. This is since the physic-chemical properties of nanoscale matter depend on particle size. Nanoparticle shape also contributes significantly to modulating their electronic properties. Several shapes ranging from rods, wires, plates and teardrop structures may be obtained by chemical methods, and triangular nanoparticles have been synthesized by using a seeded growth process [73]. Perfectly monodispersed metal nanoparticles are, of course, ideal. However, special properties are to be expected, even if the ideality is not perfectly realized. In this sense, for the biosynthesis of nanoparticles, understanding the biomolecules involved and biochemical processes that lead to the formation of nanoscale inorganic materials is therefore potentially appealing as a green-innovative alternative to chemical methods for nanoparticle synthesis.

1.1.4 A general approach to the possible biomolecules involved in the synthesis of copper nanoparticles

There is diverse literature regarding the bioreduction of metal nanoparticles by a combination of biomolecules found in plant extracts (enzymes, proteins, amino acids, vitamins, polysaccharides, and organic acids such as citrates) and the respective role of phytochemicals [15, 74, 75]. The phytochemicals responsible have been identified as terpenoids, flavones, ketones, aldehydes, amides and carboxylic acids by IR spectroscopic studies [76]. The main water-soluble phytochemicals are flavones, organic acids and quinones, which are responsible for immediate reduction [77]. Natural hydrocolloids (gums) isolated from trees are a new class of biomaterial used for the production of nanomaterials, which act both as reducing and capping agents in nanoparticle synthesis [15]. Different microorganisms have diverse mechanisms of forming nanoparticles. It grabs target ions from the environment on the surface or inside microbial cells and then reduces metal ions to nanoparticles in the presence of enzymes generated by cell activities. The electrostatic interaction between ions and the negatively charged cell wall from the carboxylate groups in enzymes reduces metal ions, which subsequently grow through further reduction and accumulation.

As previously mentioned, in the case of algae, the reduction process and consequent biosynthesis of CuO-NPs could be carried out by the hydroxyl groups in diterpenoids from brown algal biomass. However, this has only been reported for one macro-algal specie [16]. Finding possible reductant bioreagents in macroalgae for the synthesis of CuNPs is a current challenge. Due to the lack of knowledge on the topic, further research could be focused on available literature regarding the algal synthesis of silver and gold nanoparticles to explore those successful biochemical compounds as agents for copper metal salts.

1.1.5 Possible biomolecules involved in the synthesis of silver nanoparticles

For the algae-mediated synthesis of silver nanoparticles, Jena et al. [27] demonstrated a simple bioreductive synthesis method using the green microalga *Chlorococcum humicola*, where living cells as well as the algal extract produced silver nanoparticles when exposed to 5mM of silver nitrate solution and incubated for 48h. On this algal specie, a previous study identified five groups of substances present in extracellular products of different *Chlorococcum* species: (i) steam-volatile acids; (ii) yellow water-soluble phenolic compounds; (iii) lipophilic substances; (iv) proteins; and (v) polysaccharides [45]. In this sense, FT-IR analysis of *C. humicola* silver nanoparticles revealed that protein molecules were mainly responsible for producing biogenic silver nanoparticles, and the presence and binding of proteins with the nanoparticles could lead to their possible stabilization. The findings of this research can be closely linked to Mohseniazar et al. [55], who demonstrated that *Nannochloropsis gaditana* and *Chlorella vulgaris* have the potential to biosynthesize silver nanoparticles in a culture medium containing 1mM of $AgNO_3$ within 24 h. A better characterization was done by another study which aim was to explore the active species in the algal extract from *Chlorella vulgaris* that was responsible for the reduction of Ag ions and the resulting growth of Ag(0) into Ag nanoplates [32]. Besides, chemical modifications of the algal proteins were carried out to identify the amino acid residues in the proteins with Ag ion reduction capability and shape direction functionality. From this analysis, the authors concluded that in accordance with other references [30, 33, 56, 78], algal proteins were the active biomolecules in the algal extract for Ag nanoplates formation, being primarily responsible for the reduction of Ag(I) and the formation of Ag nanoplates. In addition, they discovered that Tyr residues in the proteins were responsible for Ag ion reduction, and the carboxyl groups in Asp and/or Glu residues were driving the anisotropic growth of

Ag nanocrystals onto nanoplates. The Ag nanoplates produced had a low polydispersity in good yield (> 55%).

According to this background, it is possible to suppose that proteins could also be involved in the biosynthesis of CuNPs, but further studies are needed to confirm this hypothesis.

1.1.6 Possible biomolecules involved in the synthesis of gold nanoparticles

Regarding algae-mediated synthesis of gold nanoparticles, Greene et al. [79] reported that the accumulation of elemental Au formed a nanometer-scale of irregular spherical gold, which was precipitated inside and outside the cells when a lyophilized suspension of *Chlorella vulgaris* was used. The composition of this algal strain contains proteins (42-58%), lipids (5-40%), chlorophyll (1-2%), fiber (9-18%), and many vitamins [80], but proteins are one of the biomolecules involved in the reduction process. This hypothesis was confirmed by Xie et al. [30], who explored a simple and controlled biological synthesis of large quantities of gold nanoplates by the room temperature reduction of gold ions for 48 h in an aqueous algal extract. The importance of this study was the identification, as far as possible, of the biomolecules involved in the nucleation and growth of gold clusters into specific shapes and sizes. For this purpose, proteins were eluted and isolated according to their molecular weight, and distinctive triangular and hexagonal gold nanoparticles in high yields (90% approx.) were obtained using a protein with approximately 28 kDa weight purified by reverse HPLC. The isolated protein or gold shape-directing protein (GSP) was then used to optimize the synthesis process. Another hypothesis suggests that fucoidans could be responsible for the reduction of gold. Mata et al. [31] demonstrated a great and faster reduction rate performance of Au(III) to

Au(0) by using *F. vesiculosus* at neutral pH, probably due to the higher stability of the algal reductant compounds at neutral pH. Also, the authors mentioned that reducing groups (OH) present in biomolecules of the algae, such as polyols or even polysaccharides and proteins, are more reactive at neutral pH than at more acidic or alkaline pH. Indeed, those algae are grown in seawater with an average pH of 7.5, and based on the O-H bond polarity, hydroxyl groups have a certain acid character and react with strong bases. This could explain the lack of its reducing ability at pH 11 reported in this study. On the other hand, in an acidic medium, the groups behave as weak bases due to the existence of unshared pairs of electrons in the oxygen atom and then become protonated. Protonated hydroxyl groups can be transformed into carbocations, provoking the loss of their reducing ability. Conversely, the formation of carbocation involves a strong electrostatic attraction to the tetrachloroaurate ion, which justifies the sorption of gold by the alga at acid pH. Thus, a decrease in pH favours the sorption of gold instead of its reduction. Other highly reactive functional groups, with similar chemical behaviour to hydroxyl groups, such as sulfhydryl (–SH), could also be involved in the reduction process. Those groups are present in polysaccharides of the algal cell walls and are responsible for their brown color (fucoidans). Algal pigments, such as fucoxanthins, a kind of carotenoid rich in hydroxyl groups, could have also participated in the gold reduction. These pigments have reductive properties and are released to solution by diffusion [81]. Moreover, in these experiments authors reported that the solutions remained colored after centrifuging the biomass in a blank experiment only with deionized water and alga. Nevertheless, these pigments would only explain part of the reduction since it still occurs, to a lesser extent, within alginate beads that have no pigments. With *F. vesiculosus*, these soluble elements could have acted as capping agents, preventing the aggregation of nanoparticles in solution, and playing a relevant role in their extracellular synthesis and shaping.

In conclusion, it is known that the potential for metal-ion reduction and capping of the newly formed nanoparticles (necessary for size stabilization) could be performed by biomolecules with carboxyl, hydroxyl, and amine functional groups. Consequently, a previous treatment to allow the secretion of biomolecules, such as cell lysis or lyophilization for increasing the permeability of the cell membrane, could be necessary. Extracellular biosynthesis is highly desirable since commercial processing equipment or chemical reactants to release the intracellular nanoparticles are avoided.

1.2 Hypothesis and research objectives

1.2.1 Hypothesis

Based on the previous background, and considering that:

- There is a lack of information about the biomolecules involved in the biosynthesis of copper nanoparticles carried out by macroalgae.
- The brown macroalgae *M.pyrifera* is a good source of biochemical compounds, which could act as a reductant agent for copper ions.
- The existing ready-to-use industry of algae biofuels, biomaterials and high-value co-products can be favoured by adding new algal bioproducts into the biorefinery concept in a decaying algae-based energy industry.

Therefore, considering these aspects, the working hypothesis is established as:

The *Macrocystis pyrifera* macroalgae biomass contains biomolecules that are capable of producing copper nanoparticles and/or copper oxide nanoparticles.

1.2.2 Research objectives

1.2.2.1 General objective

- To evaluate the formation of copper nanoparticles using a biomolecule from a *Macrocystis pyrifera* aqueous extract.

1.2.2.2 Specific objective

- To evaluate the influence of reaction parameters in the synthesis of copper nanoparticles using *Macrocystis pyrifera* free-biomass extract.

- To identify the main *Macrocystis pyrifera* biomolecule involved as reductant and capping agents in the synthesis process of copper nanoparticles.

CHAPTER II

Optimization of reaction parameters for the green synthesis of copper nanoparticles using Macrocystis pyrifera free-biomass extract

Optimization of reaction parameters for the green synthesis of copper nanoparticles using *Macrocystis pyrifera* free-biomass extract

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Abstract: The need to carry out research focused on novel ecological protocols has increased exponentially, motivated by the common aim to reduce toxic by-products from chemical methods. Amongst different living organisms studied as potential candidates for the synthesis of metallic nanoparticles, algal biomass is presented as a novel and easy-to-handle method that has been barely examined. We evaluate the influence of reaction parameters in the synthesis of copper nanoparticles (Cu-NPs) using *Macrocystis pyrifera* free-biomass non-boiled (FBNB) extract. Response surface methodology (RSM) based on a central composite design (CCD) was used to evaluate the following independent variables for nanoparticle formation in the extract: X_1: $CuSO_4$ concentration; X_2: pH; and X_3: temperature. Their effects were assessed on synthesized Cu-NP average size distribution,

zeta potential, and polydispersity index (PDI) by dynamic light scattering (DLS). Shape, size, and elemental mapping at a microstructural level were measured by scanning electron microscopy (SEM) with energy dispersive X-ray spectrometry (EDS). Results from CCD showed that predicted optimal reaction conditions for Cu-NP formation using *M. pyrifera* extract were 2.2 mM $CuSO_4$ concentration, pH8, and incubation at 25,5°C, obtaining an average size distribution, Z potential and PDI of 121 nm, -23.5 mV and 0.3, respectively. This work demonstrated that *M. pyrifera* extract is a feasible medium for the synthesis of Cu-NPs and that the control of the reaction parameters can determine the nanoparticle characteristics.

Keywords: Green synthesis, brown seaweed, aqueous-extract, optimization, copper oxide nanoparticles

2.1 Introduction

In recent decades, metal nanoparticles have accrued utmost interest due to their unique physicochemical properties. Particularly, copper nanoparticles (Cu-NPs) have gained much attention owing to their distinctive mechanical, catalytical, electrical, optical, magnetic and antibacterial properties, which have been applied in a wide range of industries and research fields [82]. For its synthesis, chemical and physical pathways are the two most important but toxic and costly methods that are typically used [83]. Therefore, there is an urgent need to explore environmentally friendly biological processes for the synthesis of nanoparticles. In this sense, the use of brown algae extracts for the green synthesis of silver and gold nanoparticles has been reported by several authors; however, the green synthesis of Cu-NPs has been barely explored [84]. The

marine algae *Macrocystis pyrifera* is a multicellular brown alga belonging to the class Phaeophyceae and order Laminariales. This seaweed can grow from depths of over 25 m to reach and spread along the surface in a dense canopy. During upwelling, *M. pyrifera* can grow at a phenomenal rate, up to 50 cm a day [38]. In Chile, this abundant alga is produced for national internal production and for exportation with a price that in 2013 reached 1.735 UDS/ton, being a volume of 81.7 tons commercialized same year [39]. In this context, *M. pyrifera* biomass may be considered a potential candidate for the green synthesis of Cu-NPs. Moreover, the existent biorefinery concept can be supported by new value-added products.

The direct control of reaction parameters is a key aspect that affects the biosynthesis and characteristics of the nanoparticles since the properties of Cu-NPs are highly dependent on their synthesis procedures. In this sense, the optimization of reaction parameters like incubation temperature, pH, precursor concentration, and the interaction between them for the synthesis of Cu-NPs using algae has not been thoroughly researched. This work aims to evaluate the influence of reaction parameters in the synthesis of Cu-NPs using *Macrocystis pyrifera* free-biomass extract.

2.2 Materials and Methods

Brown Marine *Macrocystis pyrifera* algae were obtained from Universidad de Los Lagos; samples were collected in Quenac, Chiloé Island, (Chile). In order to remove epiphytes and sand particles, biomass was washed three times with tap water, then twice with distilled water, and followed by twice double deionized water. Subsequently, algal biomass was dried in an oven at 50°C overnight. The dried biomass was powdered in a blender and stored in darkness. A 100 mM $CuSO_4$ (Loba Chemie, India) was prepared using double distilled water and kept at 4 °C in the dark for further experiments.

Preparation of *Macrocystis pyrifera* biomass-free non-boiled (BFNB) extract

Dried algal powder (4 g) was dispersed in a 250 Erlenmeyer flask with 100 mL double distilled water, sonicated for 15 min (VCX 130, SONICS) at 60% amplitude to facilitate cell lysis [30], and incubated in an orbital shaker (200 rpm) for 24 h at 50°C. Once cooled, the supernatant was separated from the biomass by using a grade 4 filter paper. The obtained BFNB extract was immediately used for experiments.

Copper/copper oxide nanoparticle green synthesis by BFNB extract

BFNB *M. pyrifera* extract was used for Cu-NPs synthesis. Subsequently, copper sulphate was mixed with algae extract to get a final volume of 10 mL in different concentrations (1, 2, and 3 mM). Immediately afterwards, solutions were adjusted to different pH values (8, 10, and 12). Experiments containing only the biomass-free extract (from each treatment), or only the $CuSO_4$ solution, were maintained as control.

Response surface methodology (RSM) based on a central composite design (CCD) was used to evaluate the following independent variables for nanoparticle formation in the extract: X_1: $CuSO_4$ concentration; X_2: pH; and X_3: temperature. Their effects were assessed on synthesized Cu-NP average size distribution, zeta potential, and polydispersity index (PDI) using *M. pyrifera* BMF extract.

Design Expert 7.0.0 software was used for data regression and graphic analysis. A design of 17 experiments was formulated for three factorial (23) designs, and three replicates at the central points were used for the second-order polynomial model. As optimization criteria, selected variable optimum values were obtained by solving the regression equation at process response desired values, which were selected for further study. Green synthesized Cu-NPs were analyzed through the observation of three different responses, such as hydrodynamic size, and zeta potential, and polydispersity index (PDI).

Particle size (DLS) analysis and Zeta Potential

The hydrodynamic diameter, zeta potential and polydispersity index (PDI) values were measured at 25 °C by dynamic light scattering (DLS) using the Zetasizer Nano ZS90 System (Malvern Instruments, Malvern, UK). To avoid aggregation, samples were sonicated for 1 min. Prior to the DLS measurement, the aqueous suspensions of nanoparticles were passed through a cellulose acetate syringe driven filter unit (0.22 µm pore size). For each experiment, a glass cuvette was used containing a 500 µL sonicated sample and 1 mL double distilled water.

Scanning Transmission Electron Microscopy (STEM)

Shape, size, and elemental mapping at microstructural level were measured by scanning electron microscopy (SEM) with EDS (SU 3500 Hitachi, Tokyo, Japan). Parameters were high Vacuum, 30KV, WD 5mm, using SE detector. Samples were prepared by placing a drop of colloidal solution on a 300 mesh nickel grid covered by carbon formvar; the excess solution was removed by blotting with filter paper and subsequently dried in air at room temperature before transferring it into the microscope.

2.3 Results and discussion

An experiment designed using a CCD-RSM model was conducted to evaluate copper sulphate concentration (1, 2 and 3 mM), pH (8, 10 and 12), and incubation temperature effect (20, 45 and 70°C) on the size distribution, zeta potential and PDI of Cu-NPs in order to establish optimal nanoparticle production.

Three sets of 17 experiments, including three central points replicate, were performed. The corresponding experimental matrix, along with the observed and predicted responses, is shown in Table 1. Analysis of the data with various models and subsequent analysis of variance (ANOVA) revealed that the quadratic model is a suitable empirical model to represent the relationship between the variables and size distribution, zeta potential, and polydispersity index (PDI). Values of the corresponding regression coefficient were calculated, and the equations were fitted to predict average size distribution, zeta potential, and PDI of Cu-NPs (Table 2). In relation to the equations, the sign of the model coefficients determines the response performance, when a coefficient shows a positive effect, the response pattern increases as the variable increases, and when a coefficient has a negative effect, the response decreases as the variable increases. Also, at a higher absolute coefficient value, a higher variable effect is obtained. Therefore, according to the equations shown in Table 2, it is possible to indicate that $CuSO_4$ concentration is the factor that most influences the response.

Table 1. Design matrix of experiments with three central points in relation to average size distribution, zeta potential, and polydispersity index (PDI) of Cu-NPs synthesized using *M. pyrifera* NBE.

No	Variable			Average size distribution (nm)	Zeta potential (mV)	PDI
	X_1 ($CuSO_4$, mM)	X_2 (pH)	X_3 (Temperature, °C)			
1	1	8	20	295.4 ± 62.1	-17.4± 5.1	0.6± 0.1
2	3	8	20	218.5 ± 95.8	-22.9 ± 3.1	0.5 ± 0.1
3	1	12	20	458.5 ± 236.6	-20.0 ± 4.7	0.7 ± 0.2
4	3	12	20	348.5 ± 148.5	-22.1 ± 8.2	0.6 ± 0.1
5	1	8	70	445 ± 220.6	-18.0 ± 1.7	0.7 ± 0.1
6	3	8	70	774 ± 480.7	-22 ± 4.1	0.8 ± 0.1
7	1	12	70	569.1 ± 464.7	-20.7 ± 6.4	0.7 ± 0.1
8	3	12	70	425.5 ± 164.8	-15.3 ± 3.1	0.7 ± 0.1
9	1	10	45	505.8 ± 225.9	-21.8 ± 4.3	0.8 ± 0.1
10	3	10	45	237.6 ± 88.0	-25.4 ± 3.3	0.5 ± 0.1
11	2	8	45	155.6± 43.7	-19.2 ± 2.8	0.3 ± 0.0
12	2	12	45	176 ± 43.9	-27.9 ± 3.6	0.4 ± 0.0
13	2	10	20	356.3 ± 100.7	-21.1 ± 2.6	0.6 ± 0.1
14	2	10	70	283.7 ± 152.4	-22.4 ± 5.1	0.6 ± 0.1
15	2	10	45	187.8 ± 35.5	-30.3 ± 2.8	0.4 ± 0.0
16	2	10	45	207.5 ± 86.9	-31.4 ± 1.6	0.4 ± 0.1
17	2	10	45	182,2 ± 93.1	-25.4 ± 3.6	0.4 ± 0.1

Table 2. Experimental equations of CuNPs formation evaluated as average size distribution, zeta potential, and PDI of nanoparticles to evaluate the combined effect of $CuSO_4$ concentration (X_1), pH (X_2), and temperature (X_3) on *M. pyrifera* NBE

Variable	Experimental equations	R^2
Average size distribution (nm)	$-213.57473 - 326.58675\ X_1 + 125.88625\ X_2 + 1.49825\ X_3 - 31.60625\ X_1X_2 - 1.29375\ X_2X_3 + 153.91981\ X_1^2 + 0.16355\ X_3^2$	0.76
Zeta potential (mV)	$72.26351 - 18.59373\ X_1 - 12.31342\ X_2 - 0.75419\ X_3 + 0.80000\ X_1X_2 + 0.045000\ X_1X_3 + 0.014500\ X_2X_3 + 1.89718\ X_1^2 + 0.48680\ X_2^2 + 5.99549E\text{-}003\ X_3^2$	0.70
PDI	$-1.19701 - 0.79533\ X_1 + 0.54381\ X_2 - 0.014204\ X_3 - 4.06250E\text{-}003\ X_1X_2 + 1.65500E\text{-}003\ X_1X_3 - 7.37500E\text{-}004\ X_2X_3 + 0.18162\ X_1^2 - 0.024595\ X_2^2 + 2.28992E\text{-}004\ X_3^2$	0.85

X_1: $CuSO_4$ concentration; X_2: pH; and X_3: temperature

Obtained data fit the empirical model with correlation coefficient (R^2) values of 0.76 for size distribution, 0.70 for zeta potential, and 0.85 for PDI (Table 2), which indicates that 76%, 70% and 85% of the total variation in the data around the average is explained by the fitted models given in the experimental equations. Previous studies conducted by Guan and Yao [85] suggest that the correlation coefficient should be at least 0.80 for a good fit of the model. Therefore, the models used are suitable for representing the relationship between the factors evaluated.

Three-dimensional response surface plots were used to demonstrate interactions between the reaction parameters and the average size distribution, zeta potential, and PDI of Cu-NPs synthesized using *M. pyrifera* BFNB extract. Response surface plots for size distribution are shown in Figure 1. Figures 1a, b and c present $CuSO_4$ concentration,

temperature, and pH effect on the average size distribution, respectively. It was shown that size distribution decreased as incubation temperature and pH decreased (Figure 1a). Additionally, $CuSO_4$ concentration is having a quadratic behaviour on size distribution, decreasing just when a medium (2 mM) concentration was used, and pH is decreased (Figure 1b); and finally, size distribution decreased as $CuSO_4$ concentration increased and temperature decreased (Figure 1c). $CuSO_4$ concentration effect on average size distribution is consistent with conclusions from other authors but related to chemical synthesis [86]. Now observing one of the few available studies related to pH during biosynthesis reactions, Caroling's research group [87] reported a synthesis of Cu-NPs using aqueous *Phyllantus embilica* (Gooseberry) extract at different pH, Surface Plasmon Resonance (SPR) band phenomena was observed through UV-vis at pH 8, 10, and a weak but detectable band at pH 12. No band was detected at pH 6.

Figures 1d, e, and f show zeta potential as a response to initial $CuSO_4$ concentration conditions, pH, and incubation temperature. There was not a clear influence of the studied variables in zeta potential values. Zeta potential shows Cu-NPs surface charge, dispersion, and stability, higher values than +30 mV or lower than -30 mV indicated good nanoparticle stability [88]. Therefore, obtained results revealed that *M. pyrifera* extract is well suited for synthesizing and stabilizing Cu-NPs since values were between -15.3 and -31.4 mV.

Reaction parameter effect on polydispersity index (PDI) is shown in Figures 1g, h and i. PDI decreased as pH and extract concentration in the medium increased (Figure 1g), and it was not affected by $CuSO_4$ concentration (Figure 1h). In the case of the interaction between $CuSO_4$ concentration and temperature, no variation in PDI was observed. In this sense, both the PDI and the zeta potential are directly related to nanoparticle size

distribution and agglomeration, and the proper value to indicate narrow size distribution should have a PDI value lower than 0.3 [89].

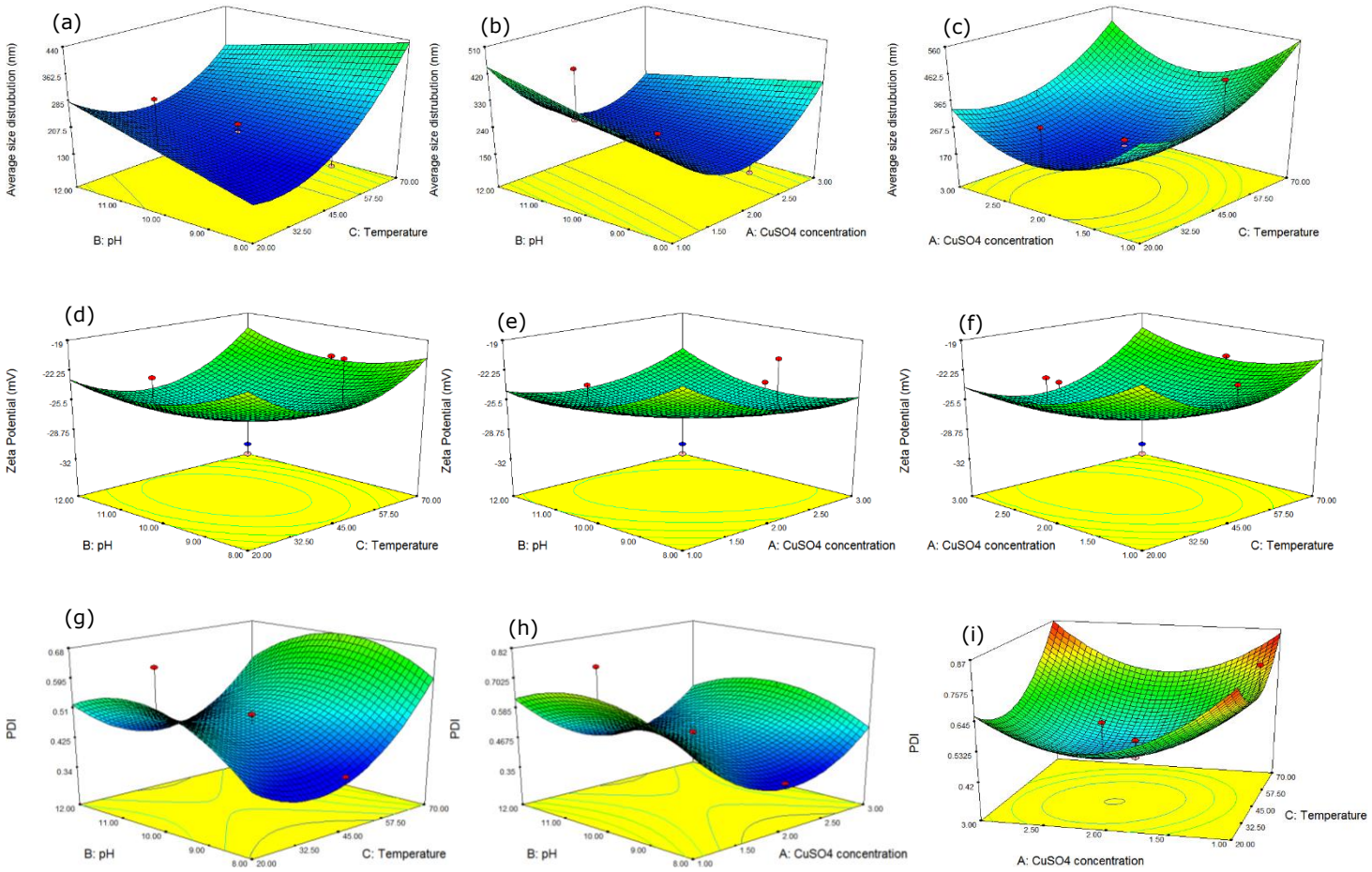

Figure 1. average size distribution 3D response surface plots as a function of pH and temperature (a), pH and $CuSO_4$ concentration (b), and $CuSO_4$ concentration and temperature (c). Z potential plots as a function of pH and temperature (d), pH and $CuSO_4$ concentration (e), and $CuSO_4$ concentration and temperature (f). PDI plots as a function of pH and temperature (g), pH and $CuSO_4$ concentration (h), and $CuSO_4$ concentration and temperature (i).

It is important to note that nano-scale products were greenly synthesized even at 70°C. In this sense, according to previous studies, *M. pyrifera* contains potential biomolecules to be used as reductant and capping agents such as fucoidans that are being degraded at that temperature. However, Cu-NPs synthesis was carried out at a high temperature, considering that polysaccharide reducing ability depends on their monosaccharide component ability to adopt an open-chain form, hence providing metal ion access to a specific functional group.

Figure 2a presents a Scanning Transmission Electron Microscopy (STEM) image, which shows that nanoparticles had a spherical morphology and ranged in size from 74 to 520 nanometers. These values are closely linked with the PDI value determined by DLS, which was 0.4, suggesting a heterogeneous size in the sample. However, the average size was 187.8 ± 35.5nm, and the nanoparticle zeta potential value from the same sample was -25.4 ± 3.6, which demonstrates a high repulsion between the synthesized nanoproducts.

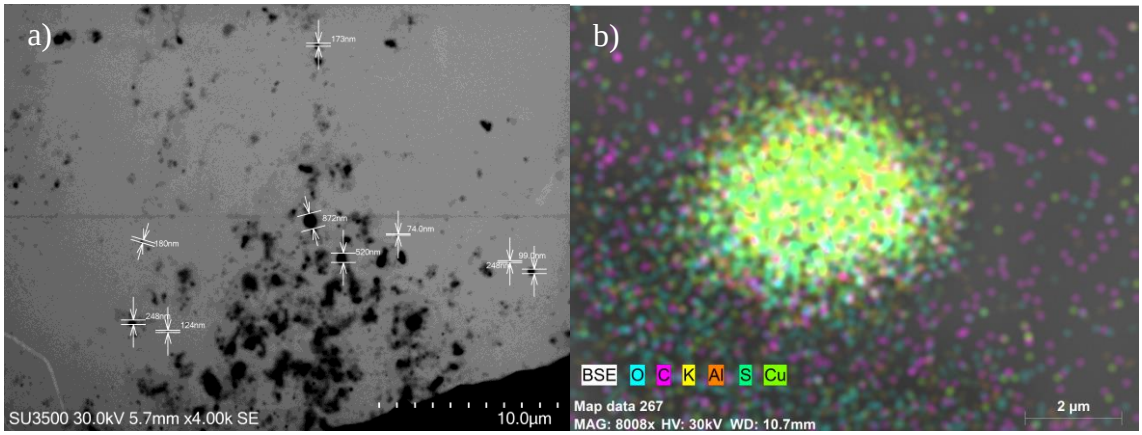

Figure 2. Green synthesized nanoparticles STEM image: a) using $CuSO_4$ 2 mM, pH10, and 45°C as reaction conditions; b) EDS Elemental Mapping from an agglomerated sample using $CuSO_4$ 2 mM, pH8, and 45°C as reaction conditions.

An energy dispersive X-ray spectrometry (EDS) Elemental Mapping from an agglomerated sample using $CuSO_4$ 2 mM, pH8, and 45°C as reaction conditions were performed on a field of view where the coalescence of nanoparticles into clusters occurred (Fig. 2b). EDS demonstrated that 2.63 % of Cu is present in the cluster, being the ionic competition among other compounds present in the same field of view (such as O, C, K, Al and S), a possible explanation to this situation. This probably means that they are involved in the early stages of nanoparticle formation (nucleation) and further aggregation, in addition to the bioreduction stage.

The importance of these parameters on the antimicrobial activity of Cu-NPs results from the fact that small nanoparticles present higher antimicrobial activity because their small size allows direct interaction with microbial membranes, generating cell death [90]. In the same way, nanoparticle monodispersity is expressed with PDI lower than 0.3 and indicates narrow size distribution [89], which is an important factor in nanoparticle antimicrobial activity. In this sense, response surface methodology (RSM) based on a central composite design (CCD) showed that predicted optimal reaction conditions for Cu-NPs formation, using *M. pyrifera* extract, were 2.2 mM $CuSO_4$ concentration, pH8, and incubation at 25,5°C, obtaining an average size distribution, Z potential and PDI of 121 nm, -23.5 mV and 0.3, respectively.

2.4 Conclusions

In conclusion, we demonstrate an approach to successfully follow an extracellular pathway for the green synthesis of copper ion bioreduction into Cu-NPs using an aqueous extract of the macroalgae *M. pyrifera*. $CuSO_4$ concentration is the parameter that is mostly affecting the average size distribution of greenly synthesized nano-scale material using *M. pyrifera* extract.

CCD-RSM model designed showed that an increase in temperature and pH reduces Cu-NPs average size distribution. Zeta potential results evidenced that stable synthesized Cu-NPs are achieved for all experimental conditions.

These results will contribute to describing an approach of possible biomolecules that are acting as reductant and capping agents during the green synthesis of Cu-NPs mediated by algae. Future studies will be carried out in order to portray the effect of a specific extract biomolecule and its possible synthesis mechanisms.

CHAPTER III

Green synthesis of copper oxide nanoparticles using protein fractions from an aqueous extract of brown algae Macrocystis pyrifera

Green synthesis of copper oxide nanoparticles using protein fractions from an aqueous extract of brown algae *Macrocystis pyrifera*

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Abstract: Amongst different living organisms studied as potential candidates for the green synthesis of copper nanoparticles, algal biomass is presented as a novel and easy-to-handle method. However, the role of specific biomolecules and their contribution as reductant and capping agents has not yet been described. This contribution reports a green synthesis method to obtain copper oxide nanoparticles (CuO-NPs) using separated protein fractions from an aqueous extract of brown algae *Macrocystis pyrifera* through Size Exclusion Chromatography (HPLC-SEC). Proteins were detected by a UV/VIS

diode array, time-based fraction collection was carried out, and each collected fraction was used to evaluate the synthesis of CuO-NPs. The characterization of CuO-NPs was evaluated by Dynamic Light Scattering (DLS), Z-potential, Fourier Transform Infrared (FTIR), Transmission Electron Microscope (TEM) equipped with Energy Dispersive X-ray Spectroscopy (EDS) detector. Low Molecular Weight (LMW) and High Molecular Weight (HMW) protein fractions were able to synthesize spherical CuO-NPs. TEM images showed that the metallic core present in the observed samples ranged from 2 to 50 nm in diameter, with spherical nanostructures present in all containing protein samples. FTIR measurements showed functional groups from proteins having a pivotal role in the reduction and stabilization of the nanoparticles. The highly negative zeta potential average values from obtained nanoparticles suggest high stability, expanding the range of possible applications. This facile and novel protein-assisted method for the green synthesis of CuO-NPs may also provide a suitable tool to synthesize other nanoparticles that have different application areas.

Keywords: Green synthesis, brown seaweed, proteins, size exclusion chromatography, copper oxide nanoparticles

3.1 Introduction

The eco-friendly synthesis of nanoparticles (NPs) is a challenging field in nanobiotechnology and is presented as a promising alternative to chemical pathways since it avoids the production of secondary contaminants that are affecting the environment [77–78]. The green synthesis of NPs uses reductant agents from bacteria, yeast, plants, algae, fungi or plants, which contain secondary metabolites such as sugars, alginates, proteins, some amino acids, and other molecules used to reduce metals in order to generate reduced ions that lead the nucleation process [93]. As a second step, the capping process may also be performed by biological agents like proteins instead of chemicals such as polyvinylpyrrolidone (PVP) to stabilize the nucleation process and obtain long-term stable NPs [90–91]. Those environment-friendly and cost-effective methods produce NPs of different compositions, sizes, morphologies, and dispersion, which may affect their final property and application [92–94]. The interest in copper-based NPs is given to their optical, conducting, magnetic, catalytic, thermal, and antibiotic activity, owing to the enhanced physic-chemical properties due to their small surface to volume ratio when compared to its bulk material [63], [83]. The use of raw aqueous algal extracts has been explored by a few authors as reductant and capping sources for the green synthesis of Cu-NPs [16], [95–98]. In this context, copper oxide nanoparticles (CuO-NPs) have been successfully synthesized using a boiled aqueous extract from the brown algae *Bifurcaria bifurcata* and *Cystoseira trinodis* within a size range from 5 to 45 nm and 6 to 7.8 nm, respectively [16, 85]. In addition, an aqueous extract from brown seaweed (*Sargassum polycystum*) was used by Ramaswamy et al. [101] to produce CuO-NPs. In a similar way, an autoclaved aqueous extract from the green microalgae *Botryococcus braunii* produced CuO-NPs in a size range between 10 and 70 nm [100]. Alternatively, Bhattacharya et al. [102] performed a slightly different

method by heating at 50 °C instead of boiling the extract in order to get an aqueous extract from the microalgae *Anabaena cylindrica*, and CuO-NPs with a particle size of 3.6 nm were obtained. It is important to note that a detailed characterization of the components of algae responsible for the reduction and stabilization process using copper as promotor is yet to be discovered. Therefore, it is relevant to lead studies towards using specific biomolecules in the green synthesis on CuO-NPs in order to expand their use for biological applications. This also may provide a better understanding to portrait its impact on the main characteristics of the CuO-NPs.

Here, we report a rapid and green synthesis method to obtain CuO-NPs using HPLC-size exclusion chromatography (SEC) separated protein fractions from an aqueous extract of the brown algae *Macrocystis pyrifera* as a reductant and capping agent. Our study suggests that using water-soluble proteins from *M. pyrifera* represents a consistent, straightforward green method to synthesize homogenous nano-scale size CuO-NPs that exhibits high-stability.

3.2 Materials and Methods

Brown Marine *Macrocystis pyrifera* algae were obtained from Universidad de Los Lagos; samples were collected in Quenac, Chiloé Island, (Chile). In order to remove epiphytes and sand particles, biomass was washed three times with tap water, then twice with distilled water, and followed by twice-double deionized water. Subsequently, algal biomass was dried in an oven at 50 °C overnight. The dried biomass was powdered in a blender and stored in darkness. A 100 mM CuSO4 (Loba Chemie, Mumbai, India) was prepared using double distilled water and kept at 4 °C in the dark for further experiments.

Preparation of *Macrocystis pyrifera* Biomass-Free Non-Boiled (BFNB) extract and protein precipitation from the algal aqueous extract

Dried algal powder (4 g) was dispersed in a 250 Erlenmeyer flask with 100 mL double distilled water, sonicated for 15 min (VCX 130, SONICS) at 60% amplitude to facilitate cell lysis [30], and incubated in an orbital shaker (200 rpm) for 24 h at 50 °C. Once cooled, the supernatant was separated from the biomass by centrifugation at 4000 rpm for 20 min, and two volumes of cold acetone (Merck, Darmstadt, Germany); were added to the previously obtained aqueous extract in a 50 mL centrifuge tube. The mix was vortexed thoroughly and incubated overnight at −20 °C. Samples were centrifuged at 4 °C for 20 min at 14,000 g, the supernatant was removed from the pellet, and 1 mL of 50 mM ammonium bicarbonate buffer (Merck, Darmstadt, Germany); was added to re-solubilize the pellet. These steps were repeated twice to ensure the removal of all undesired components from the sample. Sodium dodecyl sulfate (SDS) (Merck, Darmstadt, Germany); at a final concentration of 1% was added to dissolve remaining proteins prior to HPLC procedures.

Protein separation by size-exclusion chromatography and quantification by BCA assay.

The samples obtained in the previous activity were analyzed by size exclusion chromatography (SEC) using a Bio SEC-5 Column (Agilent Technologies, Santa Clara, CA, USA) on an HPLC (1200 Infinity series, Agilent Technologies, Santa Clara, CA, USA) connected to a fraction collector. Proteins were detected by a UV/VIS diode array detector (DAD, detection at 280 nm wavelength) (Agilent Technologies, Santa Clara, CA, USA). Running conditions were: oven temperature 25 °C, flow rate 0.5 mL/min, run time 30 min, and 50 mM ammonium bicarbonate as the mobile phase, pH 10. Time-based fraction

collection was carried out every 1 min; samples were concentrated using a vacuum centrifuge and stored at −20 °C. Bovine Serum Albumin (BSA) was used as a reference. Afterward, protein fractions were quantified using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce, Rockford, IL, USA) using BSA as a calibrant.

Biogenic synthesis and physic-chemical characterization of copper oxide nanoparticles mediated by *M. pyrifera* proteins

Samples from the protein fraction collection were used for copper nanoparticle synthesis. Those fractions without protein content were maintained as control to measure SDS impact on CuO-NPs formation. Subsequently, each fraction containing proteins were mixed with copper sulfate to get a final concentration of 2 mM. Samples were incubated at 45 °C on a rotary shaker for 48 h.

The samples obtained were further characterized by measuring the hydrodynamic diameter, zeta potential, and polydispersity index (PDI) values at 25 °C using the Zetasizer Nano ZS90 System (Malvern Instruments, Malvern, UK) through Dynamic Light Scattering (DLS). To avoid aggregation, samples were sonicated for 5 s Prior to the DLS measurement, the aqueous suspensions of nanoparticles were passed through a cellulose acetate syringe driven filter unit (0.22 µm pore size). For each experiment, a glass cuvette was used containing a 500 µL sonicated sample and 1 mL double distilled water. Fourier transform infrared (FTIR) spectral analysis was used to identify the functional groups present on the surface of the copper oxide nanoparticles. The transmission spectrum was recorded using a CARY 630 FTIR (Agilent Technologies) in the frequency range of 500–4000 cm−1. Lastly, transmission electron microscopy (TEM) images were obtained using a Tecnai F20 FEG-S/TEM operated (FEI, Hillsboro, OR, USA) at 200 kV accelerating voltage, equipped with an Energy Dispersive X-ray

Spectroscopy (EDS) detector (FEI, Hillsboro, Oregon, US). Samples were prepared by placing a drop of sample on a 300 mesh gold grid covered by carbon formvar; excess of solution was removed by blotting with filter paper and subsequently dried in air at room temperature before imaging.

3.3 Results and Discussion

In this study, we first tested the ability of different size-separated protein fractions from an aqueous extract of the brown algae *M. pyrifera* for the green synthesis of CuO-NPs. We used SEC in order to obtain 11 sample fractions (see Supplementary Materials Figure S1) and measured protein content in each fraction with a BCA assay. As can be seen in Table 1, fractions 3–9 contained measurable amounts of protein ranging from 0.72 µg (fraction 9) to 13.92 µg (fraction 7). We also collected low molecular weight (LMW) fractions (10 and 11) as well as high molecular weight (HMW) fractions (1 and 2), which did not contain protein.

Table 1. Protein quantification using the bicinchoninic acid (BCA) assay method, after recovery of 11 fractions obtained from the elution profile.

Fraction	Absorbance	Captured Mass (µg/500uL)
1	0.014	0
2	0.019	0
3	0.083	6.86
4	0.059	4.24
5	0.073	5.78
6	0.096	8.31
7	0.147	13.92
8	0.086	7.22
9	0.027	0.72
10	0.015	0
11	0.014	0

We tested each fraction for their ability to create NPs by each sample with 2 mM copper sulfate. The first color change indicative of copper reduction was observed at 24 h, with

a slight color change from light blue to light green when pellets were observed after a spin; this phenomenon was not observed in control fractions (1–2 and 10–11). The same color change phenomenon has been observed and reported by other authors when *Pterospermum acerifolium* and *Ixora coccinea* leaf extracts were used for the synthesis of CuO-NPs [99–100]. Nevertheless, both mentioned studies were focused on the use of raw leaf extract as a reductant agent, and the role of specific biomolecules was not explored. Figure 1 shows representative TEM images of the products of the $CuSO_4$ reduction by different protein fractions (3, 4, 5, 6, 7, 8, and 9) from the *M. pyrifera* aqueous extract. TEM images showed that the metallic core was between 2 and 50 nm, with spherical nanostructures present in all fractions containing proteins, while in non-protein fractions, CuO-NPs were not observed. In fraction 5 (Figure 1c), the perimeter of CuO-NPs was evidently surrounded by a layer of non-metallic material with a width of ~5 nm, suggesting that proteins are also acting as capping and stabilizing agent. EDS analysis of nanoparticles (Figure 1h) indicated the copper (Cu) composition and a strong carbon (C) signal from proteins in the synthesized nanoparticles. The gold (Au) signal is explained by the grid where the sample was deposited. Furthermore, FTIR spectral analysis provided evidence of functional groups present in CuO-NPs and is shown in Figure 2. Bands at 1638 cm^{-1} and 1540 cm^{-1} are mainly associated to C-O stretching peptide bond from amide I and N-H bending and C-N stretching vibration from amide II, respectively [105]. The peak at 3440 cm^{-1} is due to N-H and O-H stretching vibrations. Besides functional groups frequencies reported above, there is also a strong signal at 575 and 620 cm^{-1}, which belongs to the bending mode of the CuO bond [106].

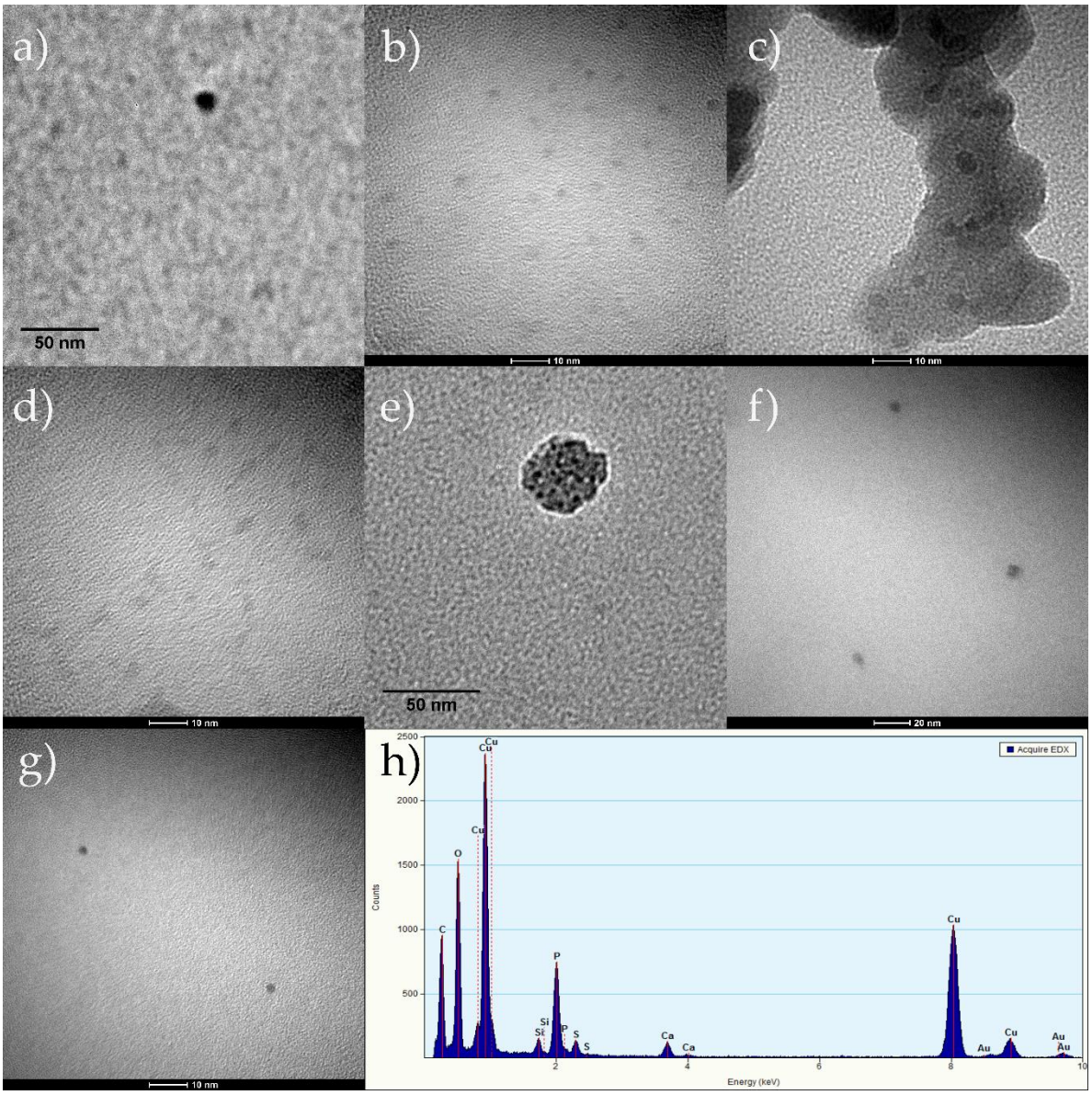

Figure 1. Transmission Electron Microscopy (TEM) images of the products of the $CuSO_4$ reduction by different protein fractions from the algal extract: (**a**) Fraction 3; (**b**) Fraction 4; (**c**) Fraction 5; (**d**) Fraction 6; (**e**) Fraction 7; (**f**) Fraction 8; (**g**) Fraction 9; (**h**) Energy Dispersive Spectroscopy (EDS) analysis spectrum of CuO-NPs.

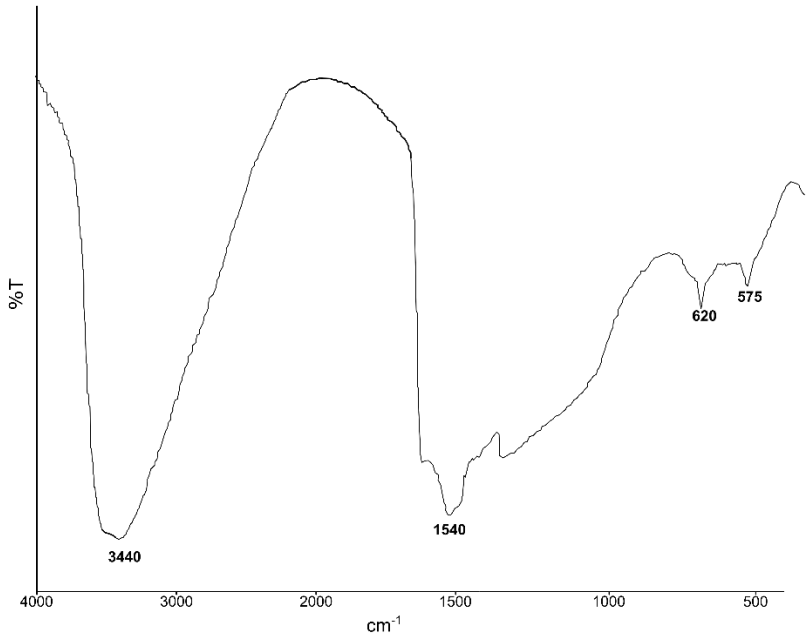

Figure 2. Fourier Transform Infrared (FTIR) spectra of CuO-NPs.

It is important to note that proteins with LMW and HMW were both able to synthesize spherical CuO-NPs. Similarly, even in fractions with a low protein content as fraction 9 with 0.72 µg of proteins, spherical CuO-NPs of 2 nm diameter were found (Figure 1g). Synthesized CuO-NPs from protein fractions 4 and 6 were very similar, with sizes ranging from 2 to 10 nm, showing well-dispersed nanoparticles along the field of view (Figure 1b, d). In all protein fractions observed, the synthesized nanoparticles were homogeneous in size, and agglomeration was not observed, although, in solution, there might be more aggregation, which may interfere with DLS measurement and raise the average size.

Protein stability capacity was supported by the DLS analysis results of CuO-NPs (Figure 3), where the Zeta Potential value obtained from samples 3 to 9 were highly negative, demonstrating a high charge repulsion between nanoparticles capping agents. Samples 1, 2, 10, and 11 without protein content were used as control, which shown no measurable NPs in solution, independently of 1% SDS and copper sulfate addition. An interesting

correlation was found with HMW proteins since they resulted in lower zeta potential values (fraction 3, −62.1 mV, Table S1) and relatively low PDI, suggesting that larger proteins in higher concentrations may benefit the capping process while LMW proteins are more unstable (Fraction 9, −26.1 mV, Table S1). Zeta potential values higher than +30 mV or lower than −30 mV exhibit high stability [107], indicating that most protein fractions can be used to create stable dispersions. Therefore, it can be proposed that proteins are acting as reductant and capping agent, conferring stability to CuO-NPs due to a protein-layered shell. Stability is a crucial aspect of nanoparticle synthesis since the lack of sufficient stability of many nanoparticle preparations has, to some extent, hindered the development of real-world applications of nanomaterials [108].

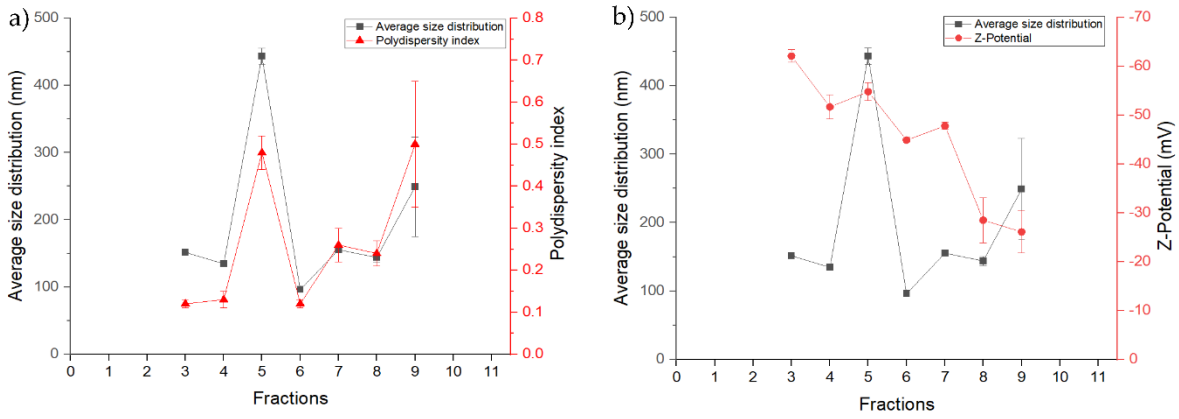

Figure 3. Characterization of CuO-NPs average size distribution, zeta potential, and polydispersity index (PDI) by Dynamic Light Scattering (DLS): (**a**) Average size distribution and Polydispersity index; (**b**) Average size distribution and Z-potential.

Results from average size distribution demonstrated nanoscale products in fractions 3, 4, 6, 7, and 8, all of them presented a PDI value below 0.3, which suggests that CuO-NPs are also highly monodispersed. Samples from fractions 5 and 9 were out of desirable size, and both PDI values are an indicative of highly polydisperse size distribution. The size

difference between TEM and DLS analyses may be due to the different principles involved in these two techniques. The DLS measurement involves the hydrodynamic state of nanoparticles, whereas it is a dry state in TEM measurement [105−106].

In terms of its biochemical composition, *M. pyrifera* biomass contain 75% carbohydrates, 13% proteins, 11% ash, and 0.7% lipids [40]. Ortiz J. and collaborators [41] explored the composition of 17 amino acids in *M. pyrifera*, showing an important contribution of water-soluble amino acids, such as glutamic acid, aspartic acid, arginine, and cysteine. It has been described that biomolecules with carboxyl, hydroxyl, and amine functional groups have the potential for metal-ion reduction and for capping in the green synthesis of nanoparticles, in replacement of chemical or physical methods [31], [42–46]. Thus, it can be explained that, even with a low protein content per fraction, glutamic, and aspartic acid represented by a various structural percentages are amino acids with significant reductant capacity at the nanoscale. Furthermore, there is strong evidence that glutamic acid and aspartic acid are capable of reducing metals for nanoparticles synthesis [107–108] while arginine has been reported as an effective capping agent of nanoparticles [113]. Moreover, all proteins that contain amino and carboxyl groups have a reported interaction with Cu^{+2}, which prevents the formed CuO-NPs from aggregating [114]. In this sense, it is relevant to note that the S signal observed in Figure 1H could indicate the presence of cysteine as a hydrophilic sulfur-containing amino acid that may lead the mechanism of reduction and stabilization for the consequent synthesis of CuO-NPs. It has been previously reported that Au(III) ions might oxidise Cys to cystine, and subsequently to sulphonic acid, driving the formation of nucleation centres that ultimately results into AuNPs dispersions [115].

Hence, it is suggested that these amino acids are playing a pivotal role in the generation of Cu reduced cations, along with a stabilization capacity given by the physicochemical interaction between the same proteins and the nano quant cations of reduced forms of Cu.

Moreover, a previous study identified five groups of substances present in extracellular products of different *Chlorococcum* species: (i) steam-volatile acids; (ii) yellow water-soluble phenolic compounds; (iii) lipophilic substances; (iv) proteins; and (v) polysaccharides [116]. In this aspect, FT-IR analysis of *C. humicola* silver nanoparticles revealed that protein molecules were mainly responsible for the production of biogenic silver nanoparticles, and the presence and binding of proteins with the nanoparticles could possibly lead to their stabilization. The findings of this research can be closely linked to Mohseniazar et al. [55], who demonstrated that *Nannochloropsis gaditana* and *Chlorella vulgaris* have the potential to biosynthesize silver nanoparticles in a culture medium containing silver nitrate in solution. A better characterization was done by another study whose aim was to explore the active species in the algal extract from *Chlorella vulgaris* that was responsible for the reduction of Ag ions and the resulting growth of Ag(0) into Ag nanoplates [44]. Besides, chemical modifications of the algal proteins were carried out to identify the amino acid residues in the proteins with Ag ion reduction capability and shape direction functionality. From this analysis, the authors concluded that, in accordance with other references [33], [56], [78], algal proteins were the active biomolecules in the algal extract responsible for Ag nanoplates formation, being primarily accountable for the reduction of Ag(I) and the formation of Ag nanoplates. In addition, they discovered that Tyr residues in the proteins were responsible for Ag ion reduction, and the carboxyl groups in Asp and/or Glu residues were driving the anisotropic growth of Ag nanocrystals into nanoplates.

Studies with *Fusarium oxysporum* showed the presence of two extracellular proteins having a molecular weight of 24 and 28 kDa, which were being responsible for the synthesis of zirconia nanoparticles [78]. In conjunction with this research focus, Xie et al. [30] explored a simple and controlled biological synthesis of large quantities of gold nanoplates through room temperature reduction of gold ions for 48 h in an aqueous algal extract. Lastly, it must be mentioned that there are agents, such as FADH2/NADH cofactors that may co-elute with the proteins, which have reported contribution as reducing agents of proteins during *in vivo* NPs [117] and they may be present in the fractions. However, these cofactors are unstable above pH 7. Thus, their concentration might not be significantly representative; instead, proteins are more probably leading the reducing power within samples.

The importance of this study was the identification, as far as possible, of the biomolecules involved in the nucleation and growth of gold clusters into specific shapes and sizes. For this purpose, proteins were eluted and isolated according to their molecular weight, and distinctive triangular and hexagonal gold nanoparticles obtained using a protein with approximately 28 kDa weight were purified by reversed-phase HPLC and then collected. Accordingly, results presented in this report, using protein fractions extracted from *M. pyrifera*, evidenced that both HMW and LMW proteins are capable of synthesizing CuO-NPs. However, due to differences between the fractions, there is still room for further optimization of CuO-NPs green synthesis.

3.4 Conclusions

This present work demonstrated a successful and straightforward strategy based on a biomolecule-assisted technique for the green synthesis of copper ion bioreduction into copper nanoparticles, using proteins precipitated from an aqueous extract of *M. pyrifera* macroalgae as both reductant and capping agent. HMW and LMW fractions were effectively used as reductant and stabilizing agent for CuO-NPs synthesis and shown some correlation between hydrodynamic size and protein size.

Reductant capacity was present in fractions with high and low protein content, indistinctively. The highly negative zeta potential average values of the obtained copper nanoparticles suggest high stability, which also showed a protein size correlation. Thus, it is fundamental to lead more research to completely elucidate biomolecules role and their impact on the green synthesis of CuO-NPs. Hence, these preliminary results on the characterization of CuO-NPs mediated by protein fractions may indeed expand the range of possible applications. This eco-friendly method should also be extendable to the preparation of different metal or biocompatible metal nanomaterials.

Supplementary Materials: The following are available online at https://www.mdpi.com/2227-971 7/9/1/78/s1

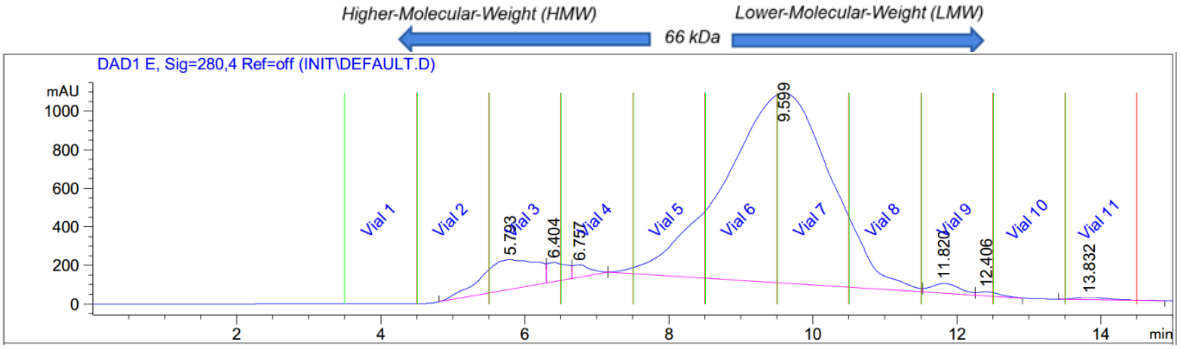

Figure S1. Chromatogram of HPLC-SEC using proteins from the *M. pyrifera* aqueous extract. Lines indicate the time-based fraction recovery of 11 fractions obtained from the elution profile.

Table S1. Characterization of CuO-NPs average size distribution, zeta potential, and polydispersity index (PDI) by Dynamic Light Scattering (DLS).

Fraction	Average size distribution (nm)	Polydispersity Index (PDI)	Z-Potential (mV)
1	-	-	-
2	-	-	-
3	151.60 ± 0.23	0.12 ± 0.01	-62.1 ± 1.29
4	134.60 ± 0.47	0.13 ± 0.02	-51.7 ± 2.50
5	443.50 ± 11.62	0.48 ± 0.04	-54.8 ± 1.80
6	96.14 ± 1.06	0.12 ± 0.01	-44.9 ± 0.51
7	155.30 ± 3.21	0.26 ± 0.04	-47.8 ± 0.76
8	114.00 ± 6.48	0.24 ± 0.03	-28.5 ± 4.62
9	249.10 ± 74.25	0.50 ± 0.15	-26.1 ± 4.30
10	-	-	-
11	-	-	-

CHAPTER IV

General discussion, concluding remarks and future directions

4. General discussion, concluding remarks and future directions

4.1 General discussion

Nanobiotechnology is an emerging field in science in which nature act as a "bio-laboratory," where plants, yeast, bacteria, fungi, and algae are used as a valuable and diverse pool of biomolecules that play a pivotal role in the synthesis process of nanoparticles with distinct shapes and sizes thereby acting as forerunners for the design of greener, safe and eco-friendly protocols. In this sense, the green synthesis of nanomaterials is an increasingly developing multidisciplinary field in the scientific sector, which has attracted great interest because of its abundant applications [118]. The synthesis of nanoparticles mediated by algal biomass and their compounds offers an alternative approach to chemical and physical pathways, which have been reported with an ample variety of harmful by-products during or after the synthesis and are often harder to manage [61].

Therefore, green synthesis of nanoparticles and nanomaterials have exhibited a far greater advantage during their synthesis due to cleaner chemicals and easier to handle wastes (e.g., exhausted algae biomass), which are easier to biodegrade or can even be integrated into circular industrial cycles as primary raw materials after nanomaterial retrieval including, bioethanol production and biofertilizer [119]. Thereupon, green biological synthesis of nanomaterials is a promising pathway to generate impact research and industrial applications that may contribute to reducing the environmental impact behind its production process.

Moreover, regarding the green synthesis of nanomaterials, there has been extensive work regarding Au and Ag nanomaterials where both have shown diverse applications such as colorimetric diagnostics and antimicrobial effects, respectively [112, 113]. The recent

scientific advances associated with the identification of specific biomolecules, the possible role in the synthesis mechanisms, and the characterization of nanoproducts obtained have been useful to enhance the process understanding for the synthesis of gold and silver nanoparticles. Conversely, Cu counterpart has been, till the submission of this thesis, insufficiently studied and most studies are based on only the presence or absence of Cu-NPs, but there is a lack of more in-depth studies involving specific biomolecules for green synthesis of Cu-NPs and their fine characterization of their overall impact on the process.

In this context, the brown seaweed *Macrocystis pyrifera* was selected as a potential candidate for the green synthesis of CuO-NPs. There are several studies using brown algae to biosynthesize metallic NPs supported by the premise that their biomolecules could act as reductant and capping agent, but further research is needed to identify a specific one for the synthesis of Cu-NPs. Moreover, *M. pyrifera* has been thoroughly characterized by Ortiz [97, 98], which facilitated the later discussion of results.

An extensive review of the green synthesis of metallic NPs was conducted. We elucidated from several reports that there is a vast potential in numerous micro and macroalgal biomolecules with known metal-ion reduction and capping proficiency to generate Au and Ag NPs, mainly due to their carboxyl, hydroxyl and amine functional groups [122]. Thus, there is a necessity for algae raw material to be pretreated to facilitate the release of different kinds of biomolecules (e.g., proteins and carbohydrates), including cell lysis and lyophilization.

Moreover, it is important to mention that extracellular biosynthesis is highly recommended as well as using extracts, due to the NPs retrieval is far greener and easier

to perform than its intracellular counterpart, which may need commercial processing equipment or chemical reactants to carry on the release-capture process [123].

Consequently, we demonstrated an approach to successfully follow a simple extracellular pathway for the green synthesis of CuO-NPs using *M. pyrifera* extract. In this regard, we evaluated $CuSO_4$ concentration, pH, and temperature in a CCD-RSM model. Interestingly, $CuSO_4$ concentration played a critical role in CuO-NPs formation, size, and stability, where significantly increasing or reducing $CuSO_4$ concentration negatively affected CuO-NPs formation.

This effect may be explained due CuO ion quantum dots chemical availability, where a low concentration reduces the kinetic energy and clustering spontaneous energy within the solution, i.e., reducing the possibilities of each CuO quantum dots to reach and complex with other quantum dots to generate a CuO-NP; thus, the overconcentration biomolecules also start to complex with these CuO-NPs, increasing the size of detected CuO-NPs [124]. Conversely, high $CuSO_4$ concentration quickly depleted the biomolecules reduction and capping capabilities, forcing left-over CuO ions to destabilize formed CuO-NPs, which then represent the reduction and capping capacities [125], increasing the average-size distribution of detected CuO-NPs in solution (Figure 1b, 1e, 1h). A similar effect could be observed with the PDI.

Moreover, zeta potential showed a similar effect previously discussed with average-size distribution and PDI, where the centered conditions showed the best results. Yet, the effect was significantly less noticeable, and the Z-potential displayed values above the -30 limit, which indicates low-stable CuO-NPs in solution [88], which may also hinder the NPs size distribution over time.

It is important to mention that overall, NPs synthesis is a highly complex interactive process between several different conditions, which finally impact the kinetic chemistry within the solution. For example, pH and temperature are known to affect particle kinetics, i.e., movement, which ultimately affects quantum dots formation and aggregation [126].

In addition, another important effect is the co-occurrence of different biomolecules, which also may be interacting within the extract of *M. pyrifera*. It is known that proteins can interact with carbohydrates, where many have a high reduction power and vice versa, which may also ultimately hamper the NPs overall formation process where it is needed to reduce Cu ions, cluster and stabilize [127]. In this context, many proteins that may have reduction and stabilization capabilities might have got lost during interacting with other biomolecules than with Cu ions, reducing the final yield and quality of NPs.

Due to the previously mentioned, we focused on extracting and purifying a biomolecule of interest, which have been several times reported with dual reduction-stabilization capabilities for NPs biosynthesis: proteins [128]. In this regard, there are several studies that point out amino acid residues as excellent dual-agents for NPs biosynthesis. To carry out this idea in our work, we precipitated soluble proteins present in *M. pyrifera* extract with acetone incubation at -20°C and darkness.

Additionally, after acetone incubation and protein extraction, we separated these proteins through HPLC-SEC and obtained several fractions. Interestingly, proteins containing no detectable proteins had no CuO-NPs synthesis when CuSO4 was added. Furthermore, all fractions containing proteins were capable of biosynthesizing CuO-NPs. We also observed some interesting correlations, which gave some new insights.

These correlations were observed that CuO-NPs made by proteins had average-size distribution correlated to their PDI almost exactly (Figure 3a). Meanwhile, another correlation was observed between the Z-potential and protein molecular weight, where LMW proteins showed lesser Z-potential, which indicates a less stable NPs. These can be explained by the amino acid residues in the proteins, where LMW proteins may have significantly fewer amino acidic residues of glutamic acid, aspartic acid and cysteine, which represent the greater % of the total protein content of *M. pyrifera* [41].

Larger proteins are also more likely to co-elute with co-factors, such as NADH2/FADH, which have an important reducing power. However, these co-factors tend to degrade on pH above 7; thus, their presence might be low, but they might still be playing a reducing role in the overall process [117].

Finally, we must mention that the overall process of using protein fractions of *M. pyrifera* to biosynthesize CuO-NPs must still be improved and optimized. However, the current results highlight the potential of *M. pyrifera* biomass as an alternative, easy and inexpensive method to produce CuO-NPs. Furthermore, showing that HMW proteins exhibit higher stability and nanoscale average-size distribution may direct future research to elucidate the optimal MW range and the optimal percentage of specific amino acidic residues necessary for a specific metallic NPs within a size range, showing promising applications of *M. pyrifera* biomass for bionanotechnological procedures including as diagnostics or microfluidic systems integration.

4.2 Concluding remarks and future directions

- Our findings confirm the feasibility to produce CuO-NPs even when using a raw aqueous extract from *M. pyrifera*. However, the high complexity of biomolecules present in an aqueous extract derives to an unspecific clustering that may hamper the final size of nanoproducts. The control of reaction parameters is a crucial aspect to determine the nanoparticle characteristics within a heterogeneous biomolecules solution incubated with copper sulphate.

- There is a clear role within pH, $CuSO_4$ concentration and temperature, which ultimately affects final CuO-NPs key characteristics (e.g., size, stability, dispersion). Although this not novel, *M. pyrifera* has shown a clear tendency with centric treatment values, exhibiting a clear potential as CuO-NPs precursor biomaterial, which need further characterization, isolation, purification and therefore more basic research to fully explore all its biomolecules potential.

- In a joint effort of our collaborators to further explore *M. pyrifera* biomolecules potential, our research also provided the first evidence that water-soluble proteins present in *M. pyrifera* brown algal biomass are actively participating in a dual role as reducing and later as a stabilizing agent of the synthesized nanoparticles. This evidence underlines the importance of studying specific biomolecules to understand the synthesis process.

- Upon further purification and incubation of water-soluble proteins present in *M. pyrifera* were grouped within two major segments, namely HMW and LMW proteins. Interestingly, HMW proteins exhibited a major negative Z-potential, which decreased with LMW protein fractions. This gives a first insight were acidic amino residues and their presence percentage play a critical role, where apparently HMW proteins are richer in glutamic acid, aspartic acid and cysteine, which have been reported with a pivotal dual-role for NPs reduction and stabilization process. Therein, HMW proteins are significantly

more stable than LMW proteins. Accordingly, we must also mention that PDI and average size distribution correlated in every point, which may indicate an important control factor, where the focus to generate nanoscale CuO-NPs must be on average size distribution, i.e., smaller CuO-NPs will exhibit a lower PDI; thus, a more homogenous solution will be obtained. This can be reached by further optimizing our current parameters.

As future directions, our next step is to further characterize *M. pyrifera* proteins to explore their potential to obtain smaller NPs, which might increase their applications and efficiency. In this sense, this may be accomplished in numerous ways, including further fraction concentration and sub-fractionation to have a better insight of the HMW proteins and obtain an optimal MW range for CuO-NPs synthesis. Another pathway might be the characterization of each protein fraction and purify the most abundant protein per fraction to then sequence it, synthesize it and explore their potential as CuO-NPs precursors, to then chose the most optimal candidates.

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