UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: BIOSYNTHESIS OF SELENIUM NANOPARTICLES BY USING THE GREEN MICROALGA CHLAMYDOMONAS REINHARDTII

Trabajo de integración curricular presentado como requisito para la obtención del título de Ingeniero Biomédico

Autor:

Jaramillo Torres Francisco

Tutor:

PhD. – Dahoumane Si Amar

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Esta tesis está dedicada a:

A mi madre Mónica Torres, quien pasó toda su vida junto a mí, apoyándome y alentándome todos los días de mi vida y en cada decisión que he tomado. Sé que siempre tuve y siempre tendré su amor infinito.

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Francisco Eliseo Jaramillo Torres

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Francisco Eliseo Jaramillo Torres

Resumen

Las nano partículas de selenio (SeNP) han atraído la atención de los científicos de materiales debido a su alta actividad biológica y baja toxicidad. Las nano partículas amorfas de selenio han demostrado propiedades únicas como fotoeléctricas, semiconductoras y anticancerígenas. El presente estudio describe la bio producción de SeNP utilizando recursos de la micro alga verde unicelular *Chlamydomonas reinhardtii* (CR) a través de un proceso sencillo de un solo paso. Esto se hizo utilizando los cultivos vivos (enteros) de CR o el sobrenadante cosechado. Esta micro alga se cultivó durante 3 semanas en el medio de crecimiento Bold's Basal (BBM), se incubó a ~ 23 ° C con un fotoperiodo de 16 h de luz / 8 h de oscuridad. Para evaluar la viabilidad de la producción de SeNP utilizando cultivos de CR, se introdujeron tres precursores de selenio en los matraces a una concentración final de 10^{-2} M: tetracloruro de selenio (SeCl4), ácido selenoso (H2SeO3) y selenito de sodio (Na2SeO3). Los resultados más notorios se obtuvieron usando ácido selenoso (H2SeO3). Esto fue confirmado por el cambio de color de verde a rojo anaranjado, la presencia de una banda de absorción ~ 500-600 nm usando espectroscopia UV-Vis y la presencia de NP esféricos con un diámetro promedio de 66 nm, usando microscopía electrónica de transmisión (TEM). Además, este proceso resultó ser dependiente de la luz.

Palabras Clave:

Amorfas, biosíntesis, cultivos vivientes, microalgas, nanopartículas, selenio.

Abstract

Selenium nanoparticles (SeNPs) have attracted tremendous attention of material scientist owing to their high biological activity and low toxicity. Amorphous selenium nanoparticles have demonstrated unique photoelectric, semiconducting and anti-cancer properties. The present study depicts the bioproduction of SeNPs using resources of the unicellular, green microalga *Chlamydomonas reinhardtii* (CR) via an easy, single-step process. This was done either using the whole living cultures of CR or the harvested supernatant. This microalga was grown for 3 weeks in Bold's Basal Medium (BBM), incubated at ~23 °C with a photoperiod of 16 h light / 8 h dark. To evaluate the feasibility of Se-NPs production by CR, three precursors of selenium were introduced to the flasks at a final concentration of 10-2 M: selenium tetrachloride (SeCl4), selenous acid (H2SeO3) and sodium selenite (Na2SeO3). Most notorious results were obtained using selenous acid (H2SeO3). This was confirmed by the change in color from green to orange-red, the presence of an absorption band ~500-600 nm using UV-Vis spectroscopy and the presence of spherical NPs with an average diameter of 66 nm, using transmission electron microscopy (TEM). Moreover, this process resulted to be light-dependent.

Key Words:
Amorphous, biosynthesis, living cultures, microalgae, nanoparticles, selenium.

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CHAPTER I

INTRODUCTION

Selenium Nanoparticles (SeNPs)

Selenium (Se) is a micronutrient metalloid and indirect semiconductor element that exhibits good photoelectrical conductivity (Mort, 1968), nonlinear optical properties, and photocatalytic activities for organic hydration and oxidation reactions (Nath et al., 2004). Also, it is known that Se was used to create high-efficiency photovoltaic solar cells (Kunioka & Nakada, 1982), rectifiers, photography exposure, dietary and even xenography (Cheng et al., 2012). Moreover, it is recognized that dietary Se compounds possess a fundamental role in human health, because Se regulates the enzymatic function of the cells and accomplishes the cell cycle including cell's apoptosis (Schrauzer & Surai, 2009). Furthermore, Se is known as a potent nutritional antioxidant when it is incorporated into selenoproteins (Hoffmann & Berry, 2008). In addition, selenium shows a protective effect at various stages of carcinogenesis. The efficacy of selenium compounds is based on the stimulation of apoptosis and inhibition of tumor cell migration and invasion (Zeng & Combs, 2008). Finally, Se nutritional deficiency is related to viral infections, immune health diseases and juvenile cardiomyopathy (Beck et al., 2003).

Selenium nanoparticles (SeNPs) have attracted attention owing to their unique properties and potential applications in the industry and biomedical field. It is proven that SeNPs possess unique mechanical, optical, electrical, biologic and chemical properties. It is proved that SeNPs act as an antioxidant against acetaminophen toxicity in the liver and brain (Mohammed & Safwat, 2013). SeNPs exhibit chemotherapeutic and chemopreventive features; in combination with antibiotics, their anticancer efficacy is improved suggesting that SeNPs could be used as a nanomedicine (Wadhwani et al., 2017). Other studies confirmed the antioxidant and lower cytotoxicity effect of SeNPs compared to selenium dioxide (Forootanfar et al., 2014). Moreover, SeNPs therapeutic effects have been reported against oxidative stress and inflammation disorders, such as arthritis, diabetes, cancer, and nephropathy (Khurana et al., 2019).

Biosynthesis

There are two general approaches to obtain nanoparticles and nanostructures, the top-down and bottom-up routes. Arole & Munde, (2014) states that the top-down method starts with larger particles and uses methods to reduce or slicing them until obtaining nano-sized particles. Most physical methods are related to top-down approach. On the other hand, the bottom-up approach starts from molecular or atomic compounds that self-assemble by physical principles or external driving forces to obtain nano-structured and self-organized systems. Usually, the bottom-up approach is related to chemical and biological methods of NP synthesis.

For the chemical synthesis of SeNPs, highly toxic chemicals have been used, such as dinitrobutylphenol (DNBP) in the presence of potassium permanganate ($KMnO_4$) (Iranifam et al., 2013). In most cases, these chemicals could generate dangerous wastes and toxic byproducts. In the case of physical synthesis of SeNPs, specialized equipment and qualified staff are needed; the procedure is tedious and made of several steps. For instance, YAG laser is required for the pulsed laser ablation to obtain nanoparticles (Quintana et al., 2002).

The most common biological ways to synthesize inorganic NPs rely on the use of plants and their extracts along with microorganisms, namely bacteria, fungus and algae. Fulfilling the Principles of Green Chemistry, these biological methods are eco-friendly, less toxic, and cost-effective in comparison with chemical and physical methods (ACS, s.f.). In 2007, Singaravelu et al. reported the first case were marine alga, *Sargassum wightii Greville*, was used as a biofactory for the production of AuNPs (Singaravelu et al., 2007) demonstrating therefore the potential of algae in nanotechnology.

Microalga for nanotechnology

Barsanti et al. defined that algae might be multicellular or unicellular photosynthetic organisms found in fresh and marine water, including lakes, rivers, oceans, and even wastewater (Barsanti et al., n.d.). Algae can tolerate a wide range of temperatures, pH, salinities, and light intensity values. They can grow under a variety of conditions, such as reservoirs or desert conditions, and also can grow alone or in symbiosis with other organisms. According to Khan et al, 2018, algae could be classified into different divisions, such as *Rhodophyta* (red algae), *Phaeophyta* (brown algae), and *Chlorophyta* (green algae) (Khan et al., 2018). Moreover, they are classified by size, such as macroalgae or microalga.

Macroalgae are multicellular, large-size algae and they could be visible with the naked eye. On the other hand, microalga are microscopic single cells (or multicellular) and filamentous. They may also be prokaryotic, like cyanobacteria, or eukaryotic, such as the *Chlorophyta*. Microalga provide various and different applications; they are rich in carbohydrates, carotenoids, and essential fatty acids. For this reason, they are used in dietary supplements, pharmaceuticals and cosmetics (Das et al., 2011). Moreover, microalga has been exploited for the production of inorganic NPs as detailed in Table 1.

Table1. Compilation of some algae strains that synthesized NPs.

MiCroalga strain	Type of NPs	Size (nm)	Reference
Chlamydomonas reinhardtii.	Silver (Ag^+)	13-31	(Rahman et al., 2018)
Chlamydomonas reinhardtii.	Cadmium Sulphide (CdS)	3.06	(Rao & Pennathur, 2017)
Gracilaria Corticata	Gold (Au^+)	45-57	(Naveena & Prakash, 2013)
Spirulina platensis	Silver (Ag^+)	30-50	(Sharma et al., 2015)
Fucus vesiculosus	Gold (Au^+)	30-50	(Mata et al., 2009)
Turbinaria Conoides	Silver (Ag^+)	96	(Rajeshkumar et al., 2012)
Klebsormidium flaccidum	Gold (Au^+)	9.0	(Dahoumane et al., 2012)

Most of the nanoparticles showed in Table 1 possess antibacterial, antimicrobial and anticancer activities.

Chlamydomonas reinhardtii

As seen before in Table 1, there are many ways to synthesize various nanoparticles and nanomaterials, using a variety of algae. But there are a few cases where it is reported the production of NPs by using the microalga *C. reinhardtii* (Cr). *C.reinhardtii* is about 10 micrometers in size, its morphology is ovoid, and it also can grow under mixotrophic conditions (autotroph-heterotroph). Moreover, there are a number of applications of the microalga Cr, including lipids for biodiesel production, heavy metal bioremediation, as a bioreactor of recombinant proteins for medical use, and carotenoids for pigment production (Solís & Valencia, n.d.). Besides that, it is used as a model for genetic studies, it was also proved that this strain of green, freshwater microalga was characterized for its ability to produce nanomaterials (Dahoumane et al., 2017). Furthermore, there are available various strains of Cr with different physical and biological characteristics such as size, cell wall,

motility, etc. (Fan et al., 2017). There was reported the production of bimetallic alloy nanoparticles composed of gold and showed well-controlled compositions, by using living cultures of Cr.(Dahoumane et al., 2014). Another study reported the production of silver NPs by using both the cell-free extract and collected whole cells of *C. reinhardtii* (Barwal et al., 2011).

The novelty of using living microorganisms facilitates the production of nanostructures, recycling materials, reducing the wastes produced and the cost and incrementing the production by implementing a scalable process. There was not any study case that reported the synthesis of SeNPs by using the microalga *C. reinhardtii*. Also, it has been exposed that SeNPs possess several applications in nanomedicine and nanomaterials, for this reason, it is important to do more research about how to optimize the production of SeNPs.

CHAPTER II

MATERIALS & METHODS

Chlamydomonas reinhardtii

Whole culture of wild type of *C. reinhardtii* was purchased from the Chlamydomonas Resource Center, University of Minnesota, St. Paul, MN, USA. The green microalga used to run the experiments was *Chlamydomonas reinhardtii*, this specie is a model used in labs, with a total volume of 400 mL per flask. The microalga was cultured in a liquid medium containing Bold's Basal Medium (BBM) for ~15 days with a photoperiod of 16 h light and 8 h dark with at room temperature (RT ~23 °C). The BBM growth medium and equipment used were autoclaved, ensuring working in sterile conditions.

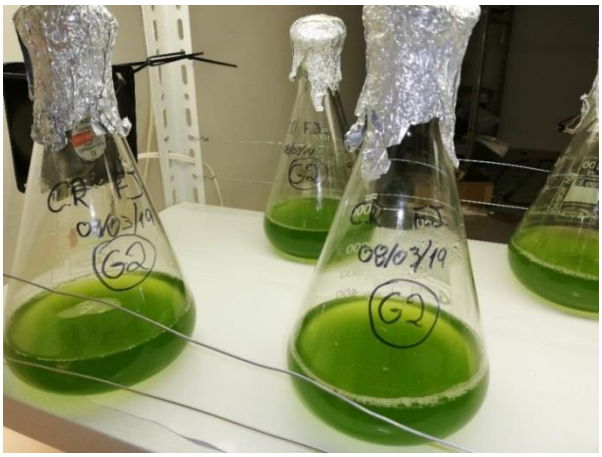


Figure 1. Image taken from the culture flask of *C. reinhardtii* in the laboratory of Yachay Tech University.

Composition and preparation of Bold's Basal Medium

Bold's Basal Medium (BBM) is an inorganic salt medium used for culturing free-living planktonic freshwater algae and is used at the Yachay Tech University for culturing *Chlamydomonas reinhardtii.*

Preparation of Stock solution

Each chemical used in the preparation of the stock solution (before prepare BBM), should be prepared separately to avoid precipitation of the constituents of some chemicals in the aqueous solution as indicated in Table 2.

Table 2. List of chemicals used for preparation of the tock solutions employed on BBM culture.

Stock Solution No.	Chemical Name	Formula	Weight (g)	Distilled Water (mL)
1	di-potassium hydrogen orthophosphate	K_2HPO_4	1.875	250
2	Potassium di-hydrogen orthophosphate	KH_2PO_4	4.375	250
3	Magnesium sulphate	$MgSO_4.7H_2O$	1.875	250
4	Sodium nitrate	$NaNO_3$	6.250	250
5	Calcium chloride	$CaCl_2.2H_2O$	0.625	250
6	Sodium chloride	NaCl	0.625	250
7	EDTA tetrasodium Potassium hydroxide	EDTA-Na_4 KOH	5.000 3.100	100
8	Ferrous sulphate Sulphuric acid concentration (wt per mL = 1.84g)	$FeSO_4.7H_2O$ H_2SO_4	0.498 0.1	100
9	Boric acid	H_3BO_3	1.142	100
10	Zinc sulphate	$ZnSo_4.7H_2O$	0.353	25
11	Manganese chloride	$MnCl_2.4H_2O$	0.058	25
12	Cupric sulphate	$CuSo_4.5H_2O$	0.063	25
13	Cobaltous nitrate	$Co(NO_3)_2.6H_2O$	0.020	25
14	Sodium molybdate	$Na_2MoO_4.2H_2O$	0.048	25

BBM preparation

To prepare 1 L of BBM, given volumes of stock solutions 1-14 should be added to deionized water (DIW). Once all chemicals are added, DIW is added up to complete 1 L volume of medium:

10mL of each stock solutions 1-6

1mL of each stock solution 7-9

0.1mL of each stock solutions 10-14

After the chemicals are added in this order, the flask is placed on an auto stirrer for ~30 min to ensure the mixing of the medium. After stirring, the pH measured should be in the range between 6.4 - 7.0. Finally, the BBM is autoclaved. (Bold, 1949)

Principle of autoclaving: Autoclave Sterilizers are principally used to decontaminate specific biological waste and to sterilize growth media, instruments and lab ware. It can sterilize solids, liquids, hollows, and instruments of various shapes and sizes. A very basic autoclave is similar to a pressure cooker; both use the power of steam to kill bacteria, spores and germs resistant to boiling water and powerful detergents (Panta et al., 2019).

Chemicals

To study the ability of *C. reinhardtii* resources to promote the production of selenium nanoparticles (SeNPs), 3 precursors were used: selenium tetrachloride ($SeCl_4$) 98% from SIGMA-ALDRICH Lot #H5473, selenous acid (H_2SeO_3) 98% from SIGMA-ALDRICH Lot #STBG8251 and sodium selenite 98% (Na_2SeO_3). They were stored at RT until use. Aqueous stock solutions of these precursors at 1 mM were prepared. The final concentration of these salts in reaction mixture was 1 mM; this was done by ten times dilution in a volume of 100 mL of deionized water. Therefore, the reaction mixture was made of 90 mL of *C. reinhardtii* and 10 mL of the stock solution of the given selenium precursor.

The materials used for the culture preparation and experiment design are listed below. For the experiment using the whole culture of *C. reinhardtti*, six 1-L flasks of 400 mL (to

grow and replicate the microalga) and thirty-two 250-mL flasks containing 100 mL of the reaction mixture (to develop the experiments), three pipettes of 10 mL and two test tubes of 50 mL.

Instruments

Characterization techniques

1. Digital pictures of cuvettes

To assess the evolution of macroscopic aspect of the reaction mixtures, photographs were taken on a regular basis, as a change in color is an indication the reaction had occurred. In fact, colloidal SeNPs are red in color. Moreover, Digital pictures were taken when the experiment starts, then D+3, D+5 after the experiment was launched. Also, there were taken photos of the controls in light and in dark conditions.

2. Ultraviolet-visible (UV-Vis) spectrophotometry

UV-Vis spectrophotometer principle is based on the Beer-Lambert Law. This law states that whenever a beam of light passed through a solution with an absorbing substance, there is a direct proportionality between the decreased rate of the radiation intensity along with the thickness of the absorbing solution, and the concentration of the solution and the incident radiation. There is established that the greater the number of the molecules that are capable of absorbing light at a specific wavelength, the greater the extent of the absorption of light.

This law is expressed as the equation:

$$A = \log(I_0/I) = ECI$$

Where A represents the absorbance, I_0 stands for the intensity of light upon a sample cell, it is referred to the departing of light intensity of the sample cell, C refers to the concentration of the solute, L stands for the length of the sample cell and E is referred to the molar absorptivity (Macheroux, 1999).

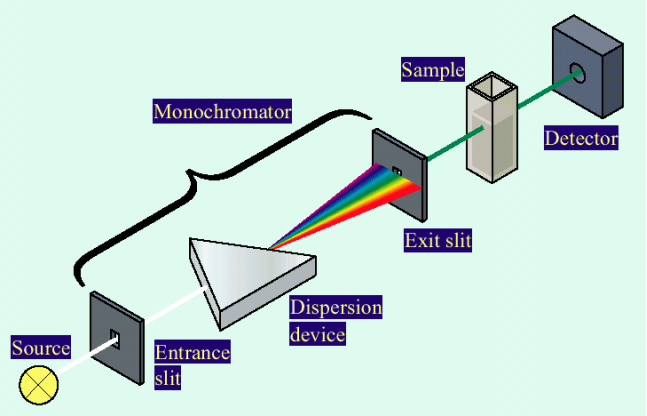


Figure 1 Scheme of operation of UV-Vis spectrophotometry. (Oliveira, A. & Araújo, M. 2011)

3. TEM images

Transmission electron microscopy (TEM) is a microscopy technique where a beam of electrons is transmitted through an ultra-thin specimen until form an image, interacting with the specimen as it passes through it. An image is formed from the interaction of the electrons with the sample as the beam is transmitted through the specimen. Its working principle is related to the nature of the electrons as they exhibit less wavelength when bombarded at high velocity(Bauer, 1988).

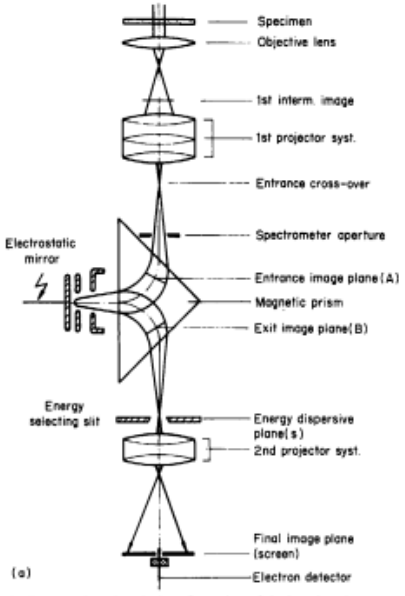


Figure 3. Beam path and optical configuration of the image electron energy loss spectrometer

4. X-ray diffraction

XRD working principle is based on constructive interference of monochromatic X-rays and a Crystalline sample: The X-rays were generated by a cathode ray tube, then they were filtered to produce monochromatic radiation, collimated to concentrate, and directed toward the sample. The interaction of the incident rays with the sample produces constructive interference (and a diffracted ray) when conditions satisfy Bragg's Law ($n\lambda=2d \sin \theta$). This law relates the wavelength of electromagnetic radiation to the diffraction angle and the lattice spacing in a Crystalline sample. The characteristic x-ray diffraction pattern generated in a general XRD analysis provides a unique "fingerprint" of the Crystals present in the sample. When properly interpreted, by comparison with standard reference patterns and measurements, this fingerprint allows identification of the Crystalline form (Bunaciu et al., 2015).

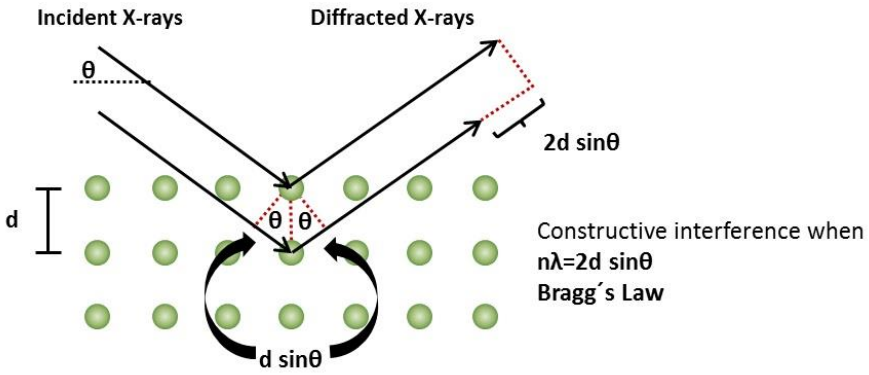


Figure 4. Schematic representation of the Bragg equation.

Experiments

Characterization techniques 1, 2 and 3 will be done at 0 h, D+2, D+3.

To characterize the samples obtained by the experiments, first, there were employed digital pictures of the Erlenmeyer of 100 mL to record the change in color done after the experiment. Then there was measured the pH of each solution using the Hanna 054504 pH meter, and record the values after 3 days. Also, UV-Vis spectroscopy was used to analyze the spectra of each experiment and to determine if there is the presence of selenium in the samples. The UV-Visible spectra of each solution were measured by PerkinElmer LAMBDA 1050+ UV/Vis/NIR spectrophotometers. XRD patterns were obtained on PANALYTICAL EMPYREAN with an X-ray generator operation at 45kV and 40 mA and a wavelength between 5-200 nm. Finally, TEM images were obtained on FEI-TECNAI G20 SPIRIT TWIN miCroscopes. The TEM sample was prepared by dropping a sample suspension in ethanol and on a Cu tip coated with a carbon film. The TEM sample was prepared by dropping a sample suspension in ethanol on a Copper tip, coated with a carbon film.

Hypothesis

Algae and microalga are capable of synthesizing a large variety of nanomaterials. It is proven that C. reinhardtii can promote silver and gold nanoparticles by enzymatic reduction. We hypothesize that the wild type strain of the *C. reinhardtii* can promote the synthesis of SeNPs (especially when H_2SeO_3 is used as a precursor), via a reduction process of Se precursors.

Outline for the Project

Procedure

1. CR is cultured for 15 days.

2. After 15 days, the Whole Living Cultures (WLC) from all flasks used is mixed in a sterile container to have a homogenous culture with the same cell density.

3. Cultures will be redistributed into the sterile 250-mL flask (in 90 mL volume).

4. Selenium precursor is added to the flasks in 10 times dilution to make up the final volume of each flask at 100 mL.

For $[SeCl_4]_f$ = 1 mM, $[SeCl_4]_0$ = 10 mM

For $[Na_2SeO_3]_f$ = 1 mM, $[Na_2SeO_3]_0$ = 10 mM

For $[H_2SeO_3]_f$ = 1 mM, $[H_2SeO_3]_0$ = 10 mM

5. Except for CD1, CD2 and CD3 (controls in dark), the flasks are kept under the same photoperiod stated above (16 h light / 8 h dark).

Table 3. Detailed design of experiment using whole living culture of CR

Flask name	**Composition of flask**	**V(BBM) mL**	**V(CR) mL**	**V($Se4^-$) mL**	**V(DIW)* mL**
E1-1	CR + 1 mM $SeCl_4$	0	90	10	0
E1-2	CR + 1 mM $SeCl_4$	0	90	10	0
E1-3	CR + 1 mM $SeCl_4$	0	90	10	0
E2-1	CR + 1 mM Na_2SeO_3	0	90	10	0
E2-2	CR + 1 mM Na_2SeO_3	0	90	10	0
E2-3	CR + 1 mM Na_2SeO_3	0	90	10	0
E3-1	CR + 1mM H_2SeO_3	0	90	10	0
E3-2	CR + 1mM H_2SeO_3	0	90	10	0
E3-3	CR + 1mM H_2SeO_3	0	90	10	0

C1-1	CR + DIW	0	90	0	10
C1-2	CR+ DIW	0	90	0	10
CD1	CR + 1 mM $SeCl_4$	0	90	10	0
CD2	CR + 1 mM Na_2SeO_3	0	90	10	0
CD3	CR + 1mM H_2SeO_3	0	90	10	0
C2	CR + 1mM $SeCl_4$	90	0	10	0
C3	CR + 1 mM Na_2SeO_3	90	0	10	0
C4	CR + 1 mM H_2SeO_3	90	0	10	0

Abbreviations: E: Experiment; C: Control; CD: Control in dark; CR: *Chlamydomonas reinhardti*i; DIW: distilled water

* each concentration

Procedure by employing the supernatant of *C. reinhardtii*

The procedure by which we obtain the microalga is exact as the procedure described before. In the case of using the supernatant of CR, the content of the flask of CR was centrifuged at 6000 rpm during 6 min, the pellet was discarded and separated from the supernatant and then organized as it is seen in Table 4, in this experiment there was used 80 mL of microalga's supernatant and 20 mL of the precursor at final concentration of 1 mM.

Table 4. Experiment design for supernatant of CR.

Flask name	Composition of flask	V(BBM) mL	V(SCR) mL	V(Se^{4+}) mL	V(DIW)* mL
Es1-1	SCR + 1 mM $SeCl_4$	0	80	20	0
Es1-2	SCR + 1 mM $SeCl_4$	0	80	20	0
Es1-3	SCR + 1 mM $SeCl_4$	0	80	20	0
Es2-1	SCR + 1 mM Na_2SeO_3	0	80	20	0
Es2-2	SCR + 1 mM Na_2SeO_3	0	80	20	0
Es2-3	SCR + 1 mM Na_2SeO_3	0	80	20	0
Es3-1	SCR + 1mM H_2SeO_3	0	80	20	0
Es3-2	SCR + 1mM H_2SeO_3	0	80	20	0
Es3-3	SCR + 1mM H_2SeO_3	0	80	20	0
Cs1-1	SCR + DIW	0	80	0	20
Cs1-2	SCR+ DIW	0	80	0	20
CDs1	SCR + 1 mM $SeCl_4$	0	80	20	0
CDs2	SCR + 1 mM Na_2SeO_3	0	80	20	0

CDs3	SCR + 1mM H_2SeO_3	0	80	20	0
Cs2	SCR + 1mM $SeCl_4$	80	0	20	0
Cs3	SCR + 1 mM Na_2SeO_3	80	0	20	0
Cs4	SCR + 1 mM H_2SeO_3	80	0	20	0

Abbreviations that are used later in the texts: SCR: Supernatant of *Chlamydomonas reinhardtii*; BBM: culture medium; V: volume; Es: experimental flask using the supernatant, Cs: control flask of supernatant; CDs: control in dark of supernatant; DIW: deionized water; D: day. The experiments were kept at room temperature and with a photoperiod of 16 h light and 8 h dark.

General Objective

The purpose of this research was to evaluate the feasibility of obtaining selenium nanoparticles by using the green microalga *C. reinhardtii* Wild Type (WT) using three different selenium salt precursors.

Specific objectives

1. Study the impact of the Se precursor on the ability of living cultures of Cr to promote the production of SeNPs.
2. Study the impact of the Se precursor on the ability of supernatant of Cr to promote the production of SeNPs.
3. In both cases, study the impact of light on SeNP production using Cr resources.
4. Characterize the produced SeNPs.

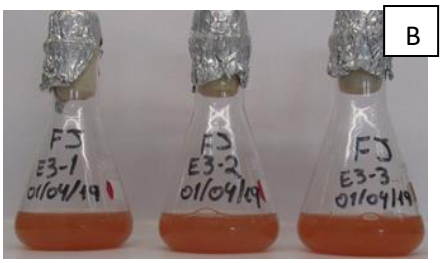
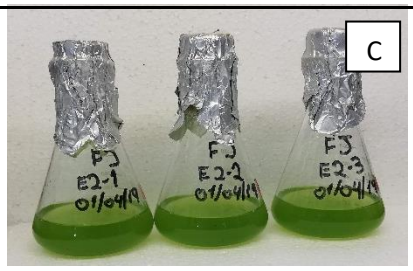
CHAPTER III

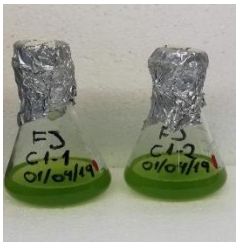
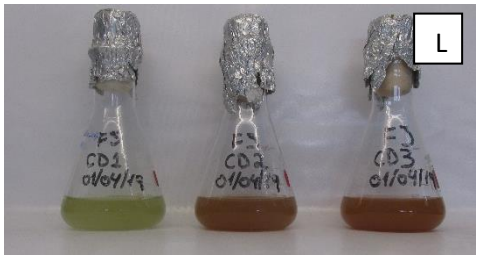
RESULTS

Macroscopic aspect

The macroscopic aspect of the cultures was analyzed by taking photos of the flask, recording the evolution of the synthesis, as listed below in Table 3.

Table 3 Evolution of the macroscopic aspect.

Content of the flasks	**Picture day 1 (01/04/2019)**	**Picture at day 5 (06/04/2019)**
CR + H_2SeO_3		
CR + Na_2SeO_3		
CR + $SeCl_4$		

CR+DIW	 G	 H
BBM + Precursors (H_2SeO_3, Na_2SeO_3 and $SeCl_4$)	 I	 J
CR+ Se precursors in dark conditions	 K	 L

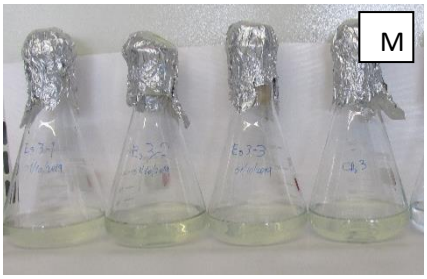
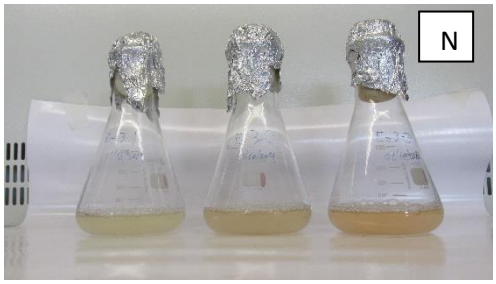
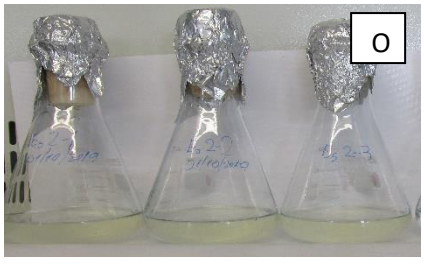
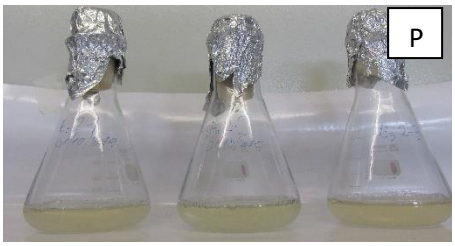
Under illumination, the most notable change in color comes from CR + H_2SeO_3, it changed gradually from vivid green to red-orange color within a few days and remained the same for months (Table 3 (A, B)). This changed color is a visual indication of SeNP formation. In the case of CR + Na_2SeO_3, the flask did not change the color and remained green. Demonstrating the absence of SeNP formation along with the viability of the cells. In the case of CR + $SeCl_4$, the cultures went whitish depicting both mass cell death and absence of SeNP formation.

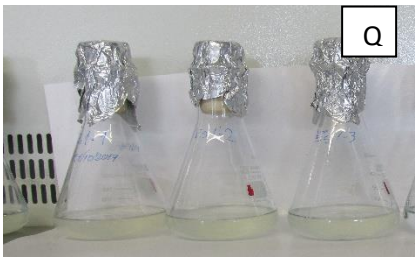
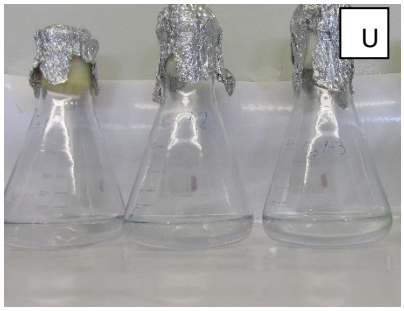
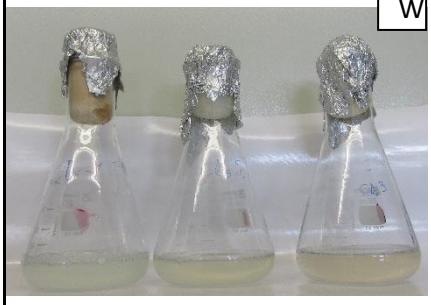
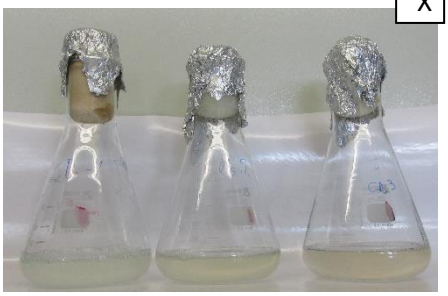
In dark conditions, the most remarkable color change comes from the flask that contained CR+H_2SeO_3, the solution changes from green to red and remained the same for months (Table 3 (E, F)). Finally, the flasks that contained CR+ Na_2SeO_3 changed the color from green to lighter orange-red and kept this color for months (Table 3 (C, D)). Unlike under

illumination where CR+H_2SeO_3 gave rise to SeNPs, both CR+ Na_2SeO_3 and CR+H_2SeO_3 promoted the production of SeNPs in dark. However, $SeCl_4$ did not promote the production of SeNPs in dark as it was under light. The flasks that contained BBM + salt precursors and CR+ DWI and that were illuminated did not produce any changes (Table.3 (G, H, I, J)). In other words, there is production of SeNPs if there are present three elements: light, H_2SeO_3 and Cr. In dark conditions, there is observed that Cr, H_2SeO_3 and Na_2SeO_3 can promote the production of SeNPs.

Furthermore, for the experiments using the supernatant, there was recorded photos to evaluate the macroscopic aspect and the evolution of the color, to know if there is the presence of SeNPs.

Table 4. Sum up of the pictures taken from the experiments using the supernatant of *C. reinhardtii.*

Content of the flasks	**Picture day 1 (01/10/2019)**	**Picture at day 3 (14/10/2019)**
SCR + H_2SeO_3		
SCR + Na_2SeO_3		

SCR + $SeCl_4$	 Q	 R
SCR+DIW	 S	 T
BBM + Precursors (H_2SeO_3, Na_2SeO_3 and $SeCl_4$)	 U	 V
SCR+ Se precursors	 W	 X

In the case of CRs + H_2SeO_3, there is a slight change in the color (Table 4 (M, N)). It suggested that there was partial production of SeNPs. For the flasks that contained CR+ Na_2SeO_3, the result was the same as before, the slight color change was visible. In this case there was suggested the partial production of SeNPs (Table 4 (O, P)). In the case of CR+ $SeCl_4$, there is any change and the color remains whitish. This result suggested that the

cultures died (Table 4 (Q, R)). The experiments that carried the use of supernatant demonstrated that there is no possibility to produce SeNPs.

UV-Vis spectroscopy

The spectra of each solution were recorded in the range between 400 to 800 nm to visualize the presence/absence of the spectra band of selenium NPs. UV–vis spectroscopy determines the "absorption maxima" of nanoparticles, depending on the concentration of the precursor and other components of reaction mixtures.

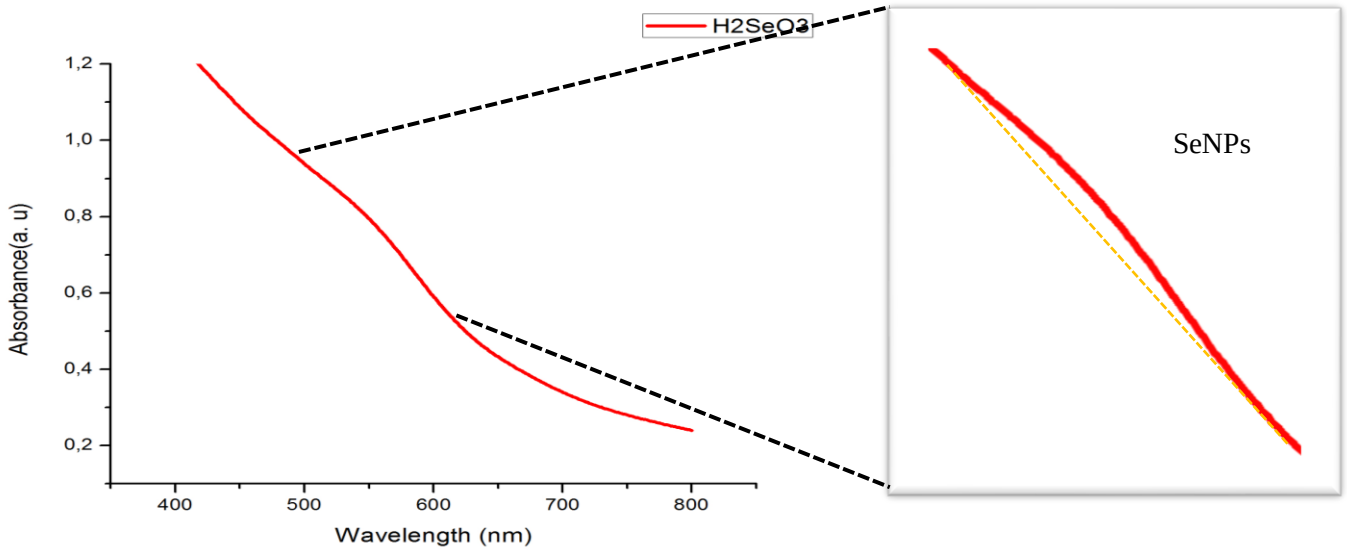


Figure 5. UV-Vis spectra of CR+ H_2SeO_3.

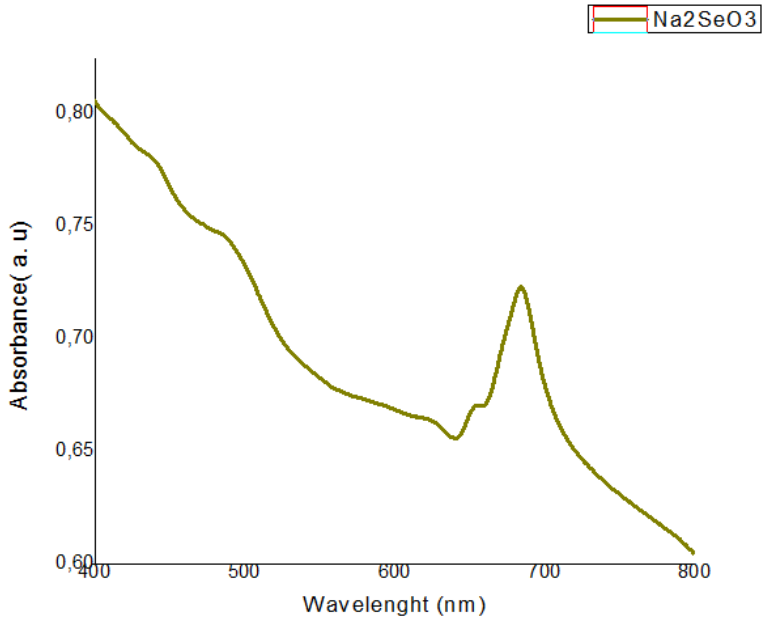


Figure 6. UV-Visible spectra of CR+ Na_2SeO_3 presents two peaks: the first between 400 to 500 nm and the second from 650 to 700 nm.

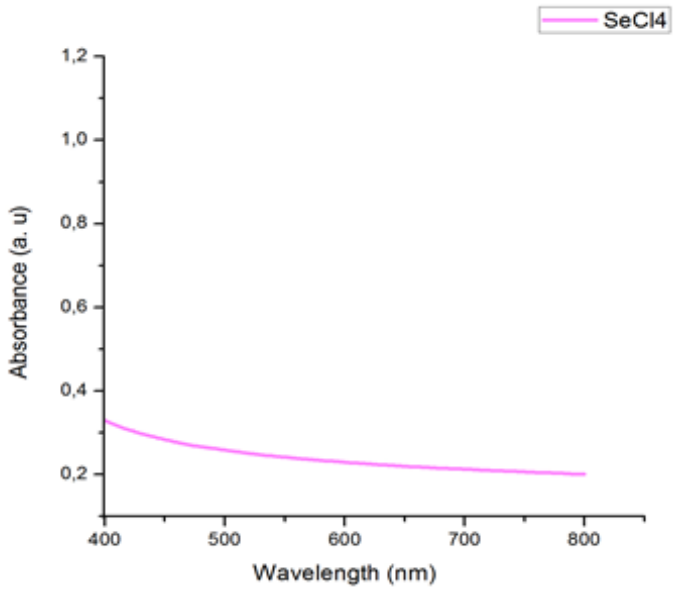


Figure 7. UV-Vis spectra of CR+$SeCl_4$ (selenium tetrachloride $[10]^{-3}$M with *C. reinhardtii).* There are no peaks seen in the spectra record.

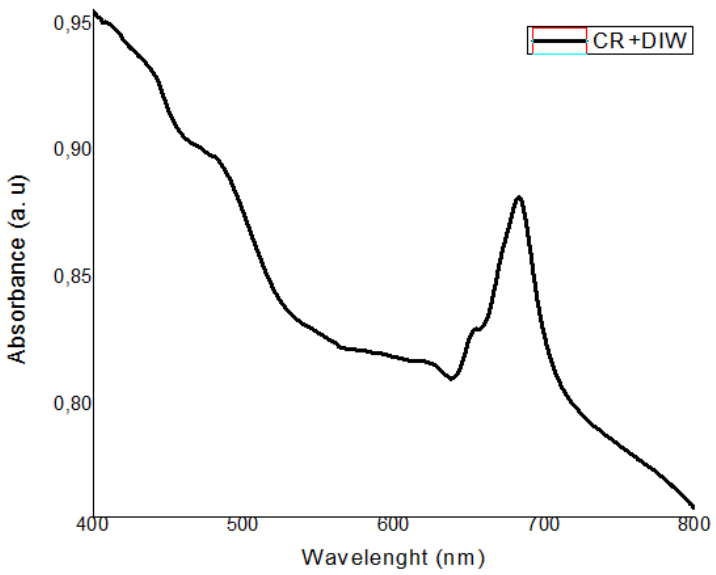


Figure 8. UV-Vis spectra of CR+DIW

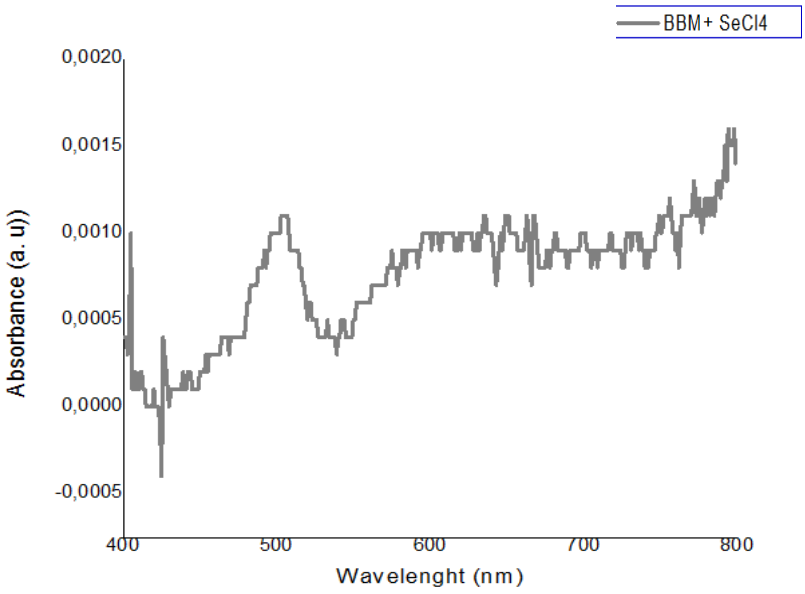


Figure 9. UV-Vis spectra of BBM+$SeCl_4$.

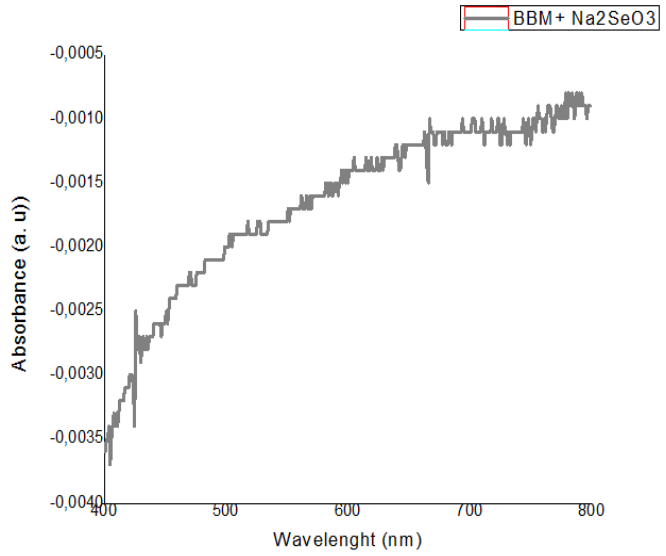


Figure 10. UV-Vis spectra of BBM+ Na_2SeO_3

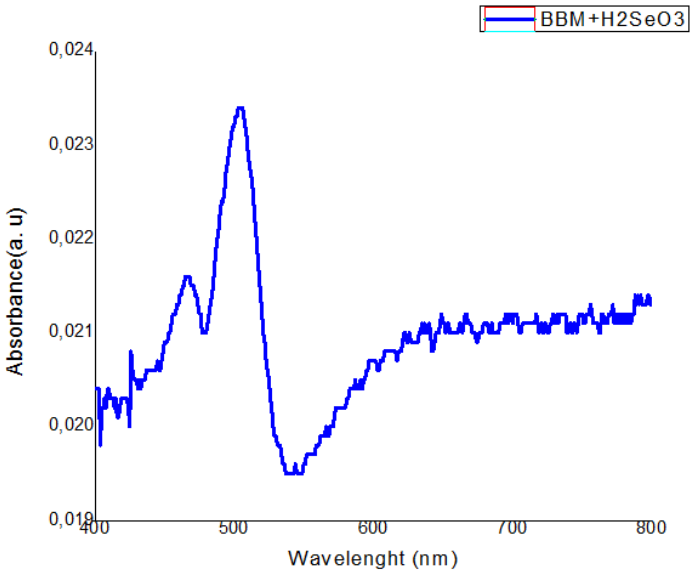


Figure 11. UV-Vis spectra of BBM+ H2SeO3

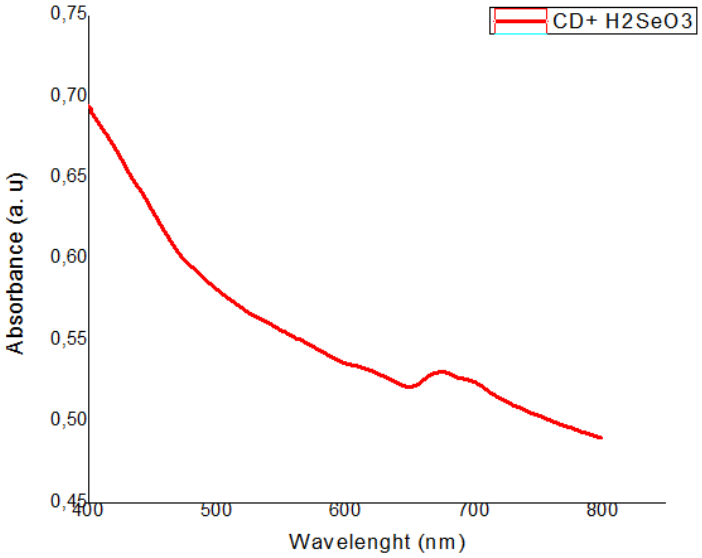


Figure 12. UV-Vis spectra of CR+H2SeO3 in dark conditions

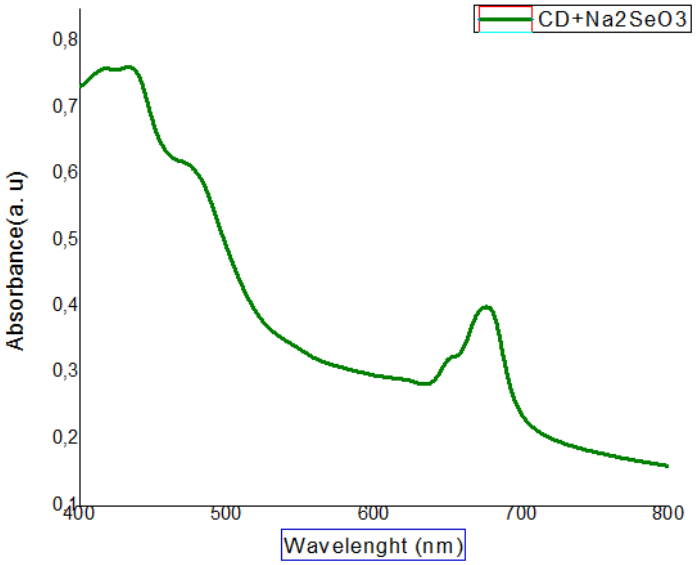


Figure 13. UV-Vis spectra of CR+Na_2SeO_3 in dark conditions

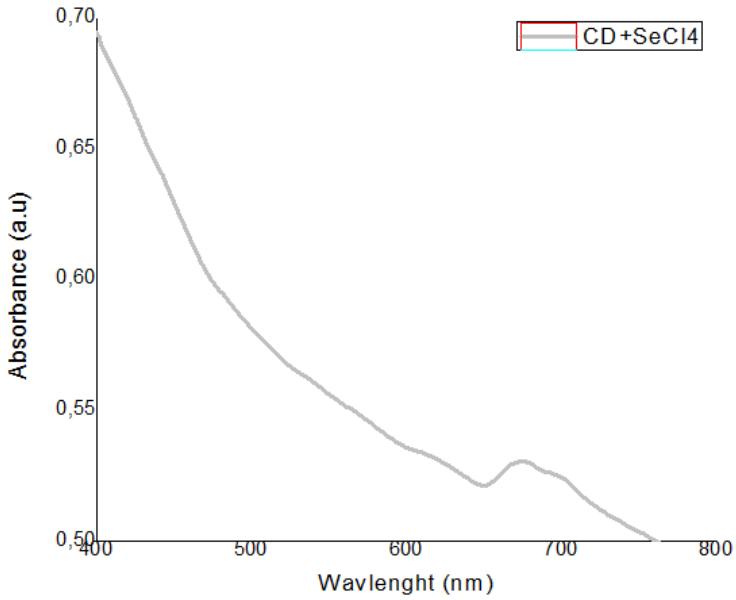


Figure 14. UV-Vis spectra of CR+SeCl4 in dark conditions

The formation of selenium nanoparticles was mainly authenticated from UV-Vis spectrophotometry from CR+H_2SeO_3 (Fig 5.), in this case, there is present a "shoulder" in the range between 500 to 600 nm, suggesting the presence of SeNPs. In the case of UV-Vis absorption of sodium selenite (Fig 6.), there is present a peak corresponding to photopigments. For the experiment using CR+ $SeCl_4$ (Fig 7), there was no present peaks, suggesting that the cells died. Also, there were recorded the UV-Vis spectra of the controls such as CR+ DWI (Fig 8) and BBM + Se precursors (Fig 9, 10 and 11), the spectra showed any representative peak and suggests that nothing happen. control in the dark that contained CR + Se precursors (Fig 12, 13 and 14), did not show peaks related to SeNPs.

Moreover, there was recorded the UV-Vis spectra of the experiments using the supernantant. The absorbance was measured in the range between 400 to 800nm.

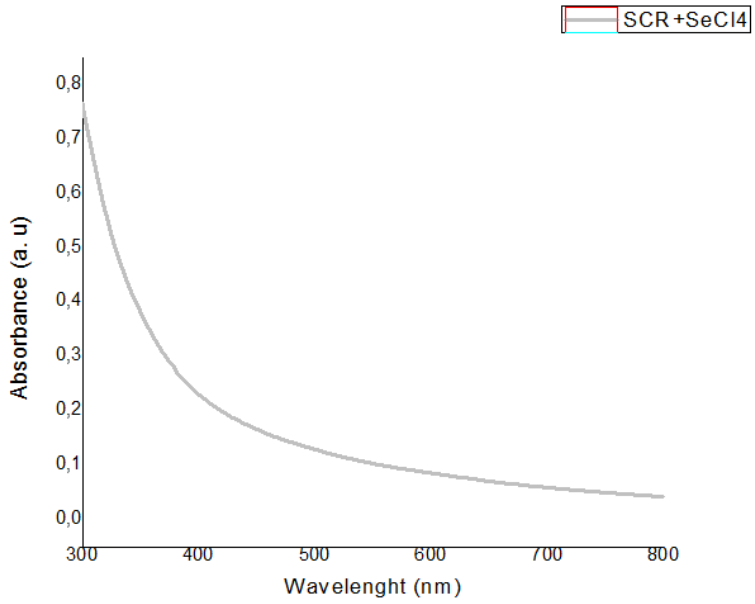


Figure 15. UV-Vis spectra of the experiment SCR+$Secl_4$

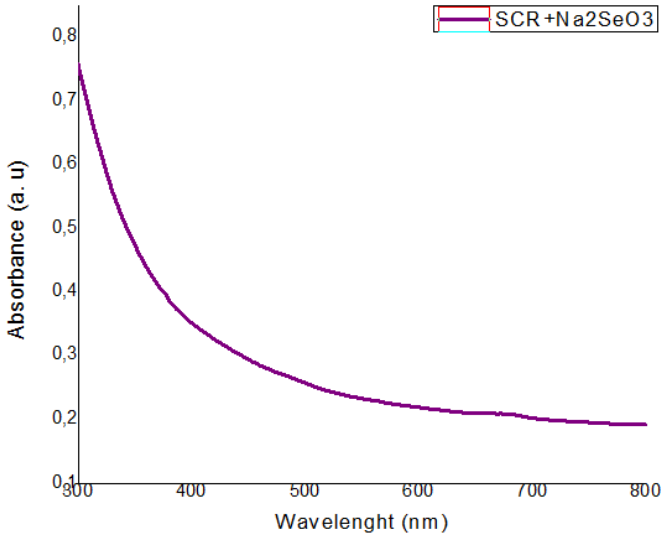


Figure 16. UV-Vis spectra of the experiment SCR+Na2SeO3

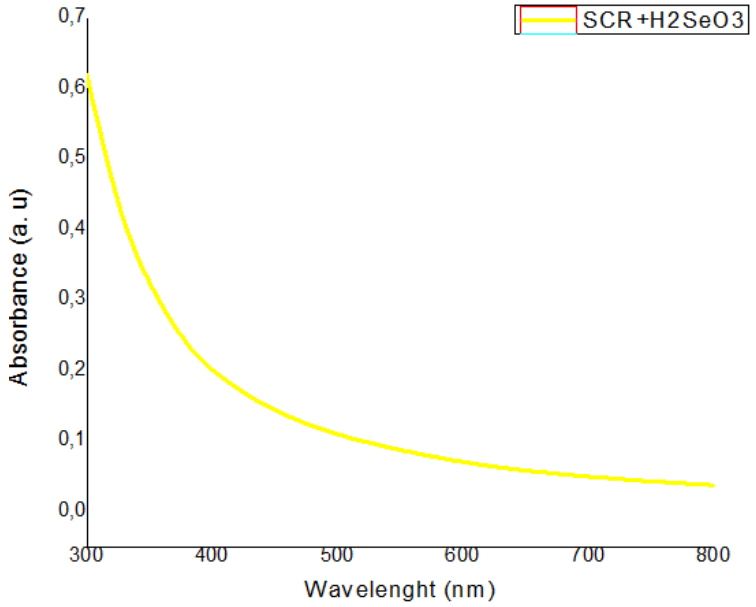


Figure 17 . UV-Vis spectra of the experiment with supernatant SCR+H_2SeO_3.

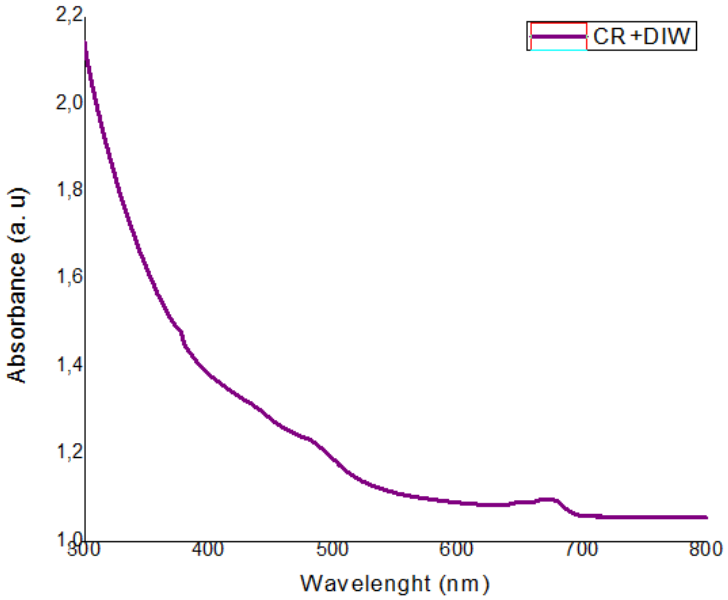


Figure 18. . UV-Vis spectra of the control using the supernatant SCR+ DWI.

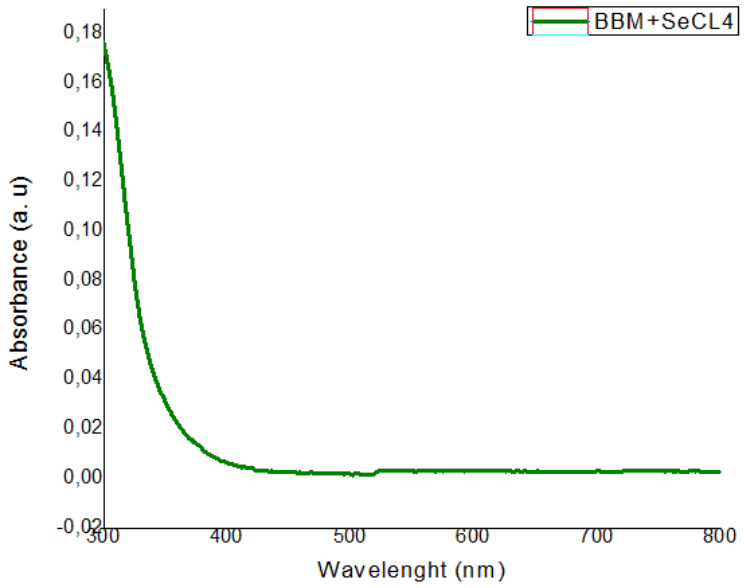


Figure 19. UV-Vis spectra of BBM+$SeCl_4$

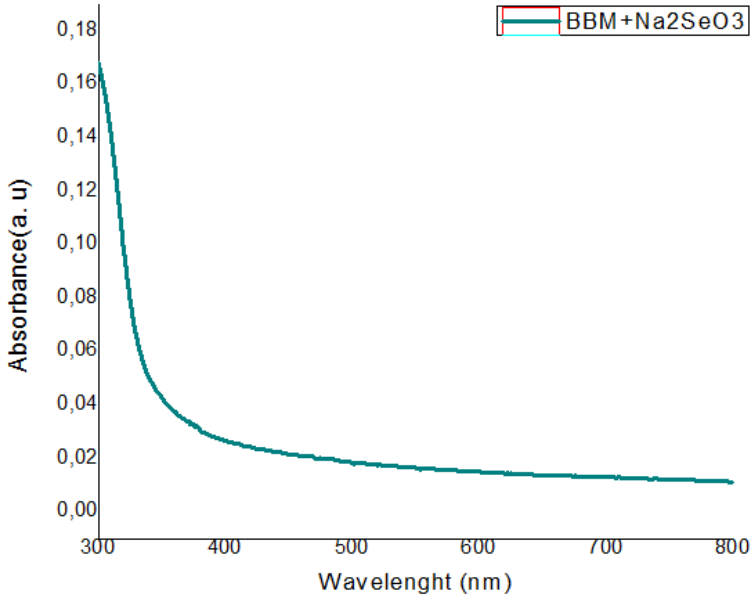


Figure 20. UV-Vis spectra of BBM+Na_2SeO_3.

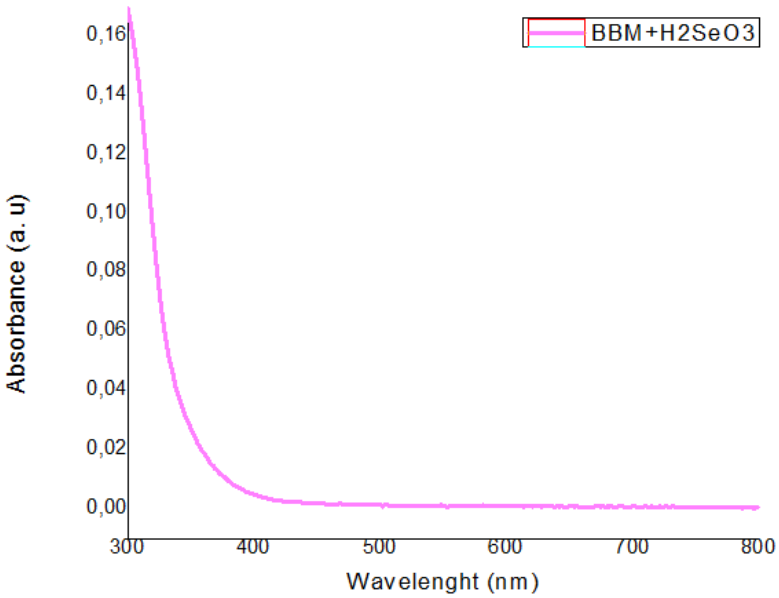


Figure 21. UV-Vis spepcctra of the cotrol BBM+ H_2SeO_3

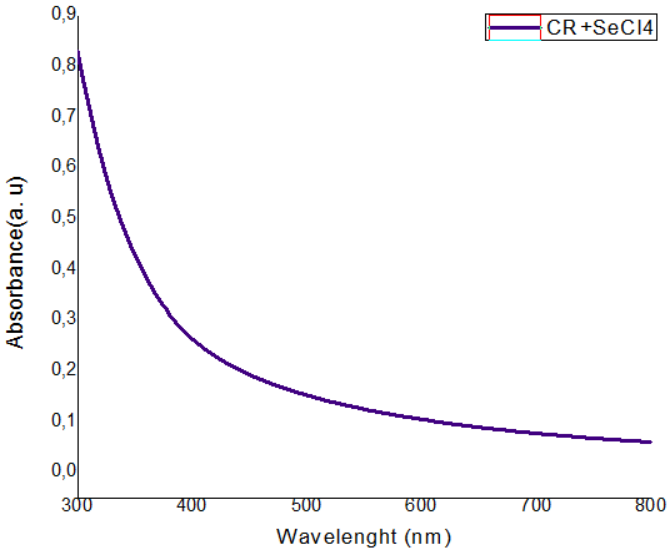


Figure 22. . UV-Vis spectra of the control in dark of $CR+SeCl_4$

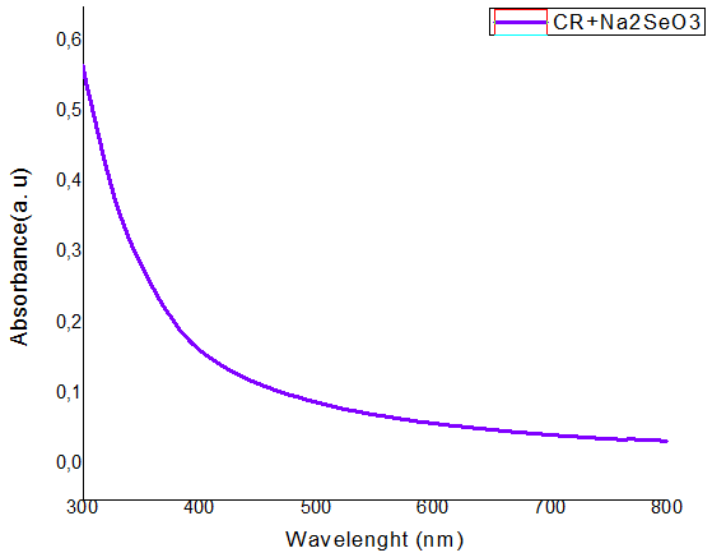


Figure 23. UV-Vis spectra of the control in dark of CR+ Na_2SeO_3

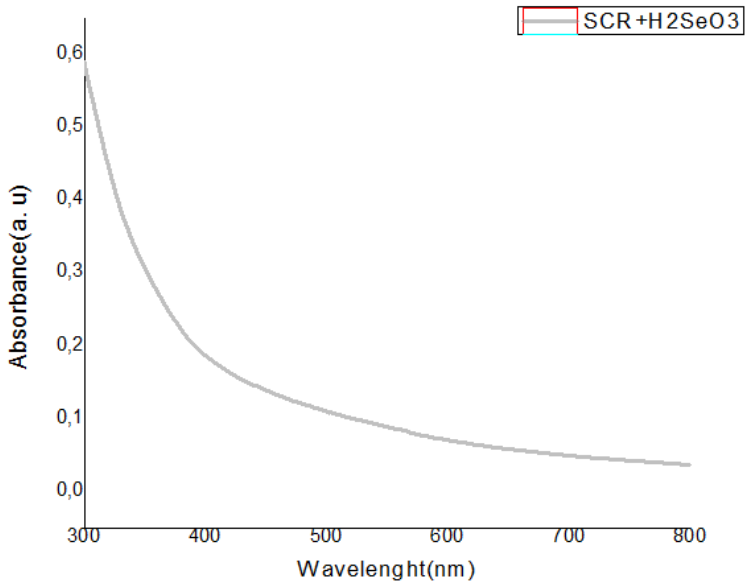


Figure 24. UV-Vis spectra of the control in dark of CR+H_2SeO_3

There is seen the absorbance of SCR+ $SeCl_4$ (Figure 15). It is not shown any peak, suggsting that the cells were died. In the case of SCR+ Na_2SeO_3 (Figure 16), there was depicted the same result as mentioned before, there is no present any peak, it means that there is no Se presence in the sample. For the flask that contained SCR+ H_2SeO_3 (Figure 17), there is no presence of peaks corresponding to the absorbance of SeNPs. The UV-Vis

spectra of the controls: SCR+DWI (Figure18), did not shown any peak related to SeNPs, only to photopignments.For the cultures that contained BBM+ Se precursors(Figure (19-21)), there is no peaks related to SeNPs nor photopigments. Finally there is the spectra of the controls in dark (Figures 22-24). In this case, there is not observed any representative peak, suggesting that there are not production of SeNPs.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a technique, which can provide valuable information about particle size, surface morphology, etc. Therefore, the structure and morphology of the synthesized selenium nanoparticles were also determined by the TEM technique. TEM images were obtained for culture flasks in dark control and culture flask in light.

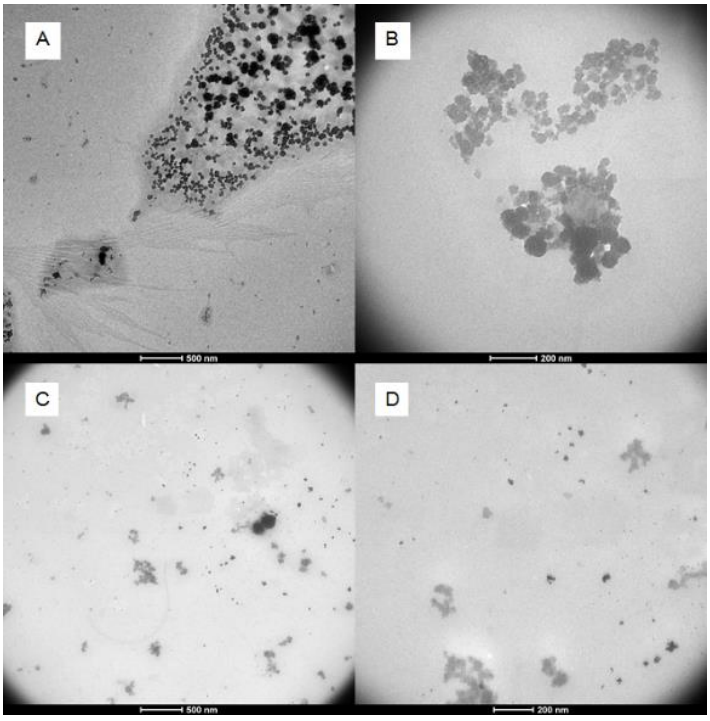


Figure 25. A and B are the TEM images obtained from the flask that contains C. reinhardtii cultured with CR+Na_2 SeO_3 [10]-3M without light. A) TEM of the cells in dark observed at 500 nm scale. B) TEM of the cells in dark observed at 200 nm scale .C and D are the TEM images of the cells cultured with CR+ Na_2SeO_3 1 mM in light conditions. C) TEM of the cells in light observed at a 500 nm scale. D) TEM of the cells in light observed at a 200 nm scale.

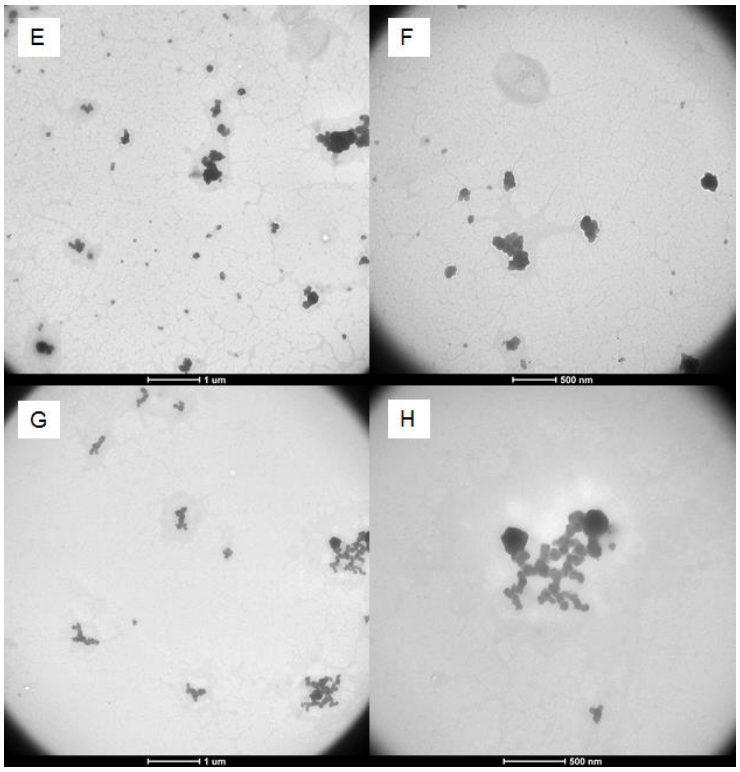


Figure 26. E and F are the TEM images obtained from the flask that contains CR+ H_2SeO_3 1mM in dark conditions. E) TEM of cells in dark at 1 um scale. F) TEM of the cells in dark at 500 nm. G and H are the TEM images obtained from the flask that contains CR+ H_2SeO_3 1mM, cultured in light conditions. G) TEM image of cells in light conditions at a 1um scale. H) TEM image of the cells in the light condition at a 500 nm scale.

The samples that contained CR+ Na_2SeO_3 (Figure 25), showed the presence of aggregated SeNPs, in dark conditions (Figure 25(A, B)); and dispersed SeNPs, in light conditions (Figure 25 (C, D)). On the other hand, the samples that contained CR+ H_2SeO_3 (Figure 26), showed the presence of SeNPs in dark conditions (Figure 26(E, F)). In the case of CR+ H_2SeO_3 under light conditions (Figure 26(G, H)), there is seen the presence of SeNPs. The software used to measure the average diameter of the nanoparticles was Image J. The average diameter calculated, using selenous acid at 10mM, was 66,14 nm.

X-ray diffraction (XRD)

XRD technique is used to analyze the composition structure of the substance (Crystallinity/amorphous) and phase of selenium nanoparticles.

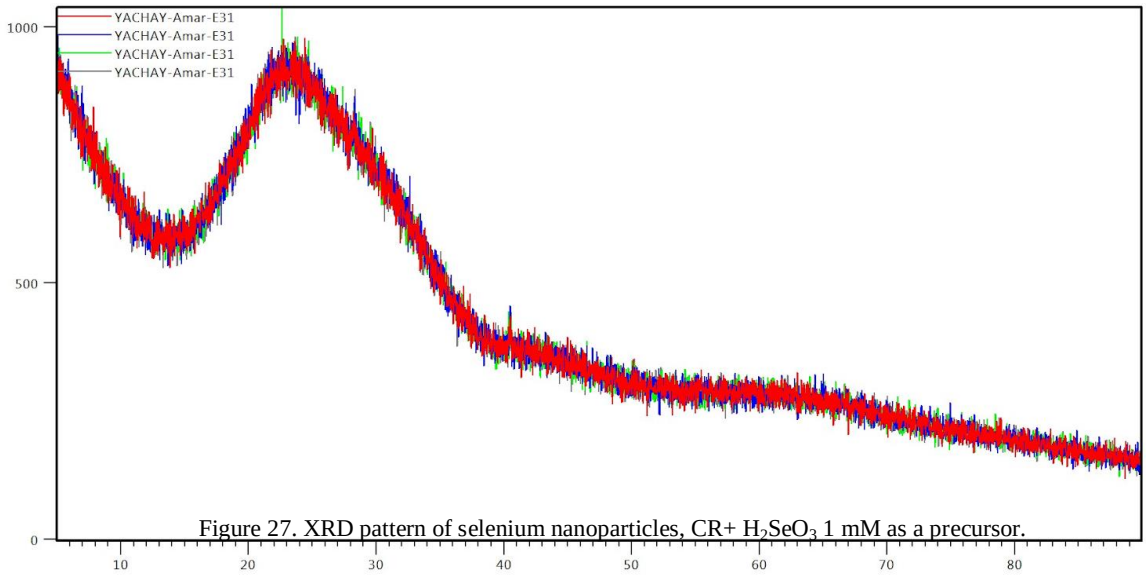


Figure 27. XRD pattern of selenium nanoparticles, CR+ H_2SeO_3 1 mM as a precursor.

The sample that contained CR+H_2SeO_3, was analyzed to determine the crystallinity or amorphous, resulting in the absence of a representative peak related to the crystalline selenium. This suggest that the SeNPs obtained were amorphous in shape

CHAPTER IV

DISCUSSION

Pictures from cultures

The results from the experiments were easily determined based on the color change from green to red-orange (Table 3 (A, B)). The color change of the experiment CR+H_2SeO_3, evidenced the reduction of selenite to elemental selenium, it was corroborated by the literature that reported the red color due to the presence of selenium nanoparticles (Fernández-Llamosas et al., 2016). On the other hand, the samples that used $SeCl_4$ precursor changed from green to white, depicting the death of the cells and the absence of any SeNP formation (Table 3 (E, F)). This contrasts with what was reported via a chemical synthesis in which this salt could produce SeNPs at a final concentration of 5 mM (Gangadoo et al., 2017). To the best of my knowledge, no biological methods explore the use of this salt to produce SeNPs. Finally, the flasks that contained CR+Na_2SeO_3 do not change the color over time while the cells remain viable, suggesting that the concentration of this salt could be increased to check whether this has an impact on SeNP production. Via a chemical method, it is reported a case in which the final concentration reached 75 mM (Kalishwaralal et al., 2015). It would be interesting to study why that microalga does not catalyze the synthesis of SeNP starting from sodium selenate while keeping its viability (Table 3 (C, D)). Is it the impact of the pH or of Se precursor concentration? The pictures taken from the controls did not show any SeNP formation proving therefore that the process of SeNP production was carried out by the living cultures of the microalga (Table 3(G, H, I, J, K, L)). Unlike the light-driven production of AgNPs using Cr (Rahman et al., 2018), using dark controls of Cr + H_2SeO_3 and Na_2SeO_3 produced partially SeNPs. This showed that the production of SeNPs using Cr is not a complete light-dependent process.

Furthermore, the experiments done with the supernatant plus Se precursors did not show any important change in color demonstrating once again the need of cells to produce SeNPs starting from the precursor. Compared with the AgNPs reported by Rahman et al, who showed the production of stable NPs by using the supernatant of Cr. For instance, the

experiment using SCR+H_2SeO_3, (Table 3(M, N)) does not change the color over the pass of time. In the case of SCR+ Na_2SeO_3, the result is the same as it is seen in (Table 3(O, P)), it does not change over time. This showed that there is no production of SeNPs. For the experiment of SCR+ $SeCl_4$, the result is a whitish color that does not change the color over time (Table 3 (Q, R)). Also, there were recorded the images of the control flasks using DWI (Table 4 (S, T)), where any color change was visible. There was taken photos of BBM + Se precursors (Table 4 (U, V)) and control in dark conditions (Table 4 (W, X)), in both cases nothing happens and there is not visible any change in color. In this case, the experiment using the supernatant of Cr showed that there is no possible the production of SeNPs.

UV-Vis spectroscopy

To corroborate the results observed in the flasks, there was measured the UV-Vis absorbance of the three precursors and the controls in the range comprised between 400 to 800 nm. There are few reports from biological routes that observe elemental selenium absorbance at a specific wavelength, but these are extensively varied and not corroboratory. For example, selenium nanoparticles obtained from *K. pneumoniae* present two peaks at 218 and 248 nm (Fesharaki et al., 2010). Furthermore, the synthesis of SeNPs using lemon leaf extract presented a maximum peak absorption at 395 nm (Prasad et al., 2013).There is a case reported the production of SeNPs obtained by using the microbe *Bacillus cereus*absorption, and possess a maximum peak at 590 nm (Dhanjal & Cameotra, 2010). In this case, the SeNPs synthesized using *C. reinhardtii* with H_2SeO_3, shown an increase in the range between 500-600 nm, and there is visible a "shoulder" in the range mentioned (Figure 5). The UV-Vis spectrum of the culture using CR+H_2SeO_3 is in accordance with the literature and it is assumed that it has a red tone because the particles were bellowed the 100 nm in size (Wadhwani et al., 2017). The UV-Vis spectra corresponding to the cultures that used CR+ Na_2SeO_3 (Figure 6) present two peaks that correspond to chlorophyll A (400-450 nm) and photo-pigments (650-700 nm) presents in the microalga (Milne et al., 2015). Moreover, the UV-Vis spectra corresponding to the cultures that contain CR+ $SeCl_4$ does not present any representative maximum peak (Figure 7), suggesting that the cells were dead, because there is no present any spectra band corresponding to photosynthetic

pigments of the microalga nor for SeNPs. The spectra band of the precursors used was compared with the controls spectra of each one, also in dark conditions (Figures 8-14).

There measured UV-Vis Spectra from the experiments that used supernatant showed that the experiments fail for the synthesis of SeNPs. In the case of SCR+$SeCl_4$ (Figure 15) there is no present any peak neither photosynthetic pigments nor SeNPs. It could be because the cells were death or the concentration of the precursor was not the correct. Moreover, the experiment of SCR+ Na_2SeO_3 (Figure 16) shows that there is no present any peak related to the presence of selenium absorption, and it is similar to the experiment of SCR+H_2SeO_3 (Figure 17). In both cases there is no present any peak related to SeNPs. These results showed the necessity to use cells to obtain SeNPs. The controls of the experiments that used supernatant, helped us to evaluate any change in color of the flasks (Figures 18-24), but there is no present any change in the color, suggesting that the production of NPs was not accomplished.

Transmission Electron Microscopy (TEM)

TEM images of the sodium selenite in dark and in light were shown in (Figure 25 (A-D)) and showed what happens with the partial production of nanoparticles. There is seen the agglomeration microscopic particles inside the microalga's cell, in dark conditions, suggesting the partial production of SeNPs (Figure 25 (A, B)). TEM images of CR+ Na_2SeO_3 in light conditions showed monodispersed and spherical nanoparticles, assuming that those are from selenium partial reduction by *C. reinhardtii* (Figure 25 (C, D)). Wadhwani et al. (2017) reported the synthesis of SeNPs with an average diameter of 78 nm, using sodium selenate at 1.5 mM. This information, compared with the results obtained in the laboratory, suggests that the concentration of sodium selenate was higher, obtaining aggregated SeNPs

The structure and morphology of SeNPs, produced by CR+H_2SeO_3 at 1 mM, were confirmed by TEM images and show that the NPs were regular in shape (Figure 26 (E, F)). Also, the calculated average diameter of SeNPs was 66.14 nm. This nanoparticle diameter is in good agreement with what was reported in the literature. There are many cases reported, that associate the diameter of the NPs to the concentration of the salts. For

example, Gangadoo et al., (2017) stated that high concentration of reducing agents compared to metal salt, produces smaller sizes of SeNPs (<70 nm). Alagesan & Venugopal (2019) reported a diameter size of SeNPs in the range from 45 to 90 nm, using the extract of *W. somnifera* and selenious acid 5mM. The nanoparticle size obtained in this experiment is in agreement with the reported literature.

X-ray Diffraction

The XRD pattern of SeNPs, employing CR+H_2SeO_3 at 1 mM, confirmed the amorphous nature of the NPs (Figure 27), by not giving any sharp peak, as only spherical nanospheres were present in the sample and were amorphous in nature. In contrast, Sarkar et al., (2015) reported Crystalline SeNPs that exhibit characteristic peaks of "2Θ" values at 23, 30 and 43. This information corroborates the amorphous nature of synthesized SeNPs using selenous acid at 1 mM as a precursor.

Rahman et al described the production of silver nanoparticles (AgNPs) using the microalga *C. reinhardtii.* They used the whole living cultures, washed cells and supernatant of the microalga to obtain highly stable AgNPs (Rahman et al., 2018). In comparison with the results obtained in the laboratory of Yachay Tech, they could not produce NPs in dark conditions like it was done by the use of Se precursors (CR+ H_2SeO_3 and CR+ Na_2SeO_3), but in our case, we cannot produce NPs with supernatant.

Nanoparticle	Algae specie	Size (nm)	Working condition
Selenium	*Chlamydomonas reinhardtti*	66	Light

CHAPTER V

CONCLUSIONS

A novel, faster and eco-friendly method has been presented to synthesize amorphous selenium nanoparticles in the presence of *C. reinhardtii*, with an average diameter size of 66 nm: 3 precursors were used ($SeCl_4$, Na_2SeO_3, and H_2SeO_3) at the same concentration (1 mM). From which, H_2SeO_3 demonstrated to be the precursor which exhibited positive results for the production of SeNPs. To corroborate the presence of SeNPs different characterization techniques were used such as UV-Vis spectroscopy, TEM, and XRD that determined the spectra band, the morphology, and Crystallinity, respectively.

UV-Visible spectroscopy showed a graphic that is in accordance with the literature reviewed. The UV-Vis spectroscopy of CR+H_2SeO_3 revealed the presence of an absorption band due to SeNPs between 500-600 nm. The UV-Vis spectroscopy of CR+$SeCl_4$ did not present any absorption peak, suggesting that the cell culture died, presumably by the concentration of the salt used. The UV-Vis spectroscopy for Na_2SeO_3 samples, revealed the presence of photo pigments and chlorophyll A, but did not reveal any peak related to the presence of SeNps.

TEM images determined the shape and size of the synthesized SeNPs. By using software to analyze the TEM images, it was possible to calculate the average diameter of the nanoparticles, resulting near the 66 nm for CR+H_2SeO_3 sample. Also, TEM images of H_2SeO_3 were taken in dark and in light conditions to show the development of SeNPs. It is concluded that without light there is a partial production of nanoparticles. Furthermore, there was taken TEM images from Na_2SeO_3 in dark and in light conditions. In the case of the dark conditions, there was observed that the microalga cells were grouped and possess inside their systems nanoparticles, presumably of selenium. On the other hand, TEM images of Na_2SeO_3 in light conditions showed aggregated nanoparticles of Se with various shapes and sizes, explaining why the UV-Vis did not display any peak linked to these unstable NPs. There was not used any stabilizer for the nanoparticles, but it is necessary for

future investigation related to the study of the nanoparticles optimization. Also, it is recommended to take TEM images after few days of the first image taken, to evaluate the evolution of the particle size.

X-ray diffraction was only carried out for the sample that contained H_2SeO_3 to study the Crystallinity of the as produced SeNPs. It was demonstrated that the nanoparticles obtained did not present any sharp peak related to crystalline selenium, suggesting that they were amorphous in nature. Finally, it was concluded that the selenium salt concentration affects directly the size and shape of the nanoparticle, meanwhile the pH affects the correct metabolism of the microalga, it means the production or not of SeNPs, and the biosynthesis of SeNPs using *C. reinhardtii* is mainly a light-dependent process.

There are many aspects related to SeNPs production, that would need to be studied and developed. It is necessary to prove the synthesis production with diverse species of microalga, to choose the most optimum. Also, the light intensity plays a fundamental role in the microalga growth; for this reason, it is necessary to evaluate the optimum conditions of light intensity. Moreover, the biomass of the microalga, cell density, and kinetics need to be studied to optimize this process.

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