

TOXICITY DETERMINATION OF ENGINEERED NANOPARTICLES ON MICROALGAE

Submitted in partial fulfillment of the requirements for the award of
Bachelor of Science degree in Biotechnology.

By

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**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO & CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

**Accredited with Grade 'A' by NAAC
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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the Bonafide work of **Susmita. A (40770053)**, who carried out the project entitled "**Toxicity Determination of Engineered Nanoparticle on Microalgae**" under our supervision from **September-2022 to March-2023**.

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DECLARATION

I, **Susmita. A**, hereby declare that the Project Report entitled “**Toxicity Determination of engineered nanoparticles on Microalgae**” done by me under the guidance of **Dr. V. Ramesh Kumar** (Internal) and **Dr. T. Stalin Dhas** (External) at **Sathyabama Institute of Science and Technology, Chennai, Tamilnadu, India**, is submitted in partial fulfillment of the requirements for the award of **Bachelor of Technology degree in Biotechnology**.

A rectangular box containing a handwritten signature in blue ink that reads "Susmita".

12/05/2023
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SIGNATURE OF THE CANDIDATE

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ABSTRACT:

The purpose of the study is to determine the toxicity of CS-Se NPs on cultivated microalgae, which are smaller species in the marine ecosystem, and to investigate how they affect *Nannochloropsis sp.* Nanoparticles are released into the environment, which pollutes it and has an impact on them. We chose to use CS-Se NPs because they are a crucial part of human nutrition and have anti-oxidative and chemoprotective properties that boost the immune system, increase male fertility, and slow the ageing process. In this study, sodium selenite, chitosan, and ascorbic acid were used to synthesize selenium, which was then characterized using spectroscopic and microscopic methods. A characteristic peak in UV-vis spectroscopy at 260 nm indicates the selenium(Se) absorbance range and may be used in conjunction with other techniques to determine the stability, functional group, and shape of selenium. By exposing CS-Se NPs to microalgae, we were able to measure the cytotoxicity, which was 75.189 % at 1000 g/mL using the MTT test, the lipid peroxidation, which was 88.86 % at 1000 g/mL using the modified TBARS assay, and the ROS estimation, which was 22.030 % done using the DCFH-DA assay. These findings clearly establish the microalgae (*Nannochloropsis sp.*) as being sensitive to selenium nanoparticle toxicity.

Keywords:

Marine microalgae, Nanoparticle, selenium toxicity, cytotoxicity.

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Abbreviation

- | | |
|--|---|
| 1. CS-Se NPs | - Chitosan - Selenium Nanoparticles |
| 2. DCFH-DA | - 2',7'-Dichlorofluorescein Diacetate |
| 3. DLS | - Dynamic Light Scattering particle size analyzer |
| 4. FTIR | - Fourier-Transform Infrared spectroscopy |
| 5. MDA | - Malondialdehyde |
| 6. MTT | - (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). |
| 7. Na ₂ Sia ₃ .9H ₂ O | - Sodium Metasilicate Nonahydrate |
| 8. NaH ₂ PO ₄ 2H ₂ O | - Sodium Phosphate |
| 9. NaNO ₃ ⁻ | - Sodium Nitrate |
| 10. NPs | - Nanoparticles |
| 11. PBS | - Phosphate buffered saline |
| 12. ROS estimation | - Reactive Oxygen Species estimation |
| 13. TBA | - Tertiary butyl alcohol |
| 14. TBARS Assay | - Thiobarbituric acid Reactive Substance Assay |
| 15. TEM | - Transmission Electron Microscopy |
| 16. TMS | - Trace Metal Solution |
| 17. UV - vis spectroscopy | - Ultraviolet-Visible Spectroscopy |

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CHAPTER-1

INTRODUCTION

The development of nanotechnology has rapidly advanced with the widespread use of nanoparticles (NPs) in numerous fields. Particles with at least one dimension between 1 and 100 nm are referred to as nanoparticles [11]. Since the Earth's formation, there have been ultra-small particles on the planet. Nanometer-scale particles are naturally produced by geological eruptions or hydrothermal processes, but man-made, extremely small particles have started to exist in the environment.

The contemporary word "nanomaterials" (NMs), which is more usually cited as nanoparticles (NPs) in this revision [3]. Nanoparticles are frequently used in biomedical research, electronic industries, paint and coating industries, etc. However, using nanoparticles has so many benefits, environmental toxicity is a major issue that could lead to unplanned health or environmental risks.

Manufacture, transportation, usage, and dumping of NPs will release them into the environment, and they'll eventually settle on soils and water bodies. In actuality, a huge number of industrial chemical producers of NPs release wastewater into the sea or into rivers. Yearly, a huge number of NPs are released into the water body. By several complicated processes like adsorption, desorption, suspension, sedimentation, and interaction with minerals, organic matter, biofilm, and other complicated features of the water environment, NPs pose a risk to the aquatic ecology and human health in a water environment [11].

The aquatic environment, for example, is expected to be the final recipient of discharged nanoparticles, and various critical evaluations have focused on the environmental hazard assessment of engineered NPs and their harmful effects in fish and invertebrates. Furthermore, because their surface qualities are so important for aggregation behavior, data on their mobility and interactions with aquatic biota, such as algae, are still limited [5].

In this study, I have used selenium (Se) nanoparticle because it is an important component in human nutrient with oxidative and chemoprotective effects that improve the immune system, improve male fertility, and reduce ageing. Selenium exists naturally in soil at quantities ranging from 0.05 to 0.09 mg.kg⁻¹, although its concentration in most aquatic settings is in the nanomolar range.

Dissolved Se in aquatic environments is typically contributed by industrial and agricultural sources, and the environment cycles through chemical reactions and biological changes mediated by microalgal communities [12].

Selenium is a nonmetal with the atomic number 34 and the atomic mass 78.96, as well as the electronic configuration [Ar] 3d¹⁰ 4s² 4p⁴. It exists in five oxidation states in nature: (-II), (-I), (0), (+IV), and (+VI). SeO₄²⁻ and SeO₃²⁻ are soluble oxyanions known as selenate and selenite, respectively. Selenite is a weak acid that can exist in solutions such as H₂SeO₃, HSeO₃⁻, or SeO₃²⁻.

The bioavailability of selenium species in the environment, a factor connected with numerous environmental variables, and the bioaccumulation of selenium in some organisms, such as plants, influence selenium toxicity and deficiency. Many studies performed over the last 30 years have documented accurate physicochemical processes maintaining selenium bioavailability in multiple environments.

Inorganic selenium oxygen anions have been utilized in fertilizers as nutritional supplements, and the bioavailability of organic selenium compounds to various plants and animals has been extensively researched. Developments in drug delivery systems have recently focused some attention on the bioavailability of nanoparticles of elemental selenium, a chemical species formerly thought to be physiologically inert.

Microalgae are a diverse group of unicellular or multicellular photosynthetic microorganisms that are capable of converting sunlight and carbon dioxide into biomass and oxygen through the process of photosynthesis.

They have received increasing attention in recent years due to their potential as a sustainable source of food, fuel, and other valuable products (Chen *et al.*, 2019). Microalgae can grow rapidly and efficiently under a wide range of environmental conditions, and they can produce a wide variety of bioactive compounds, such as carotenoids, polyunsaturated fatty acids, and phycobiliproteins, which have important applications in the food, pharmaceutical, and cosmetic industries (Lee *et al.*, 2018).

According to a study by Chen *et al.*, (2019), microalgae have several advantages over traditional crops as a source of biofuels, including higher photosynthetic efficiency, higher oil content, and lower land use requirements. Moreover, microalgae can be grown on non-arable land using wastewater, which makes them a potentially sustainable and environmentally friendly source of energy.

Microalgae are a diverse group of unicellular or multicellular microorganisms that inhabit diverse aquatic environments, including freshwater, seawater, and brackish water. They are photosynthetic organisms that can convert light energy into chemical energy through photosynthesis, and they are considered one of the most promising sources of sustainable biomass for biofuels, food, feed, and pharmaceuticals (Chisti *et al.*, 2007).

According to a study by Chisti (2007), microalgae have several advantages over other sources of biomass for biofuels and other applications, including their high growth rates, high lipid content, and ability to grow in non-arable land and saline water. Moreover, microalgae can also sequester CO₂ from industrial flue gases and wastewater, contributing to mitigating climate change.

Overall, microalgae represent a promising and versatile source of biomass that can address many of the environmental and economic challenges of the 21st century. With continued research and development, microalgae-based technologies could play a significant role in achieving a sustainable and equitable future for humanity.

In this study, CS-Se NPs were synthesized and characterized using various spectroscopic and microscopic techniques. The toxicity of CS-Se NPs was assessed on a dominant marine diatom, *Nannochloropsis oculata* growth inhibition, enzymatic assays, ROS production and morphological changes using various microscopic analysis.

CHAPTER – 2

REVIEW OF LITERATURE

The ultrasonic method was used in this study to create neodymium tungstate nanoparticles for the first time. It is crucial to research how these materials affect aquatic species given the use of nanoparticles in many technologies and the potential environmental effects of these substances. The effectiveness of these nanoparticles on *D. salina* alga was investigated to accomplish two objectives. The first objective was to reach a level of nanoparticle concentration that prevented algae growth and was hazardous to the environment. The second objective was to achieve a nanoparticle concentration that promoted algae growth and, as a result, increased the output of valuable pigments for industrial use [Hassanpour M et al., 2021].

Due to their distinct physical properties, chemical composition, and mechanical properties, cobalt nanoparticles (Co NPs) can penetrate aquatic environments and have a wide range of applications. As primary producers, microalgae are crucial to maintaining the equilibrium of the aquatic ecosystem. It was discovered that the release and accumulation of toxicants, which at first disturbed the growth of marine microalgae, influenced higher trophic levels. In this study, three different kinds of marine microalgae, *Platymonas subcordiformis*, *Chaetoceros curvisetus*, and *Skeletonema costatum*, were selected for algal growth inhibition tests to examine the potential toxicity of Co NPs. The tolerances of the three marine microalgae to Co NPs were compared. In comparison to *Platymonas subcordiformis*, the cell sizes of *Skeletonema costatum* and *Chaetoceros curvisetus* were smaller and had greater specific surface areas (*Chlorella*). Additionally, Co NP size distribution, SEM images, and the effects of Co NPs on algae shading were obtained. Algal growth inhibition tests were carried out following Co^{2+} exposure to further investigate the toxic mechanism of Co NPs on microalgae. Co^{2+} in the F/2 seawater medium was determined. This is one of the first extensive studies on the potential toxicity of Co NPs and their toxic

mechanism on marine microalgae that we are aware of, to the best of our knowledge [Chen X *et al.*, 2018].

The catalytic abilities of Ag-AuNPs have been researched. By co-reducing chloroauric acid, silver nitrate, and sodium citrate in an aqueous solution, these bi-metallic Nanomaterials can be created. In this review, researchers do research on the fate, behavior, and toxicity of silver nanoparticles (Ag NPs), gold nanoparticles (AuNPs), and silver-gold nanoparticles (Ag-AuNPs) on photosynthetic marine microbiota, as well as a comparison with freshwater species. Along with some final thoughts on knowledge gaps and suggested future research in this field, some recent methods used in the green (plant-mediated, including algae and microalgae) biosynthesis of Ag NPs and AuNPs are studied [I. Moreno-Garrido *et al.*, 2015]

By studying the aquatic toxicity of Cd S and ZnS NPs produced by a recently developed xanthate-mediated methodology using a microalgae-bioassay approach, this study aims to contribute to the mentioned field of research. Aquatic test organisms that are widely dispersed in the ocean and serve as the base trophic level of food chains include microalgae, which can be highlighted. To make it easier to design safer NPs and introduce new regulations, it is crucial to investigate the toxicity effects on such species. Any processes or substances that affect their population can have negative effects on higher trophic levels [K. Pikula *et al.*, 2020].

The other claims that the primary goal of this research was to examine the impact of ZnO nanoparticles on freshwater (*Chlorococcum sp.* and *Scenedesmus rubescens*) and marine (*Dunaliella tetractolecta* and *Tetraselmis suecica*) microalgae grown in various media with numerous zinc concentrations. Saline species were grown on salt-modified BG-11 and F/2 medium, while freshwater species were grown on modified Blue Green medium (BG-11) and Bold's Basal medium (BBM). Due to their aggregation and mobility behavior, ZnO NPs' surface properties were also examined. Since the various culture media may affect both the behavior of nanoparticles and the growth of algae,

interactions between NPs and algae were also studied. According to our knowledge, this is the first investigation into the comparative toxicity of Zn O NPs on microalgae grown in various culture media [Aravantinou et al., 2015].

In this study, we compared the impact of bulk Zn O and Zn O nanoparticles on *Coelastrella terrestris* growth kinetics. This is a relatively lengthy study investigating the impact of Zn O exposure on the life cycle of *Coelastrella terrestris* because we monitored the dynamics of toxicity over the course of 25 days. By estimating the IC₅₀, we have tried to find a clear relationship between the dose concentration and the number of exposure days. The metabolic, physiological, and morphological changes caused by the treatment during the growth phases of the algae are analyzed. These modifications are brought about by the inclusion of Zn O in bulk and nanoform [P. Saxena et al., 2019].

In this research project, the effects of DOM (Suwannee River humic acid) on the toxicity of silver nanoparticles (AF-Ag NPs) to the freshwater algae *C.vulgaris* are studied. These nanoparticles were formed from the leaf extract of the medicinal herb *Allium fistulosum*. During the 72-hrs exposure, the biomass (number of algal cells/mL) and chlorophyll a (photosynthetic pigment) contents of *C.vulgaris* were monitored in both the presence and absence of DOM. Additionally, after being exposed to the treatments (AF-Ag NPs and AF-Ag NPs + DOM), *C. vulgaris* underwent morphological changes. Our findings may offer fresh perspective on how organic matter influences the toxic effects of AF-Ag NPs to *C.vulgaris*.

This article proves that the toxicity in the tested organisms was caused by both Ag NPs and free Ag⁺ ions. In contrast to larger Ag NPs, smaller Ag NPs were more toxic, according to other reports. The contradictory results reported in the literature could mean that the test organisms represent only a few of the variables affecting Ag NPs' harmful effects. To evaluate the impact of both Ag NP size and concentration, the current study will examine the effect of Ag NPs of two different sizes (50 and 100 nm) at four different concentrations (10, 50, 100, and

200 mg/L). We started to look at the microalga's chlorophyll concentration, viable cell concentration, (ROS) formation, and extra- and intracellular changes [L. Hazeem *et al.* , 2019].

Ag NPs demonstrated a potent killing impact on *Prototheca sp* algae as all species' minimal algicidal concentrations (MAC) matched the associated MIC values exactly (MAC = MIC, 1-4 mg/L), except for *Prototheca sp* (MIC > 8 mg/L). The concentrations required to inhibit pathogenic *Prototheca sp* (MIC, 1-4 mg/L) were lower than the levels required to detect any toxicity in epithelial cells (CC20 > 6 mg/L). The potential of Ag NPs as a brand-new therapeutic tool for the treatment of *Prototheca sp* infections is highlighted by the study.

Chlorella vulgaris is a green microalga which can grow in both freshwater and seawater and has a short development period, making it excellent for toxicological testing and for the observation of toxicity at the level of cell membranes. This algae species has been discovered to have the exceptional ability to remove a wide range of environmental contaminants. *Chlorella vulgaris* has been used in this study to explore the toxicity and oxidative damage caused by cadmium selenide NPs. The collected data can be used to analyze the ecological safety of nanomaterials (Siemens X-ray diffractometer D5000 Germany) with Cu K radiation ($= 1.54065 \text{ \AA}$). In order to examine the morphology of NPs, SEM (MIRA3 FEG-SEM, Tescan, Czech Republic) was used. A Bruker IR spectrometer was used to capture FT-IR spectra. (Tensor 27, Bruker, Germany) [A. Movafeghi *et al.* , 2019].

The evaluations primarily summarized the behavior and fate of nanoparticles in the environment, including interactions between NPs and organic materials in the environment, as well as interactions between NPs and organisms, and highlighted the negative impacts of NPs on creatures such as algae, plants, and fungi. Some evaluations have noted the toxicity of NPs to microalgae and their behavior in the environment. Bioassay approaches for microalgae nanotoxicology were discussed, and suggestions for further research were made. The effect of NPs on algae was the subject of this article. To that

end, this review will comprehensively summarize and analyze NP adsorption and distribution in algal cells, as well as the ecotoxic effects of NPs on algae and influencing factors [F. Wang *et al.* , 2019].

The goal of this paper is to provide a systematic theoretical basis for biotechnological applications by analyzing Se uptake, accumulation, biotransformation, and the subsequent effect on culture parameters of green algae cultured in the laboratory. Green algae have no negative impact on human health in general, and because they can ingest and change Se, they are excellent carriers for bioavailable organic Se forms that might be utilized as a food supplement. A promising new branch of algal biotechnology appears to be the production of Se-enriched microalgal biomass as a dietary supplement [Z. Gojkovic *et al.* , 2015].

The aim of this review is to provide a summary of the most relevant biogeochemical processes influencing selenium bioavailability in soils, waterways, and sediments. Emphasis has been focused on the structural properties of the adsorption processes, as well as a review of present research about elemental selenium nanoparticles [A. Fernández-Martínez *et al.* , 2009].

CHAPTER – 3

AIM AND OBJECTIVE

To evaluate the toxicity of Chitosan - Selenium nanoparticles on microalgae species.

- Nanoparticle synthesis and characterization
- Maintenance of microalgae species.
- Treatment of microalgae with nanoparticles and analysis of Cytotoxicity, Lipid peroxidation and ROS formation.

CHAPTER – 4

MATERIALS AND METHODOLOGY

4.1 Synthesis of Chitosan - Selenium nanoparticles:

Selenium was prepared using heat assisted method by reducing sodium selenite 10^{-3} mM [Na_2SeO_3] 100 mL, Chitosan 0.5 g [$\text{C}_5\text{H}_{10}\text{N}_9\text{O}_{39}$] with 1 % (w/v) acetic acid solution 100 mL and stirring in Magnetic stirrer with heat until it dissolves, then combine the sodium selenite and chitosan solution in same flask. A stock solution of 50 mL of Ascorbic acid 10^{-3} mM [$\text{HC}_6\text{H}_7\text{O}_6$] was prepared and added dropwise to the CS-Se NPs (200 mL) under while stirring in 700 rpm with heat. The color changes from transparent from brick red or bright orange color indicates the completion of reduction and formation of the CS-Se NPs. The reduced solution was centrifuged at 8000 rpm for 15 mins in 25°C , separate the CS-Se NPs pellets and stored separately for further use.



Fig 4.1: Synthesized Chitosan-Selenium Nanoparticle

4.2 Characterization of Selenium nanoparticles:

The absorbance maxima of synthesized CS-Se NPs were observed by using UV- vis spectrophotometer (UV-1800 Shimadzu Japan) and identifying the

UV-vis highest peak. For identifying chemical bonds in the solution by producing infrared absorption spectrum by scanning the sample using FTIR Agilent Cary 630- ATR-FTIR [Fourier Transform Infrared Spectroscopy]. Particle size (HORIBA, Japan) and Zeta potential analysis was done to find the particle size of CS-Se NPs and to find the stability of CS-Se NPs in colloidal solution. [TEM] Transmission Electron Microscope characterization was done to analyze the quality, shape, size distribution and morphology of nanoparticles.

4.3 Preparation of F/2 media:

The F/2 media was prepared following the method of Guillard and Ryther. (Guillard et al 1962) (Guillard., 1975).

Table 4.1: Preparation of stock trace metal solution

COMPONENTS	Gram/L H ₂ O
Na ₂ EDTA.2H ₂ O	4.36 g
FeCl ₃ .6H ₂ O	3.15 g
CoCl ₂ .6H ₂ O	10.0 g
ZnSO ₄ .2H ₂ O	22.0 g
MnCl ₂ .4H ₂ O	180.0 g
CuSO ₄ .5H ₂ O	9.8 g
Na ₂ MoO ₄ .2H ₂ O	6.3 g

All these components were weighed carefully and added to 500 mL distilled water in a sterile container and mixed well. The solution was made up to 1000 mL final volume and was labelled as trace metal stock solution. The solution was autoclaved at 121 °C, 15 psi pressure for 15 mins and stored at 4 °C for future use.

Table 4.2: Stock Salt solution Preparation

Chemicals	Quantity in grams/L H ₂ O
NaNO ₃	75.0 g
NaH ₂ PO ₄ .2H ₂ O	5.0 g
Na ₂ SiO ₃ .9H ₂ O	30.0 g

All these components were weighed carefully and added separately to 500 mL distilled water in separate sterile containers and mixed well. The solutions were made up to 1000 mL final volume. The solutions were autoclaved at 121 °C, 15 psi pressure for 15 mins and stored at 4 °C for future use.

Vitamin stock solution and antibiotic stock solution were prepared separately under sterile conditions.

Table 4.3: Vitamin stock preparation

Component	Quantity (g/L H ₂ O)
Biotin	1.0 g
Cyanocobalamin	1.0 g
Thiamine HCl	0.2 g

About 200 mg of thiamine HCl was added to 950 mL distilled water and 1 mg each of biotin and Cyanocobalamin stock solutions were added and the final volume was made up to 1 mL with distilled water. The vitamin stock solution was filter sterilized and stored at 4 °C for further use.

Table 4.4: Antibiotic stock Preparation

Components	Quantity (g/L H ₂ O)
Gentamycin	10 g
Amphotericin	10 g
Streptomycin	10 g

The antibiotic stock was prepared by adding . 1 g of the above in 1 mL distilled water and mixed thoroughly. The solution was filter sterilized and stored at 4 °C.

Table 4.5: Preparation of complete f/2 media

Chemical	Quantity in mL/L H ₂ O
NaNO ₃	1 mL
NaH ₂ PO ₄ ·2H ₂ O	1 mL
Na ₂ SiO ₃ ·9H ₂ O	1 mL
Trace metal solution	1 mL
Vitamin solution	1 mL
Antibiotic solution	1 mL

To prepare the complete F/2 media, 950 mL of sea water was filtered. To this filtered natural sea water, 1 mL of each salt stock solution was added and mixed thoroughly. 1 mL trace metal solution was added to this, and the media was autoclaved at 121 °C 15 psi pressure for 15 mins. To the autoclaved media

1mL each of antibiotic solution and vitamin solution were added under sterile conditions and used for micro algal inoculation.

4.4 MICROALGAE INOCULATION

The isolated micro algal samples were inoculated in the autoclaved F/2 media under sterile condition. The microalgae species *Nannochloropsis sp* were inoculated carefully under sterile conditions to avoid possible contamination. The cultures were maintained at 25 ± 2 °C under optimum light intensity 1000 Lux and 16:8-hour light-dark cycle.

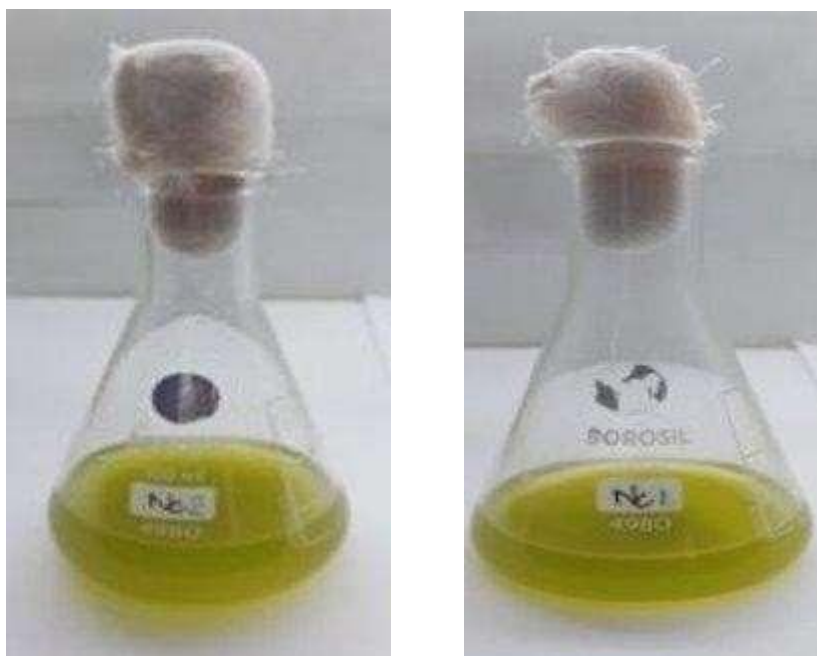


Fig 4.2: Nannochloropsis sp

After growth of algae, treat them with different concentration (with distilled water for 2 mL) of CS-Se NPs and Triton X (for Comparision) which is prepared in Eppendorf tube and store them for further use.

4.5 CYTOTOXICITY ASSAY

The cytotoxicity and cell viability of selenium nanoparticles in cell proliferation were examined using the MTT dye [The yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] reduction test. An

insoluble purple formazan product that is measurable using a microplate reader (Synergy™ H1 Hybrid Multi-Mode Reader, Biotek, USA) was created when metabolically active cells decreased MTT. A maintained algal culture, CS-Se NPs, and Triton X were added to a 96-well plate before we began; we gave them 24 hrs to react before taking the first reading. Before taking readings, add MTT dye to a 96-well plate and wait for four hours. Eventually, i have taken readings at regular intervals for 48, 72, and 96 hrs. This interacts with the culture's mitochondria and creates purple – colored pigment. It helps to find the cell viability of the microalgae after reacting with nanoparticles. % increase in MTT was plotted against concentration $\mu\text{g/mL}$.



Fig 4.3: MTT well plate with color change.

4.6 Lipid Peroxidation Assay

The MDA (Malondialdehyde) assay is a commonly used method for measuring lipid peroxidation, a process in which free radicals attack and damage lipids in cell membranes, leading to production of MDA as a lipid peroxidation product. Microalgae should treat with CS-Se NPs and Triton X for 4 to 6 hrs in before starting the assay, then treat them with 500 μL of 0.1 M Hydrochloride and incubate them for 20 mins at room temperature (25 °c). Further 900 μL of TBA (Thiobarbituric Acid) of 0.025 M was added and incubate them for 65 mins at

37 °c. After it incubated add 400 μ L PBS (Phosphate Buffered Saline) of 10 mM and analyze the fluorescence by taking readings at 520 nm and 549 nm (excitation and emission) using Synergy™ H1 Hybrid Multi-Mode Reader (Biotek, US). Percentage increase in MDA was plotted against concentration μ g/mL.

4.7 ROS estimation:

DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate) assay is a quantitative method commonly used to measure intercellular reactive oxygen species (ROS) levels of nanoparticles treated with microalgae. It is based on the principle that DCFH-DA, a non-fluorescent compound, is taken up by cells and converted into the highly fluorescent compound DCF (2',7'-dichlorofluorescein) in the presence of ROS. CS-Se NPs and Triton X treated algal culture was incubated in 96 well plate. After incubation, 5 μ L of DCFH-DA (100 μ M) was added to the well plate and again incubate them in dark for 30 mins. Fluorescence was measured using Synergy™ H1 Hybrid Multi-Mode Reader at wavelength 530 nm (excitation maximum) and 485 nm (emission maximum). ROS was calculated and graphs were plotted.

CHAPTER – 5

RESULTS AND DISCUSSIONS

1. Characterization of selenium nanoparticles

1. UV- vis spectrophotometer:

A UV-vis spectrophotometer is a scientific instrument used to measure the absorption or transmission of ultraviolet (UV) and visible (vis) light by a nanoparticle. The basic component of a UV-vis spectrophotometer typically includes a light source that emits UV and visible light, a monochromator that detector that measures the intensity of light before and after it passes through the Selenium NPs. The UV- Visible spectrum (Fig 5.1) revealed an absorbance maximum at 252.5 nm which is characteristic of the reduction of selenium salt to CS-Se NPs. The Selenium prepared by reducing agent contains carboxylic COO⁻ group so, the absorbance maximum range should be between 200 nm to 300 nm.

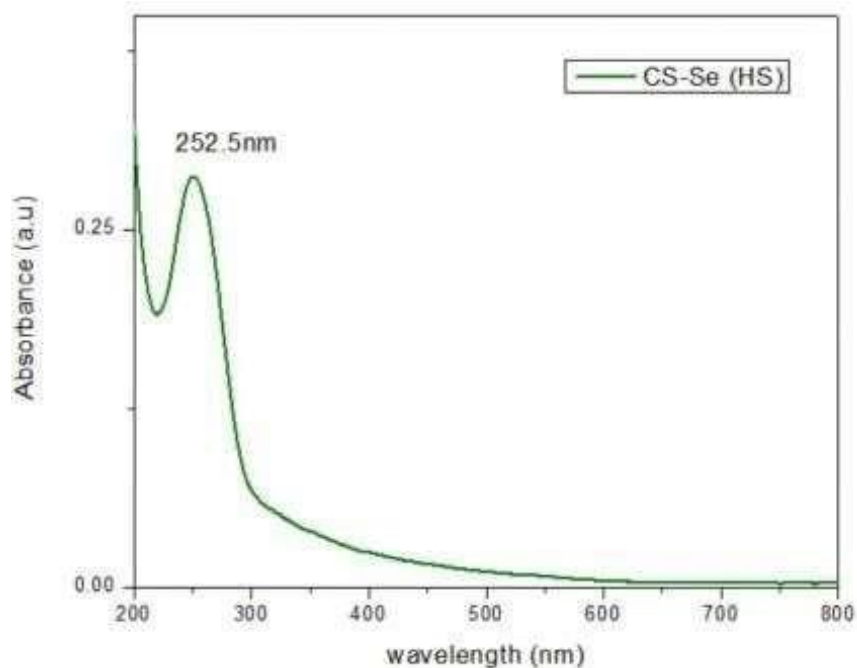


Fig 5.1: UV absorption spectrum of CS-Se NPs.

5.1.2 Fourier-Transform infrared spectroscopy (FTIR):

FTIR spectroscopy is used to determine the interaction of reducing and stabilizing agents on the surface of nanoparticles. Identification of organic, inorganic and polymeric materials utilizing infrared light for scanning the nanoparticle. In FTIR spectroscopy, infrared radiation is passed through a CS-Se NPs, and the result transmitted or reflected radiation is analyzed to obtain information about the molecular composition and structure of the selenium nanoparticles. FTIR is used in a variety of applications, including identifying and characterizing organic and inorganic compounds, determining functional groups in molecules, analyzing polymers, and investigating protein structure.

In the FTIR Spectrum (Fig 5.2), peaks at 1603.57 cm^{-1} and 1655.70 cm^{-1} correspond to asymmetrical and symmetrical stretching vibrations of COO^- . The peak obtained at 1378.19 cm^{-1} indicates the symmetric stretch of COO^- in Chitosan. The shift in peak to 1393.52 cm^{-1} indicated the involvement of COO^- groups in the synthesis of CS-Se NPs. The band at 3412 cm^{-1} corresponds to the O-H stretching. The IR band obtained at 2849.31 cm^{-1} , is indicative of the presence of Aliphatic C-H vibrations. The IR band obtained at 2369.42 cm^{-1} is indicative of the presence of Aliphatic C-H vibrations. The IR band at 672.91 cm^{-1} corresponds to =C-H out of plane bending $(\text{CH}_2)_n$ $(\text{CH}_2)_n$.

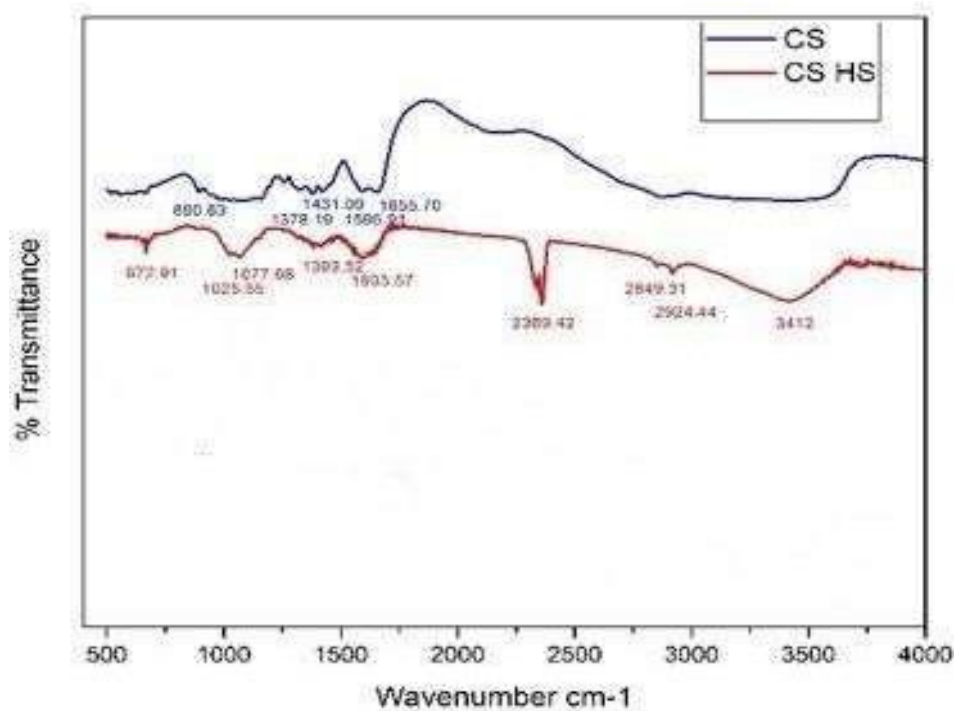


Fig 5.2 FTIR Spectrum of Chitosan and Synthesized CS-Se NPs

5.1.3 Particle size and Zeta potential:

Particle size and zeta potential are important parameters that are used to characterize colloidal systems, such as nanoparticles, emulsion and suspension. Particle size refers to the dimension of the particles in a colloidal system. Particle size can affect the stability, surface area, and reactivity of nanoparticles. It is used to find the particle size of nanoparticles.

Zeta potential is also known as electrokinetic potential, is the electric potential difference between the surface of a particle and surrounding medium in a colloidal system. Zeta potential characterization is used to find the stability of selenium nanoparticles.

The prepared CS-Se NPs was spherical and polydisperse with an average size of 164 nm. Particle size measurement (Fig 5.3) resulted in an average size of particle with 164 nm. Zeta potential (Fig 5.4) of the prepared CS-Se NPs was found to be 59.6 mV indicating significant stability of nanoparticles in colloidal solution.

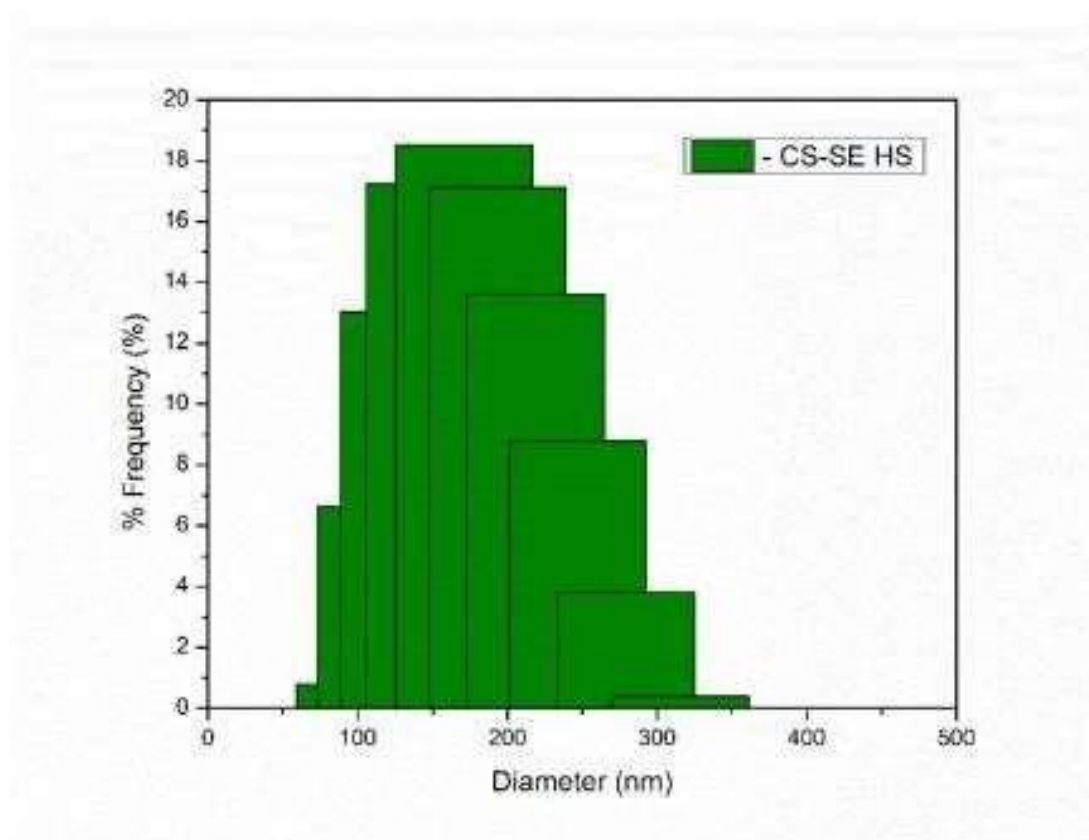


Fig 5.3 Particle Size Distribution graph of CS-Se NPs

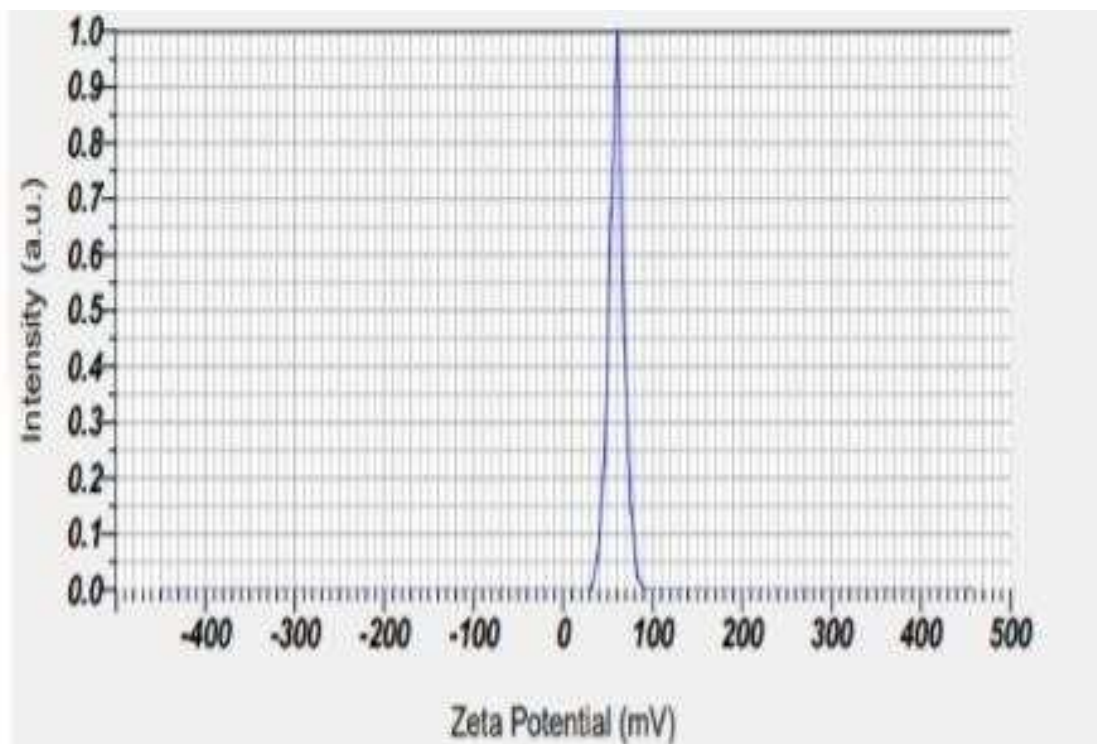


Fig 5.4 Zeta potential measurements of CS-Se NPs.

5.1.4 Transmission electron microscope (TEM):

A transmission electron microscope (TEM) is a type of electron microscope that uses a beam of electrons to illuminate a thin specimen, allowing for high- resolution imaging of the specimen's internal structure at the nanometer scale. A TEM works by passing a beam of electrons through a thin specimen, such as a Se nanoparticle. The electrons in the beam interact with the atoms in the nanoparticle, and the transmitted electron are collected and used to form an image. It is used to analyze the quality, shape, size, grain size, size distribution and morphology of nanoparticles. The transmission electron microscopic imaging (Fig 5.5) of the synthesized selenium- chitosan NPs revealed spherical morphology. The average size was found to be between 10-190 nm, which agreed well with the DLS (Dynamic Light Scattering) Particle sizing data. The nanoparticles were polydisperse.

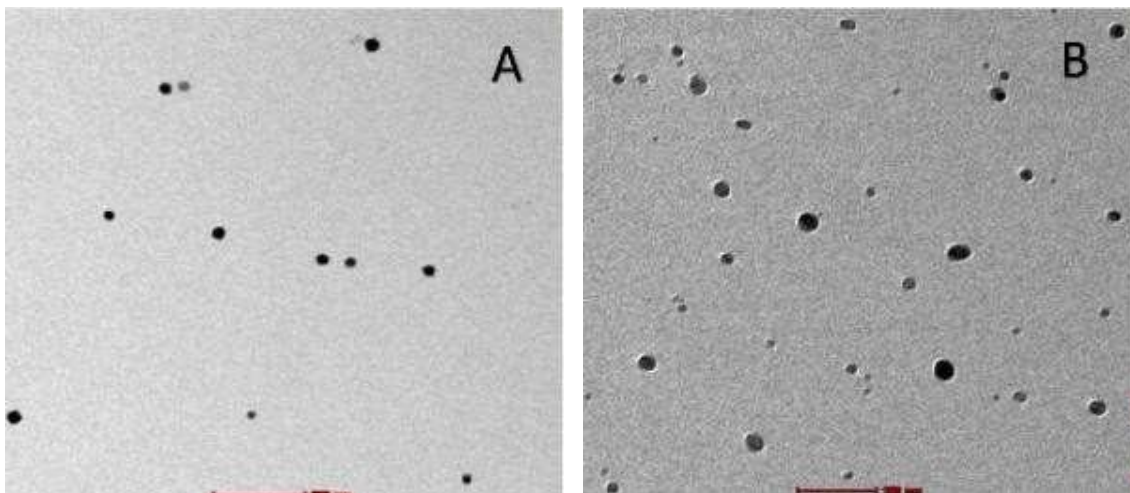


Fig 5.5 Transmission electron micrograph of CS- Se NPs

5.2 Microscopic Images

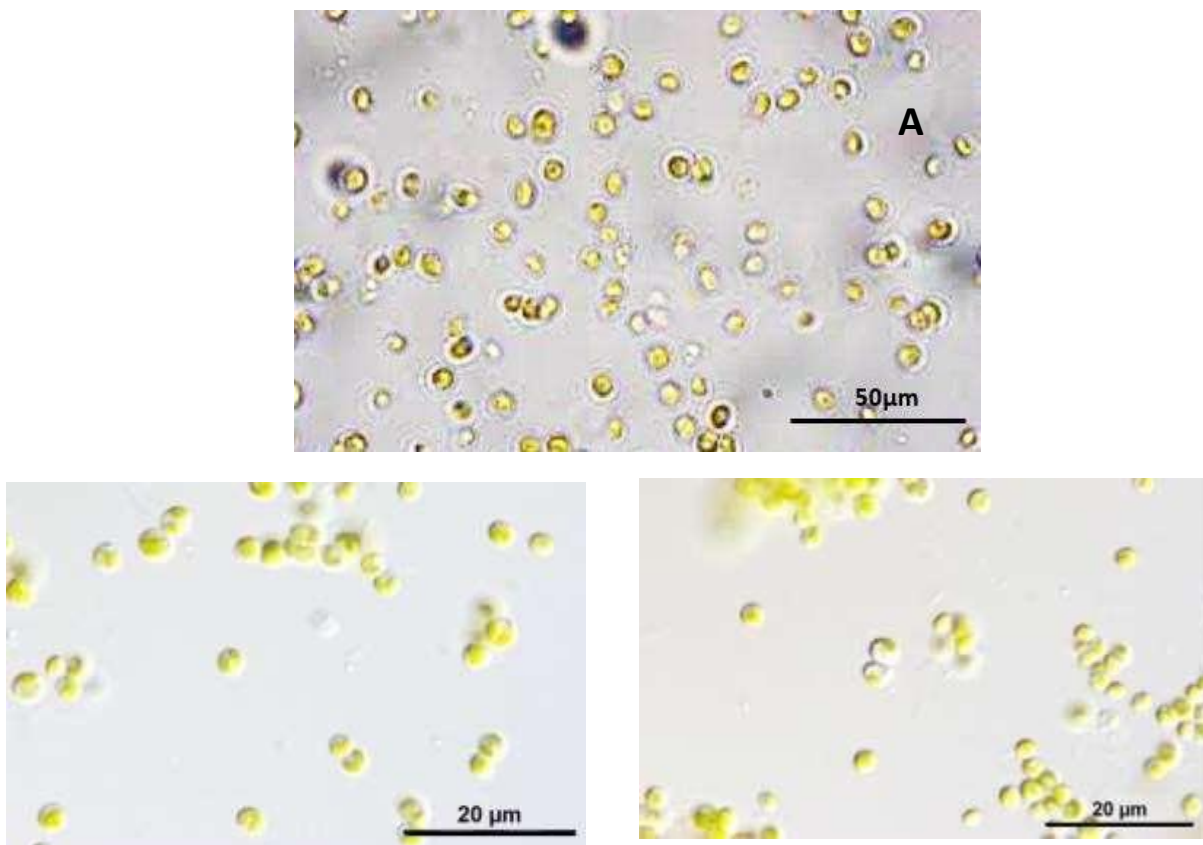


Fig 5.6 Microscopic images of Microalgae (Nannochloropsis sp)

3. Toxicity testing

1. Cytotoxicity assay:

Cytotoxicity assays are used to evaluate the potential toxicity of various environmental contaminants, such as pollutants, chemicals, and nanoparticles, on microalgae. These assays provide insights into the adverse effects of contaminants on microalgae, including cell viability, growth inhibition, and physiological responses. Cytotoxicity is evidentially increasing with respect to increase in the Selenium Nanoparticle concentration. A potential change that could account for CS-Se NPs' toxicity is the breakdown of ions. After confirmation, we believe that the possible cause could be the competition between Se ions and SO_4^{2-} ions during the transport of Se ions through the sulphate transporter, which would result in toxicity.

At the Minimum concentration of CS-Se NPs 15.625 $\mu\text{g/mL}$, cytotoxicity percentage showed at 15.625 % and the cytotoxicity % showed at 75.189 % in the Maximum concentration of CS-Se NPs 1000 $\mu\text{g/mL}$.

Table 5.1: Cytotoxicity % table

Concentration	Tx-100	CS-Se NPs
0	0	0
15.625	23.25	15.625
31.25	41.29	26.113
62.5	50.28	40.162
125	64.16	54.88
250	75.23	61.259
500	82.58	72.159
1000	90.08	75.189

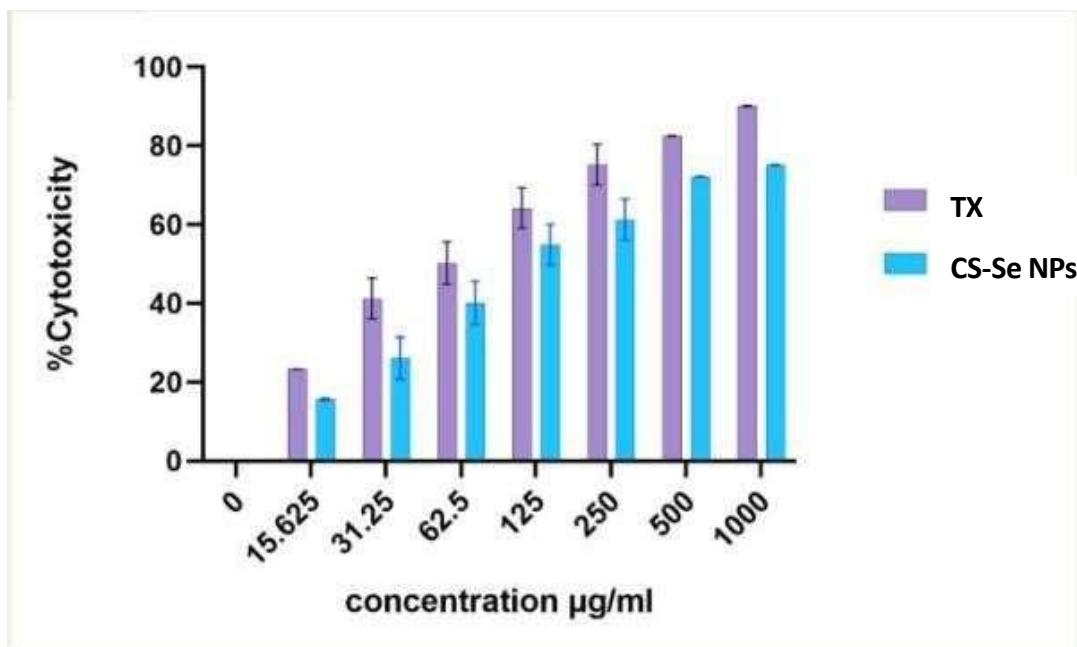


Fig 5.7: Cytotoxicity analysis of CS-Se NPs.

5.3.2 Lipid peroxidation assay:

Lipid peroxidation is evidentially increasing with respect to the increase in the Selenium Nanoparticle concentration. Lipid peroxidation assays are widely used in research and clinical settings to assess oxidative stress and lipid damage in biological samples. In this assay, malondialdehyde (MDA), a major product of lipid peroxidation, reacts with thiobarbituric acid (TBA) to form a colored complex that can be measured spectrophotometrically. The intensity of the color is proportional to the amount of MDA, which is used as an indicator of lipid peroxidation.

At the Minimum concentration of CS-Se NPs 15.625 µg/mL, Lipid peroxidation percentage showed at 16.615 % and the Lipid peroxidation % showed at 88.86 % in the Maximum concentration of CS-Se NPs 1000 µg/mL.

Table 5.2: Lipid Peroxidation % table

Concentration $\mu\text{g/mL}$	Tx-100	CS-Se NPs
0	0	0
15.625	25.25	16.615
31.25	40.62	29.62
62.5	45.126	41.72
125	67.02	54.86
250	83.09	63.92
500	87.06	81.8
1000	89.58	88.86

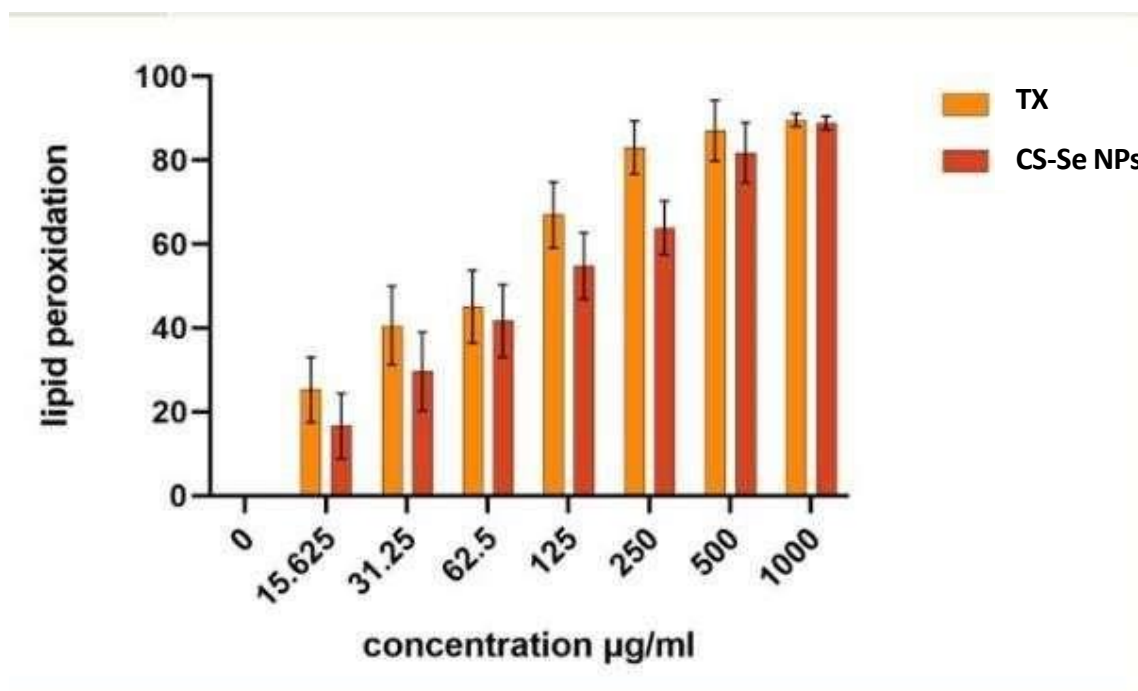


Fig 5.8 Lipid peroxidation analysis of CS-Se NPs

5.3.3 ROS estimation:

DCFH-DA test on algal cells was used to observe the involvement of CS-Se NPs, which in causing ROS. The level of ROS generation is indicated by the fluorescence intensity of 2',7'-dichlorofluorescein. All live cells generate ROS as part of their metabolic process. The reduced oxygen intermediates (H_2O_2) include hydrogen peroxide and the radical species superoxide (O_2^-) and hydroxyl ($OH^\bullet$).

At the Minimum concentration of CS-Se NPs 15.625 $\mu\text{g/mL}$, ROS estimation percentage showed at 5.567 % and the ROS estimation % showed at 22.03 % in the Maximum concentration of CS-Se NPs 1000 $\mu\text{g/mL}$.

Table 5.3: ROS estimation Table

Concentration $\mu\text{g/mL}$	TX-100	CS-Se NPs
0	0	0
15.625	4.335	5.024
31.25	19.875	99.900
62.5	29.500	26.642
125	36.167	92.405
250	45.911	93.265
500	95.106	94.058
1000	96.455	95.970

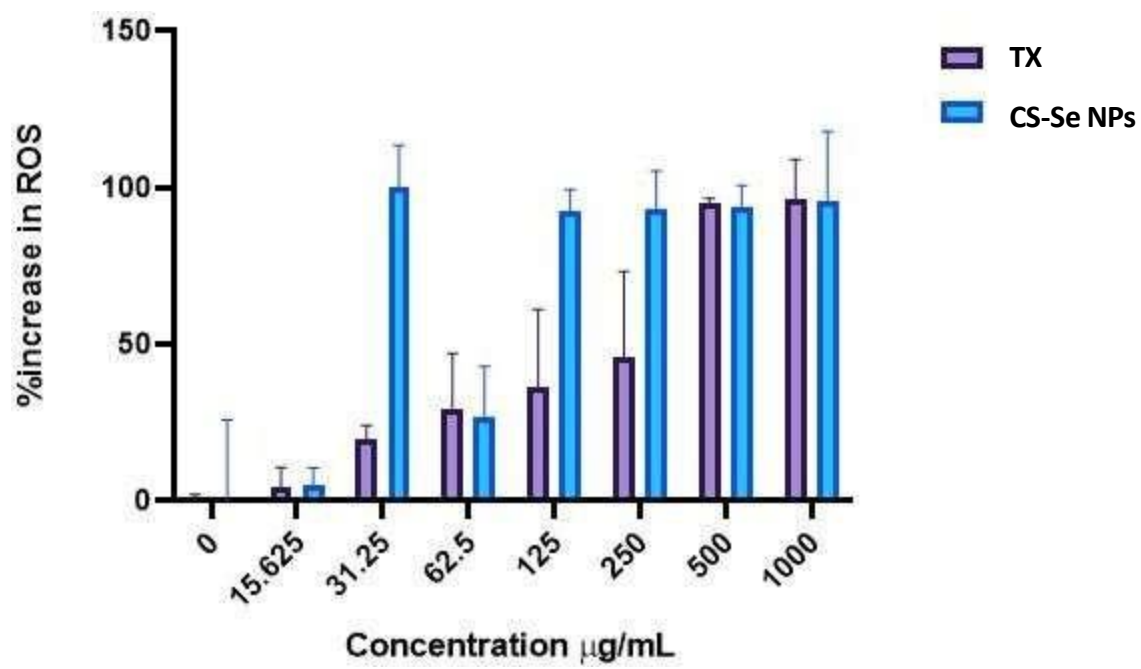


Fig 5.9 ROS estimation analysis of CS-Se NP

CHAPTER- 6

SUMMARY AND CONCLUSION

Selenium was synthesized and characterization was done using different spectroscopic and microscopic instruments. The absorbance maximum range and organic or inorganic bonds was found by the help of UV-vis spectrophotometer and FTIR using UV rays, visible and infrared rays. Microalgae exposed with selenium NPs to indicate the Toxicity determination; toxicity level depends on increases in concentration of CS-Se NPs and duration of CS-Se NPs treated with microalgae that found considering changes in the process of MDA, MTT and ROS assays. Particle size and stability was analyzed with the help of PSA and Zeta potential. According to the study, interaction with CS-Se NPs causes distortion in the morphological traits of *Nannochloropsis sp.* after 96 hrs (about 4 days). The alterations in cell viability, ROS, and MDA provide definitive evidence of CS-Se NPs toxicity.

Chapter: 7

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