Symbiosis

www.symbiosisonline.org
www.symbiosisonlinepublishing.com

International Journal of Scientific Research in Environmental Science and Toxicology

Research Article

Open Access

# Metal Nanoparticle Triggered Growth and Lipid Production in *Chlorella Vulgaris*

**Sibi G [1*], D Ananda Kumar[1], T Gopal[1], K Harinath[1], Banupriya S[1] and Chaitra S[1]**

*[1]Department of Biotechnology, Indian Academy Degree College-Autonomous, Bengaluru, India*

**Received: December 14, 2017; Accepted: December 20, 2017; Published: December 26, 2017**

***Corresponding author:** Sibi G, Department of Biotechnology, Indian Academy Degree College-Autonomous, Bengaluru, India, E-mail: gsibii@gmail.com*

## Abstract

Microalgae contribute not only to the environment through biodiesel production and water purification system but also to economy and society. Various strategies have been used to improve microalgal growth and among them the use of nanometals to facilitate microalgae culture and derive valuable products. Nanotechnology poses substantial impacts on living organisms and toxicity of metal nanoparticles on microalgae is widely reported. This study involved a positive approach to use metal nanoparticles to induce metal resistance in *Chlorella vulgaris* thereby increasing the high value products from microalgae. Four metals namely copper, magnesium, lead and zinc were used to synthesize metal nanoparticles. In the first phase, microalgae were cultivated in the presence of varying concentrations of metal nanoparticles to induce resistance against metals. In the second phase, microalgae grown under metal nanoparticles that have recorded significant increase in growth rate and biomass were selected for further cultivation in the presence of metal salts. Comparative experiments showed that metal nanoparticle exposed strain (MNPS) from nano metal containing media has exhibited more growth rate, biomass, cellular pigments and lipid production than non exposed microalgae thus indicating the positive effect of metal nanoparticles on microalgal growth.

**Keywords:** *Chlorella;* metal nanoparticles; microalgae; lipid; biomass;

## Introduction

Microalgal biomass is used as feedstock over fossil fuels by capturing $CO_2$ while converting it into reduced carbon sources [1, 2]. Cellular pigments produced by microalgae find application as medical antioxidants, anti-inflammatory agents and cosmetic agents [3, 4]. Metal and metal oxide nanoparticles are more favourable source than the native state to improve the biological functions of various organisms [5, 6]. The toxicity of nanoparticles to microalgae is known to be related to reactive oxygen species (ROS) generation inducing oxidative stress [7, 8], shading effect [9] and agglomeration [10]. Nanoparticles which induce oxidative stress could be a candidate to promote algal growth and secondary metabolites accumulation [11]. Inhibitory effects of metals were reported towards numerous fresh water and marine algae. However, the application of nanomaterials to facilitate algal cultures is still in its nascent phase.

The presence of metallic nanomaterials can alter the inhibitory effect of metals on microalgae. This work had a positive approach of using metal nanoparticles to improve the microalgal growth under two phases. In the first phase, microalgae were cultivated in the presence of varying concentrations of metal nanoparticles to induce resistance against metals. The effect of metal nanoparticles and its effect in terms of specific growth rate and biomass concentration were determined. In the second phase, microalgae that has recorded significant increase in growth rate and biomass grown under metal nanoparticles was selected for further cultivation in the presence of metal salts. Both the metal nanoparticle exposed strain (MNPS) and wild strain (WS) were cultivated separately in metal salts containing medium. Growth rate, biomass concentration, pigment concentration, contents of protein, carbohydrate and lipid of two groups (MNPS and WS) were compared to determine the role of metal nanoparticles in deriving high value products from microalgae.

## Material and Methods

### Microalgae

*Chlorella* sp isolated from effluents of metal processing units was used in this study. Standard protocols as described by Anderson [12], Stanier et al., [13] and the database http://web.biosci.utexas.edu/utex/default were used for identification of the algal isolates. Initial study involved the cultivation of microalgae in the presence of varying concentrations of metal nanoparticles (copper, lead, magnesium and zinc). The temperature of the cultivation room was kept at 20 ±1°C using an air conditioner. The relative humidity was kept at 31 ± 1%, the flasks were shaken by hand and randomly placed in a growth cabinet (27 ± 1ºC, 12:12 h light/dark cycle, Philips TL 40W cool white fluorescent lighting, 140 µmol photons/m2/s) for 15-20 days. Aeration was done for 8 hours daily using aquarium motor. This is followed by the selection of microalgae that has recorded significant increase in growth rate and biomass for further cultivation in the presence of metal salts. Both the metal nanoparticle exposed strain (MNPS) and wild strain (WS) were grown in the medium containing metal salts to determine the effect of metal nanoparticles on microalgae.

 ***Corresponding author email:** gsibii@gmail.com*

## Metal Nanoparticle Synthesis

### Copper Nanoparticle Synthesis

Copper nanoparticles (CuNP) were synthesized by addition of 0.1 M copper (II) sulfate pentahydrate solution into 120 ml of starch (1.2 %) solution with vigorous stirring for 30 min. This is followed by addition of 50 ml of ascorbic acid solution (0.2 M) under continuous rapid stirring. Subsequently, 30 ml of sodium hydroxide solution (1 M) was slowly added to the prepared solution with constant stirring and heating at 80°C for 2 h. The color of the solution turned yellow to ocher. After the completion of reaction, the solution was taken from the heat and allowed to settle overnight and the precipitates were separated from the solution by filtration. The precipitate was washed with deionized water and ethanol for three times and dried at room temperature [14].

### Lead Nanoparticle Synthesis

Lead oxide nanoparticles (PbNP) were prepared by heating 60 ml of lead (II) acetate solution (1 M) up to 90°C. This solution was added to an aqueous solution of 50 ml of 19 M NaOH and stirred vigorously. Upon adding the lead (II) acetate, the solution initially became cloudy, and then turned a peach colour, and finally a deep orange red. At this position, stirring was stopped, and the precipitate was allowed to settle and dried for overnight at 90°C [15].

### Magnesium Nanoparticle Synthesis

Magnesium oxide nano particles (MgNP) were prepared by addition of 100 g of $MgCl_2$ (500 ml) with 1 N NaOH (50 ml) and stirring for 4 hours to generate the magnesium hydroxide precipitates. The suspension was centrifuged at 3,000 rpm for 5 min to obtain the $Mg(OH)_2$ gel, washed several times with distilled water and dried at 60°C for 24 h. The dried powder was finally calcinated in air under 450°C for 2 h [16].

### Zinc Nanoparticle Synthesis

Zinc oxide nanoparticles (ZnNP) were prepared by adding zinc acetate dihydrate (1.65 g) to 84 ml of methanol under magnetic stirring and maintaining this solution 20 min under reflux. To this solution, KOH (0.983 g) solution in methanol (46 ml) was rapidly added. The zinc acetate solution in presence of KOH was refluxed again for 2 h under magnetic stirring. The solution was centrifuged at 4,000 rpm for 10 mins and the gel was recovered from the supernatant and further washed with ethanol before use [17].

## Characterization of Metal Nano Particles

Scanning electron microscopy (SEM) analysis was done using Hitachi s-4500 SEM machine. Thin films of the metal nanoparticles were prepared on a carbon coated grid by just dropping a small amount of the sample on the grid and then the film SEM grid was allowed to dry by putting it under a mercury lamp for 5 min.

## Specific Growth Rate

Specific growth rate (μ) of the microalgae was calculated according to the following formula.

$$\mu = \frac{\ln(N_t / N_0)}{T_t - T_0}$$

Where, $N_t$ and N0 are the dry cell weight concentration (g $L^{-1}$) at the end ($T_t$) and start ($T_0$) of log phase respectively.

## Biomass Concentration

Biomass (g $L^{-1}$) of microalgae grown in the presence of metal nanoparticles was determined by measuring the optical density of samples at 600 nm ($OD_{600}$) using UV-Vis spectrophotometer. Biomass concentration was then calculated by multiplying $OD_{600}$ values with 0.6, a predetermined conversion factor obtained by plotting $OD_{600}$ versus dry cell weight (DCW). DCW was determined gravimetrically by centrifuging the algal cells (3000×g, 10 min) and drying.

Biomass concentration = $OD_{600} \times 0.6$..... Eq. (1)

## Chlorophyll Estimation

Chlorophyll contents of the microalga were estimated according to Becker [18]. Algal cells were centrifuged and extracted with acetone overnight. The extract was centrifuged at 3000 x g for 5 mins and the chlorophyll content in the supernatant were determined by measuring the optical densities at 645 and 663 nm in a spectrophotometer and then calculated using the following equation.

Chl (mg/L) = 8.02 x $OD_{663}$ + 20.21 x $OD_{645}$ ..... Eq. (2)

## Carotenoids Estimation

Carotenoids were determined by following the procedure of Whyte [19]. Algal cells were centrifuged and treated with KOH (60% w/w). The mixture was homogenized and warmed to 40°C for 40 mins and extracted using ethyl ether. The solvent was evaporated followed by resuspending in acetone and the optical density was measured at 444 nm. Total carotenoids were calculated using the below equation.

Ct (mg/L) = 4.32 × $OD_{444}$ – 0.0439..... Eq. (3)

## Protein Assay

The extraction of proteins from microalgae was performed using alkali method. Aliquots of algal sample were centrifuged and 0.5 N NaOH was added to the pellet followed by extraction at 80°C for 10 mins. The mixture was centrifuged and protein content of the supernatant was estimated using Bovine Serum Albumin (BSA) as standard by Lowry et al., method [20].

## Carbohydrate Assay

Cellular carbohydrates were estimated using the anthrone method described by Gerhardt et al., [21] after hot alkaline extraction [22]. Briefly, microalgal pellets were resuspended in

distilled water and then heated in 40% (w/v) KOH at 90°C for 1 h. After cooling down, ice cold ethanol was added and stored at -20°C overnight followed by centrifugation. The pellet was resuspended in distilled water and then reacted with anthrone reagent. D-glucose was used as standard and the colour development was read at 578 nm in a spectrophotometer.

### Determination of Total Lipids

Algae cells were harvested through centrifugation and then dried for the analysis of lipid content. The lipids were extracted using a one step extraction method [23]. Dried algal cells added with distilled water were ultrasonicated and mixed with chloroform: methanol (2:1). The mixture was left for 30 mins in a water bath (30°C) and filtered through a Whatman No.1 filter paper. The filtrate was transferred to another screw cap tube containing NaCl solution (0.9%) and the purified chloroform layer was evaporated to a constant weight in a fuming hood under vacuum at 60°C. The total lipid content of dry weight was calculated using the following Equation (4).

Lipid content (%) = $(m_2-m_0)/m_1 \times 100$…….. Eq. (4)

where $m_1$ is the weight of the dried algal cells, $m_0$ is the weight of the empty new screw cap tube and $m_2$ is the weight of the new screw cap tube with the dried lipids. Lipid productivity ($g\ L^{-1}\ d^{-1}$) was determined using the following Equation (5).

Lipid productivity = Biomass productivity × Lipid content…….. Eq. (5)

### Statistical Analysis

The mean and standard deviation values for experiments performed in triplicates were calculated using SPSS statistics 21 software. Data from three replicates (n = 3) were analyzed using one-way ANOVA, and $p < 0.05$ was considered statistically significant.

## Results and Discussion

Microalgal samples collected from heavy metal processing industrial effluents were identified and found that Chlorella *vulgaris* as the predominant species. To investigate the behaviour of nanoparticles on microalgal growth and metal adsorption, microalgae were initially exposed to metal nanoparticles. Four metals namely copper, magnesium, lead and zinc were used to synthesize nanoparticles. SEM analysis of the metal nano solutions confirmed the nano sized metal particles from copper (89 nm), magnesium (82 nm), lead (76 nm) and zinc (92 nm). The metal nanoparticles of varying concentrations were used at initial screening to determine heavy metal tolerance and the concentrations which had not caused any adverse effects on the microalgal growth were selected to induce metal resistance. Accordingly, copper nanoparticles (10, 20, 30, 40 and 50 mg $L^{-1}$), magnesium nanoparticles (50, 100, 150 and 200 mg $L^{-1}$), lead nanoparticles (50, 100, 150 and 200 mg $L^{-1}$) and zinc nanoparticles (50, 100, 150 and 200 mg $L^{-1}$) were used in the study.

Varying concentrations of metal nanoparticles were added into Bristol medium and initial growth in the presence of metal nanoparticles was varying with different days of cultivation. The growth rate was increasing in the beginning followed by decline phase in most cases. The visible growth of microalgae was observed after 8 days in the presence of copper nanoparticles (CuNP), 13 days of cultivation period in the medium containing magnesium nanoparticles (MgNP), 7 days in lead nanoparticles (PbNP) and 11 days in zinc nanoparticles (ZnNP). Total cultivation period of 8 days from the initial growth day was considered for all the metal nanoparticles tested. Highest specific growth rate and biomass were observed in 20 mg $L^{-1}$, 100 mg $L^{-1}$, 50 mg $L^{-1}$ and 50 mg $L^{-1}$ concentrations for CuNP, PbNP, MgNP and ZnNP respectively during the cultivation period (Figure 1a-4b). Both the specific growth rate and biomass concentration were decreased with increasing days of cultivation in media containing CuNP, PbNP and MgNP whereas increasing trend was observed in the presence of ZnNP with longer cultivation period. Control experiments were carried out using Bristol medium without metal nanoparticles under the same cultivation conditions. In general, higher concentrations of metal nanoparticles induced growth inhibition of microalgae under the experimental conditions. In the case of zinc nanoparticles containing media, metal concentrations above 100 mg $L^{-1}$ has inhibited the growth of microalgae up to 17 days of cultivation period. The difference in the growth of microalgae in the presence of various metal nanoparticles is due to type of metals used (Cu, Mg, Pb, Zn) and size of the nanoparticles (76 nm - 92 nm). A number of environmental factors are known to influence nanoparticle behaviour including pH, ionic strength and particle concentration [24-26].

In the second phase of the experiment, microalgae which exhibited maximum specific growth rate and biomass concentration was selected and used for further cultivation. This is based on the fact that the presence of metal nanoparticles had induced metal resistance in microalgae which in turn was able to survive and produce highest specific growth rate and biomass. The second phase involved the comparison of biochemical attributes of metal nanoparticle induced metal resistance microalgae with non-metal nanoparticle exposed microalgae. For this study, both the strains were cultivated for a period of 8 days in the presence of metal salts and the concentration is similar to that of metal nanoparticles.

Growth rate, biomass and biochemical attributes of tested microalgae groups grown in the presence of metal salts were evaluated (Table 1-4). In most cases, inhibitory effects of metal nanoparticles on microalgae were reported. Toxicity of silver nanoparticles and nickel oxide nanoparticles on microalgae were reported earlier [27, 28]. The effect of zinc nanoparticles on *Chlorella* sp was studied by [29] (Chen et al., 2012). The presence of metal nanoparticles has affected the photosynthetic pigments of *Chlorella vulgaris* [30]. Higher concentrations of Manganese oxide nanoparticles had inhibitory effect on *Chlorella pyrenoidosa* [31]. Toxicity of Zinc oxide nanoparticles on microalgae was studied by Pendashte et al., [32] and found that *Chlorella* species was more sensitive than *Scenedesmus.*

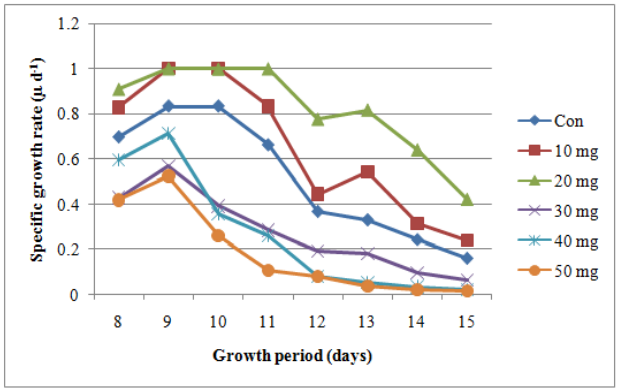


**Figure 1a:**Specific growth rate of *C. vulgaris* in the presence of copper nanoparticles

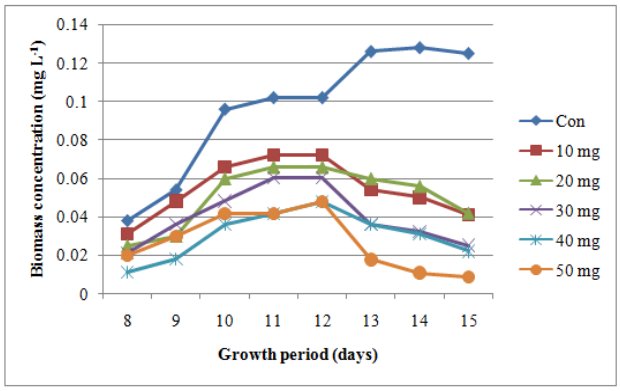


**Figure 1b:**Biomass concentration of *C. vulgaris* in the presence of copper nanoparticles

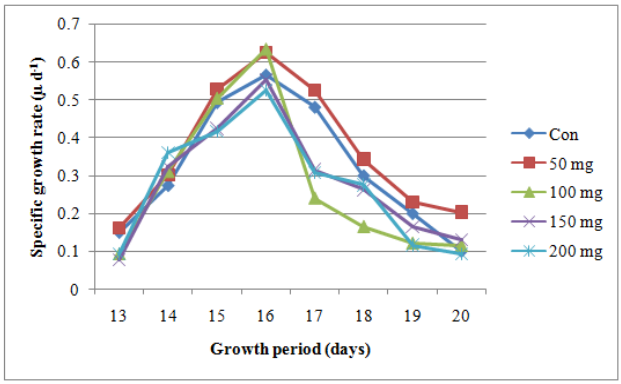


**Figure 2a:**Specific growth rate of *C. vulgaris* in the presence of magnesium nanoparticles

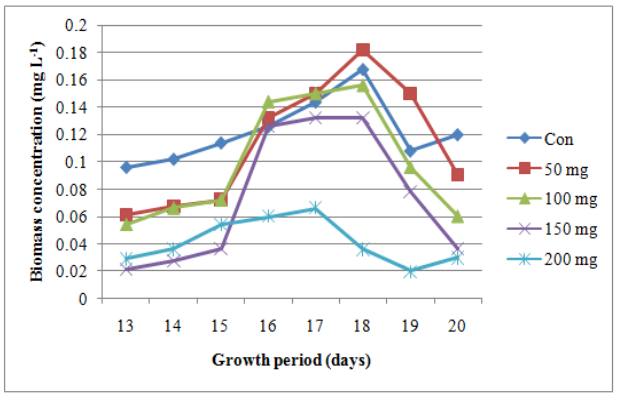


**Figure 2b:**Biomass concentration of *C. vulgaris* in the presence of magnesium nanoparticles

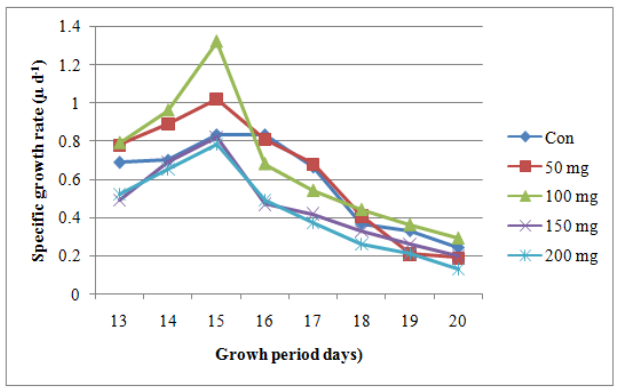


**Figure 3a:**Specific growth rate of *C. vulgaris* in the presence of lead nanoparticles

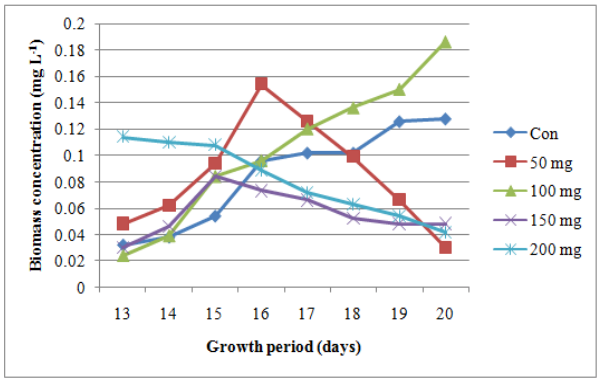


**Figure 3b:**Biomass concentration of *C. vulgaris* in the presence of lead nanoparticles

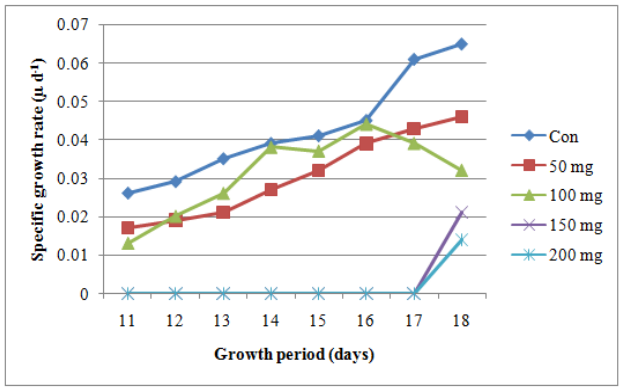


**Figure 4a:**Specific growth rate of *C. vulgaris* in the presence of zinc nanoparticles

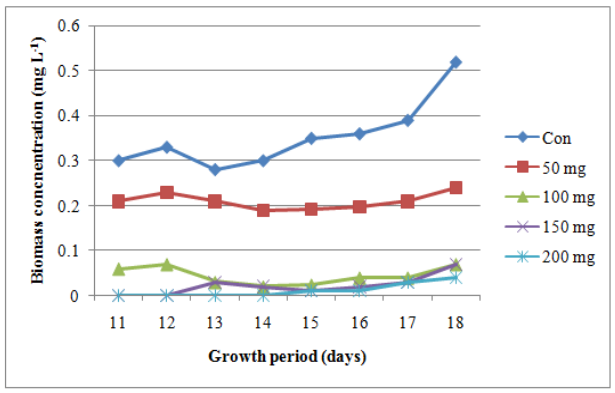


**Figure 4b:**Biomass concentration of *C. vulgaris* in the presence of zinc nanoparticles

**Table 1**: Growth rate, biomass and biochemical attributes of metal nanoparticle exposed strain (MNPS) and wild strain (WS) in the presence of copper salt

| Cu NP | | WS | | | | | MNPS | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Control | 10 mg | 20 mg | 30 mg | 40 mg | 50 mg | 10 mg | 20 mg | 30 mg | 40 mg | 50 mg |
| SGR (μ $d^{-1}$) | 1.25 ± 0.06 | 0.86 ±0.11 | 0.97 ± 0.10 | 0.91 ±0.04 | 0.82 ±0.11 | 0.80 ±0.21 | 0.91 ±0.04 | 0.99 ± 0.17 | 1.01 ±0.21 | 0.90 ±0.31 | 0.87 ±0.41 |
| Biomass (g $L^{-1}$) | 1.37 ± 0.20 | 0.94 ± 0.12 | 1.02 ± 0.71 | 1.09 ± 0.31 | 0.91 ± 0.07 | 0.86 ± 0.04 | 1.00 ± 0.51 | 1.11 ± 0.06 | 0.99 ± 0.35 | 0.92 ± 0.30 | 0.89 ± 0.09 |
| Chlorophyll (mg $L^{-1}$) | 2.83 ± 0.01 | 0.72 ± 0.63 | 0.52 ± 0.21 | 0.36 ± 0.12 | 0.29 ± 0.00 | 0.36 ± 0.05 | 1.36 ± 0.11 | 1.14 ± 0.21 | 0.96 ± 0.05 | 0.87 ± 0.02 | 0.78 ± 0.07 |
| Carotenoid (mg $L^{-1}$) | 1.79 ± 0.16 | 0.47 ± 0.06 | 0.36 ± 0.03 | 0.25 ± 0.11 | 0.24 ± 0.21 | 0.20 ± 0.02 | 0.38 ± 0.16 | 0.39 ± 0.08 | 0.43 ± 0.10 | 0.51 ± 0.23 | 0.43 ± 0.19 |
| Protein (mg $L^{-1}$) | 50 ± 0.17 | 23 ± 0.30 | 20 ± 0.35 | 11 ± 0.22 | 10 ± 0.14 | 9 ± 0.03 | 20 ± 0.29 | 22 ± 0.74 | 20 ± 0.79 | 25 ± 0.35 | 25 ± 0.15 |
| Carbohydrate (mg $L^{-1}$) | 16 ± 0.74 | 10 ± 0.09 | 14 ± 0.38 | 21 ± 0.61 | 18 ± 0.70 | 7 ± 0.34 | 30 ± 0.68 | 26 ± 0.41 | 20 ± 0.05 | 14 ± 0.36 | 10 ± 0.71 |
| Lipid (mg $L^{-1}$ $d^{-1}$) | 0.15 ± 0.10 | 0.13 ± 0.09 | 0.19 ± 0.10 | 0.18 ± 0.01 | 0.11 ± 0.13 | 0.11 ± 0.92 | 0.19 ± 0.01 | 0.28 ± 0.01 | 0.20 ± 0.33 | 0.13 ± 0.87 | 0.12 ± 0.00 |

**Table 2**: Growth rate, biomass and biochemical attributes of metal nanoparticle exposed strain (MNPS) and wild strain (WS) in the presence of magnesium salt

| Mg NP | | WS | | | | MNPS | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 50 mg | 100 mg | 150 mg | 200 mg | 50 mg | 100 mg | 150 mg | 200 mg |
| SGR (μ $d^{-1}$) | 1.25 ± 0.06 | 0.98 ± 0.14 | 0.81 ± 0.25 | 1.14 ± 0.21 | 1.02 ± 0.10 | 0.94 ± 0.08 | 0.97 ± 0.04 | 1.04 ± 0.15 | 0.85 ± 0.20 |
| Biomass (g $L^{-1}$) | 1.37 ± 0.20 | 0.82 ± 0.01 | 0.86 ± 0.44 | 1.01 ± 0.06 | 1.21 ± 0.15 | 0.89 ± 0.11 | 0.98 ± 0.34 | 1.25 ± 0.31 | 1.03 ± 0.15 |
| Chlorophyll (mg $L^{-1}$) | 2.83 ± 0.01 | 1.81 ± 0.25 | 1.45 ± 0.36 | 0.76 ± 0.44 | 0.88 ± 0.75 | 1.77 ± 0.06 | 1.81 ± 0.09 | 1.97 ± 0.10 | 1.93 ± 0.06 |
| Carotenoid (mg $L^{-1}$) | 1.79 ± 0.16 | 0.51 ± 0.27 | 0.69 ± 0.19 | 0.51 ± 0.09 | 0.64 ± 0.52 | 0.47 ± 0.11 | 0.51 ± 0.20 | 0.60 ± 0.31 | 0.69 ± 0.29 |
| Protein (mg $L^{-1}$) | 50 ± 0.17 | 50 ± 0.64 | 35 ± 0.71 | 20 ± 0.58 | 10 ± 0.09 | 35 ± 0.29 | 32 ± 0.47 | 29 ± 0.53 | 32 ± 0.68 |
| Carbohydrate (mg $L^{-1}$) | 16 ± 0.74 | 14 ± 0.54 | 18 ± 0.39 | 28 ± 0.66 | 12 ± 0.29 | 18 ± 0.05 | 15 ± 0.36 | 16 ± 0.64 | 25 ± 0.51 |
| Lipid (mg $L^{-1}$ $d^{-1}$) | 0.15 ± 0.10 | 0.21 ± 0.01 | 0.22 ± 0.41 | 0.43 ± 0.06 | 0.28 ± 0.15 | 0.22 ± 0.13 | 0.23 ± 0.10 | 0.46 ± 0.16 | 0.31 ± 0.07 |

**Table 3**: Growth rate, biomass and biochemical attributes of metal nanoparticle exposed strain (MNPS) and wild strain (WS) in the presence of lead salt

| Lead NP | | WS | | | | MNPS | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 25 mg | 50 mg | 75 mg | 100 mg | 25 mg | 50 mg | 75 mg | 100 mg |
| SGR (μ $d^{-1}$) | 1.25 ± 0.06 | 0.85 ± 0.11 | 0.89 ± 0.04 | 0.92 ± 0.14 | 1.04 ± 0.07 | 0.97 ± 0.02 | 1.04 ± 0.12 | 1.21 ± 0.07 | 1.31 ± 0.10 |
| Biomass (g $L^{-1}$) | 1.37 ± 0.20 | 0.92 ± 0.13 | 0.97 ± 0.20 | 1.04 ± 0.12 | 1.16 ± 0.04 | 0.99 ± 0.17 | 1.02 ± 0.07 | 1.27 ± 0.10 | 1.41 ± 0.11 |
| Chlorophyll (mg $L^{-1}$) | 2.83 ± 0.01 | 1.28 ± 0.16 | 1.49 ± 0.71 | 1.65 ± 0.20 | 1.81 ± 0.36 | 1.39 ± 0.04 | 1.51 ± 0.26 | 1.59 ± 0.17 | 1.72 ± 0.41 |
| Carotenoid (mg $L^{-1}$) | 1.79 ± 0.16 | 0.30 ± 0.22 | 0.30 ± 0.72 | 0.38 ± 0.68 | 0.69 ± 0.14 | 0.34 ± 0.19 | 0.35 ± 0.24 | 0.38 ± 0.51 | 0.39 ± 0.35 |
| Protein (mg $L^{-1}$) | 50 ± 0.17 | 48.8 ± 0.24 | 48.5 ± 0.54 | 61.7 ± 0.42 | 87.5 ± 0.63 | 45.5 ± 0.49 | 49.6 ± 0.64 | 58.5 ± 0.74 | 65.3 ± 0.23 |
| Carbohydrate (mg $L^{-1}$) | 16 ± 0.74 | 23.7 ± 0.10 | 12.9 ± 0.23 | 26.4 ± 0.04 | 34.3 ± 0.51 | 19.8 ± 0.08 | 21.9 ± 0.41 | 20.4 ± 0.48 | 19.7 ± 0.64 |
| Lipid (mg $L^{-1}$ $d^{-1}$) | 0.15 ± 0.10 | 0.26 ± 0.08 | 0.56 ± 0.70 | 0.65 ± 0.58 | 0.76 ± 0.62 | 0.30 ± 0.11 | 0.49 ± 0.27 | 0.55 ± 0.20 | 0.65 ± 0.04 |

**Table 4**: Growth rate, biomass and biochemical attributes of metal nanoparticle exposed strain (MNPS) and wild strain (WS) in the presence of zinc salt

| Zinc NP | | WS | | | | MNPS | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Control | 50 mg | 100 mg | 150 mg | 200 mg | 50 mg | 100 mg | 150 mg | 200 mg |
| SGR (μ $d^{-1}$) | 1.25 ± 0.06 | 1.04 ± 0.10 | 1.01 ± 0.24 | 1.21 ±0.03 | 1.06 ±0.21 | 0.94 ± 0.05 | 0.96 ± 0.11 | 0.89 ± 0.20 | 0.81 ± 0.06 |
| Biomass (g $L^{-1}$) | 1.37 ± 0.20 | 1.20 ± 0.10 | 1.17 ± 0.07 | 1.41 ± 0.31 | 1.11 ± 0.24 | 1.44 ± 0.10 | 1.40 ± 0.20 | 1.35 ± 0.20 | 1.27 ± 0.20 |
| Chlorophyll (mg $L^{-1}$) | 2.83 ± 0.01 | 1.73 ± 0.11 | 1.97 ± 0.03 | 1.60 ± 0.21 | 1.36 ± 0.66 | 1.04 ± 0.20 | 1.97 ± 0.09 | 1.59 ± 0.16 | 1.43 ± 0.57 |
| Carotenoid (mg $L^{-1}$) | 1.79 ± 0.16 | 080 ± 0.14 | 0.50 ± 0.07 | 0.84 ± 0.11 | 0.11 ± 0.01 | 0.22 ± 0.05 | 0.34 ± 0.10 | 0.67 ± 0.07 | 0.55 ± 0.04 |
| Protein (mg $L^{-1}$) | 50 ± 0.17 | 65 ± 0.09 | 72 ± 0.10 | 54 ± 0.61 | 41 ± 0.24 | 62 ± 0.81 | 42 ± 0.06 | 32 ± 0.47 | 60 ± 0.31 |
| Carbohydrate (mg $L^{-1}$) | 16 ± 0.74 | 14 ± 0.55 | 12 ± 0.26 | 9 ± 0.09 | 8 ± 0.13 | 20 ± 0.41 | 45 ± 0.38 | 46 ± 0.28 | 29 ± 0.21 |
| Lipid (mg $L^{-1}$ $d^{-1}$) | 0.15 ± 0.10 | 0.51 ± 0.77 | 0.58 ± 0.72 | 0.54 ± 0.50 | 0.37 ± 0.42 | 0.68 ± 0.22 | 0.57 ± 0.49 | 0.74 ± 0.17 | 0.53 ± 0.17 |

Copper oxide nanoparticles (CuO) have been shown to induce growth inhibition in green alga *Chlamydomonas reinhardtii* at concentrations higher than 100 mg $L^{-1}$ [33]. In this study, CuNP at a concentration of 40 mg $L^{-1}$ and above reduced the growth of Chlorella *vulgaris*. Further experiments with effect of nanometal induced metal resistance on microalgae revealed that biochemical attributes of microalgae were increased at 20 mg $L^{-1}$ concentration. Chlorophyll content of metal nanoparticle exposed microalgae was higher (1.14 mg $L^{-1}$) than wild strain (0.52 mg $L^{-1}$) revealing 54% increase in metal resistant strain (MNPS), however, the content of carotenoid was higher in wild strain. Similarly, there was a 46% increase in carbohydrate content of metal nanoparticle induced resistant microalgae. Total lipid was increased up to 32% in MNPS when compared to wild strain and 46.4% increase than control indicating the positive effect of metal nanoparticle induced resistance in *C. vulgaris.*

Magnesium at a concentration of 150 mg $L^{-1}$ has produced highest biomass and chlorophyll content but is lesser than control. Magnesium is one of the key elements required for chlorophyll synthesis [34] and higher magnesium concentration and $MgSO_4$ nanoparticles were found to enhance the lipid accumulation in *Chlorella vulgaris* [35]. This is in accordance with the present study where lipid content was significantly increased in both the groups (0.46 and 0.43 mg L-1) than control.

Higher concentrations of zinc metal had influenced the growth rate and biomass concentration of *C. vulgaris* in the study, There were variations in cellular pigments and protein contents in which WS recorded of higher content than MNPS. Increase in soluble protein content is considered as an evidence of active defense mechanism to prevent algae cells from damaging by abiotic stress [36] and increase in total protein content of the cells exposed to metal salts was observed in this study. The carbohydrate content was greatly influenced in MNPS and recorded 46 mg $L^{-1}$ which is 80% and 65% higher than WS and control. Soluble sugars are effective candidates for capturing reactive oxygen species (ROS) and scavenging the free radicals in microalgal cells exposed to environmental stresses [37]. It was noteworthy to observe that the total lipid content was 0.74 mg L-1 in MNPS whereas it was 0.54 and 0.15 mg $L^{-1}$ in WS and control respectively.

The study revealed that nanometal induced resistant strain has produced higher growth rate, biomass, cellular pigment, protein, carbohydrate and lipid content than non metal exposed strain when grown in the presence of metal salts. The data obtained were similar in most of the nanometals tested but the results were different for lead nanoparticles. In other words, growth rate and biochemical attributes were higher in WS than MNPS. Protein content of 87.5 mg $L^{-1}$ was observed in WS grown in lead containing medium which is highest than other metal salts and control. Another significant observation is the increase in lipid content of microalgae as 0.76 mg $L^{-1}$ which is also higher than any other metal salts studied. It was also noted that the lead metal in the form of lead acetate salt had positive effect on biomass and lipid content of *C. vulgaris*. The feasibility of mass culture of microalgae for biodiesel production greatly depends on high biomass productivity and lipid yield [38].

The inhibitory effects of nanoparticles on microalgae were reported widely and reports which revealed the positive effect of nanoparticles on various microalgae are also available. Increase in microalgal pigments were observed when the microalgae were cultivated in the presence of metal nanoparticle solutions [39]. The addition of lower concentrations of copper nanocarboxylates (20 to 40 mg $L^{-1}$) and selenium nanocarboxylates (0.07 to 0.2 mg $L^{-1}$) had stimulated the growth of *Chlorella* and increase in biomass along with chlorophyll content of the microalgae [40]. Both neutral and total lipid contents were increased in *Scenedesmus obliquus* in the presence of carbon nanotubes, $Fe_2O_3$ nanoparticles and MgO nanoparticles [41]. Zero-valent iron nanoparticles were found to boost the growth of several green algae [42].

In conclusion, this study evaluated the use of metal nanoparticles to induce metal resistance in C. vulgaris thereby increasing the high value products such as biomass, cellular pigments and lipid from microalgae. Initial experiments demonstrated the metal resistance development through metal nanoparticles and further experiments confirmed the positive influence of metal nanoparticles to improve microalgal growth, biomass and lipid production when grown in the presence of metal salts.

## References


1. Melis A and M Melnicki. Integrated Biological Hydrogen Production. Int J Hydrogen Energy. 2006;31(11):1563-1573. Doi:org/10.1016/j.ijhydene.2006.06.038
2. Razeghifard R. Algal biofuels. Photosynth Res. 2013;117(1-3):207-219.
3. Gordon H T, Bauernfeind J C, Furia T E. Carotenoids as food colourants. Crit Rev Food Sci Nutr. 1983;18(1):59-97. Doi:10.1080/10408398209527357
4. Orosa M, Torres E, Fidalgo P, Abalde J. Production and analysis of secondary carotenoids in green algae. J Appl Phycol. 2000;12(3-5):553-556. Doi:org/10.1023/A:100817380
5. Johnston B D, T M Scown, J Moger, C R Tyler. Bioavailability of nanoscale metal oxides TiO2, CeO2, and ZnO to Fish. Environ Sci Technol. 2010;44(3):1144-1151. Doi:10.1021/es901971a
6. Kadar E, Rooks P, Lakey C, White D A. The effect of engineered iron nanoparticles on growth and metabolic status of marine microalgae cultures. Sci Total Environ. 2012;439:8-17. Doi: org/10.1016/j.scitotenv.2012.09.010
7. Ji J, Long Z, Lin D. Toxicity of oxide nanoparticles to the green algae *Chlorella* sp. Chemical Engineering Journal. 2011;170(2-3):525-530. Doi:org/10.1016/j.cej.2010.11.026
8. He D, Dorantes-Aranda J J, Waite T D. Silver nanoparticle-algae interactions: Oxidative dissolution, reactive oxygen species generation and synergistic toxic effects. Environ Sci Technol. 2012;46(16):8731-8738. Doi:10.1021/es300588a
9. Schwab F, Bucheli T D, Lukhele L P, Magrez A, Nowack B, Sigg L, et al. Are carbon nanotube effects on green algae caused by shading and agglomeration? Environ Sci Technol. 2011;45(14):6136-6144. Doi: 10.1021/es200506b
10. Ribeiro F, Gallego-Urrea J A, Jurkschat K, Crossley A, Hasselloy M, Taylor C, et al. Silver nanoparticles and silver nitrate induce high toxicity to *Pseudokirchneriella subcapitata*, *Daphnia magna* and *Danio rerio*. Sci Total Environ. 2014;466-467:232-241. Doi: 10.1016/j.scitotenv.2013.06.101
11. Kang N, Lee B, Choi G G, Moon M, Park M S, Lim J K, et al. Enhancing lipid productivity of *Chlorella vulgaris* using oxidative stress by TiO2 nanoparticles. Korean Journal of Chemical Engineering. 2014;31(5):861-867. Doi:org/10.1007/s11814-013-0258-6
12. Anderson R A. Algal culturing techniques. Elsevier Inc Amsterdam. 2005.
13. Stanier R Y, Kunisawa R, Mandel M, Cohen-Bazire G. Purification and properties of unicellular bluegreen algae (Order Chroococcales). Bacteriological Reviews. 1971;35(2):171-205.
14. Khan A, Rashid A, Younas R, Chong R. A chemical reduction approach to the synthesis of copper nanoparticles. International Nano Letters. 2016;6(1):21-26. Doi:org/10.1007/s4008
15. Karimi H, Karimi M A, Haghdar S, Sadeghi A, Mir Ghasemi R, Mahdi-Khani S. Synthesis of lead oxide nanoparticles by Sonochemical method and its application as cathode and anode of lead-acid batteries. Materials Chemistry and Physics. 2008;108(2-3):337-344. Doi:org/10.1016/j.matchemphys.2007.09.045
16. Moussavi G, Mahmoudi M. Removal of azo and anthraquinone reactive dyes from industrial wastewaters using MgO nanoparticles. Journal of Hazardous Materials. 2009;168(2-3):806-812. Doi:org/10.1016/j.jhazmat.2009.02.097
17. Akkari M, P Aranda, H Ben Rhaiem, A Ben Haj Amara, E Ruiz-Hitzky. ZnO/clay nanoarchitectures: synthesis, characterization and evaluation as photocatalysts. Appl Clay Sci. 2016;131:131-139. Doi:org/10.1016/j.clay.2015.12.013
18. Becker EW. Microalgae: Biotechnology and Microbiology, Cambridge University Press, Cambridge, UK. 1994.
19. Whyte J N C. Biochemical composition and energy content of six species of phytoplankton used in mariculture of bivalves. Aquaculture. 1987;60(3-4):231-241.
20. Lowry O H, Rosebrough N J, Farr A L, Randall R J. Protein Measurement with the Folin Phenol Reagent. The Journal of Biological Chemistry. 1951;193(1):265-275.
21. Gerhardt P, Murray R G E, Wood W A, Krieg N R. Methods for General and Molecular Bacteriology. ASM Washington DC. 1994.
22. Leyva A, Quintana A, Sanchez M, Rodriguez E N, Cremata J, Sanchez J C. Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation. Biologicals. 2008;36(2):134-141.
23. Folch J, Lees M, Stanley G H S. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem. 1956;226(1):497-509.
24. Bai W, Zhang Z, Tian W, He X, Ma Y, Zhao Y, et al. Toxicity of zinc oxide nanoparticles to zebra fish embryo: a physicochemical study of toxicity mechanism. J Nanoparticle Res. 2010;12(5):1645-1654. Doi:org/10.1007/s11051-009-9740-9
25. von der Kammer F, Ottofuelling S, Hofmann T. Assessment of the physico-chemical behavior of titanium dioxide nanoparticles in aquatic environments using multi-dimensional parameter testing. Environ Pollut. 2010;158(12): 3472-3481. Doi:org/10.1016/j.envpol.2010.05.007
26. Tiede K, Hassellov M, Breitbarth E, Chaudhry Q, Boxall A B. Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles. J Chromatogr A. 2009;1216(3):503-509. Doi:10.1016/j.chroma.2008.09.008
27. Oukarroum A, Bras S, Perreault F, Popovic R. Inhibitory effects of silver nanoparticles in two green algae, *Chlorella vulgaris and Dunaliella tertiolecta*. Ecotoxicol Environ Saf. 2012;78:80-85. Doi: 10.1016/j.ecoenv.2011.11.012
28. Oukarroum A, Zaidi W, Samadani M, Dewez D. Toxicity of nickel oxide nanoparticles on a freshwater green algal strain *of Chlorella vulgaris*. BioMed Research International. 2017;ArticleID 9528180. Doi:org/10.1155/2017/9528180
29. Chen P, Powell B A, Mortimer M, Ke P C. Adaptive Interactions between Zinc Oxide Nanoparticles and *Chlorella* sp. Environ Sci Technol. 2012;46(21):12178-12185. Doi:10.1021/es303303g
30. Morgalev Y N, Kurovsky A V, Gosteva I A, Morgaleva T G, Morgalev Y S, Burenina A A, et al. Influence of metal-containing nanoparticles on the content of photosynthetic pigments of unicellular alga *Chlorella vulgaris* Baijer. Nano Hybrids and Composites. 2017;13:255-262. Doi:10.4028/www.scientific.net/NHC.13.255

**31.** Karunakaran G, Jagathambal M, Gusv Alexander, Kolesnikov E, Kuznetsov D. Assessment of FeO and MnO nanoparticles toxicity on *Chlorella pyrenoidosa*. Journal of Nanoscience and Nanotechnology. 2017;17(3):1712-1720. Doi:org/10.1166/jnn.2017.12927

**32.** Pendashte H, Shariati F, Keshavarz A, Ramzanpour Z. Toxicity of zinc oxide nanoparticles to *Chlorella vulgaris* and *Scenedesmus dimorphus* algae species. World Journal of Fish and Marine Sciences. 2013;5(5):563-570. Doi: 10.5829/idosi.wjfms.2013.05.05.74127

**33.** Melegari S P, Perreault F, Costa R H R, Popovic R, Matias W G. Evaluation of toxicity and oxidative stress induced by copper oxide nanoparticles in the green alga Chlamydomonas reinhardtii. Aquat Toxicol. 2013;142:431-440. Doi:10.1016/j.aquatox.2013.09.015

**34.** Shaul O. Magnesium transport and function in plants: the tip of the iceberg. Biometals. 2002;15(3):307-321. Doi: org/10.1023/A:1016091118585

**35.** Sarma S J, Das R K, Brar S K, Bihan Y L, Buelna G, Verma M, et al. Application of magnesium sulfate and its nanoparticles for enhanced lipid production by mixotrophic cultivation of algae using biodiesel waste. Energy. 2014;78:16-22. Doi: org/10.1016/j.energy.2014.04.112

**36.** Sabatini S E, Juarez A B, Eppis M R, Bianchi L, Luquet C M, Rios de Molina Mdel C. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. Ecotoxicology and environmental safety. 2009;72(4):1200-1206. Doi: org/10.1016/j.ecoenv.2009.01.003

**37.** Mohamed Z A. Polysaccharides as a protective response against microcystin-induced oxidative stress in *Chlorella vulgaris* and *Scenedesmus quadricauda* and their possible significance in the aquatic ecosystem. Ecotoxicology. 2008;17(6):504-516. Doi:10.1007/s10646-008-0204-2

**38.** Griffiths M J, Harrison S T L. Lipid productivity as a key characteristic for choosing algal species for biodiesel production. J Appl Phycol. 2009;21(5):493-507. Doi: org/10.1007/s10811-008-9392-7

**39.** Eroglu E, Eggers P K, Winslade M, Smith S M, Raston C L. Enhanced accumulation of microalgal pigments using metal nanoparticle solutions as light filtering devices. Green Chem. 2013;15:3155-3159. Doi:10.1039/C3GC41291A

**40.** Mykhaylenko N F, Zolotareva E K. The effect of copper and selenium nanocarboxylates on biomass accumulation and photosynthetic energy transduction efficiency of the green algae *Chlorella vulgaris*. Nanoscale Res Lett. 2017;12(1):147. Doi: 10.1186/s11671-017-1914-2

**41.** He M, Yan Y, Pei F, Wu M, Gebreluel T, Zou S, et al. Improvement on lipid production by *Scenedesmus obliquus triggered by low dose exposure to* nanoparticles. Sci Rep. 2017;14;7(1):15526. Doi: 10.1038/s41598-017-15667-0.

**42.** Padrova K, Lukavsky J, Nedbalova L, Cejkova A, Cajthaml T, Sigler K, et al. Trace concentrations of iron nanoparticles cause overproduction of biomass and lipids during cultivation of cyanobacteria and microalgae. J Appl Phycol. 2015;27(4):1443-1451. Doi:10.1007/s10811-014-0477-1