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Effect of Zinc Ferrite nanoparticles on the growth of *Chlorella pyrenoidosa.*

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ABSTRACT

We report the effect of $ZnFe_2O_4$ nanoparticles on fresh water micro algae *Chlorella pyrenoidosa* in a dose dependent manner. The zinc ferrite nanoparticles of particle size (14 ± 2)nm were prepared by Sol – gel technique. The structural and elemental analysis of the prepared nanoparticles was studied using XRD, TEM, FTIR and XRF. The magnetic measurements indicated superparamagnetic nature of the prepared samples. At low concentration $ZnFe_2O_4$ showed a stimulatory effect on *Chlorella pyrenoidosa,* but algal growth was found to be retarded as the nanoparticle concentration increased. It was found that the algal growth was inhibited by 47% even at a low concentration of 2µM which is a very promising result. The negative effect of $ZnFe_2O_4$ nanoparticles on *Chlorella pyrenoidosa* was manifested by the progressive depletion in algal chlorophyll content and cell count. The result indicates that $ZnFe_2O_4$ have the potential to be used both for enhancing the growth of microalgae as well as for inhibiting algal growth by proper control of its concentration. The superparamagnetic property of zinc ferrite can be exploited as a cheap bioengineering strategy for separation of algae cells. To the best of our knowledge, the effect of ferrite nanoparticles on algae has not been reported elsewhere.

Keywords: Chlorella pyrenoidosa, Zinc ferrite, chlorophyll content, superparamagnetic property.

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INTRODUCTION

Spinel ferrite MFe_2O_4 (M = Zn, Mg, Co, Mn, Ni) is one of the most widely used magnetic oxides. Production of ferrite nanoparticles has increased in the recent years due to their large range of application in technical as well as biomedical fields; as a result large amount of nanoparticles may reach the environments voluntarily or involuntarily, where their fate and behavior are largely unknown. Nanoparticles show a different toxicity profiles compared to their bulk counterpart due to their potential to interact more effectively with biological systems due to their nano size and huge surface area.

Algae being the first level of the trophic chain play a vital role in the equilibrium of aquatic eco-systems. This study is therefore aimed to explore the influence of $ZnFe_2O_4$ nanoparticles on *Chlorella pyrenoidosa*. *Chlorella pyrenoidosa* is a potential candidate to normalize body functions in patients with fibromyalgia, hypertension or ulcerative colitis [1]. However the unwanted and prolific growth of algal weeds may clog the water channels and water supply leading to fowling of water bodies.

Many studies have reported the effect of nanoparticles on algal growth. The effect of oxide nanoparticles (Al_2O_3, SiO_2, ZnO, TiO_4) to green algae *Chlorella* sp was investigated by Jing *et al.,* [2]. The evidence of surface modification of *Chlorella vulgaris* cells using magnetite particles was put forward by Prochazkova *et al.,* [3]. Oukarroum *et al.,* reported the inhibitory effect of silver nanoparticles on *Chlorella vulgaris* and *Dunaliella tertiolecta* [4]. The use of $ZnFe_2O_4$ nano particles provides a cheaper way to control algal weed together with the possibility of magnetic separation. To the best of our knowledge, no study has been reported on the effect of ferrite nanoparticles on algal growth. The result from this study would facilitate a cheaper technique both for cultivation as well as removal of *Chlorella pyrenoidosa* and other microalgae based on proper tuning of the concentration of $ZnFe_2O_4$ nanoparticles.

EXPERIMENTAL DETAILS

Synthesis of $ZnFe_2O_4$ nanoparticles

Zinc ferrite ($ZnFe_2O_4$) nanoparticles were prepared by sol-gel technique [5]. Stoichiometric ratio of AR grade Ferric nitrate ($Fe(NO_3)_3.9H_2O$), Zinc nitrate ($Zn(NO_3)_2.6H_2O$), (99.9% pure Merck) were dissolved in ethylene glycol, at room temperature. The solution was heated at 60^0C to form a wet gel. This gel was then dried at 120^0C for 6 hrs, which self-ignites to yield a voluminous and fluffy product. The product was then grounded well to yield fine $ZnFe_2O_4$ nanoparticles.

Culturing of the algae

Experiments were performed with fresh water green algae coming under the division Chlorophyta, Chlorella pyrenoidosa maintained at the Marine Botany Laboratory, Dept of Marine Biology, Microbiology & Biochemistry, School of Marine Sciences, CUSAT. 15ml exponentially growing Chlorella pyrenoidosa with cell density of 6×10^4 cells/ml was inoculated into a 250ml Erlenmeyer flasks containing 150ml Walnes medium [6] in the presence and absence of the test substance. The culture without zinc ferrite was kept as the control. Different concentrations of $ZnFe_2O_4$ 0.5µM, 1.0µM, 1.5µM, 2.0µM were added to the test culture. The cultures were incubated at the optimum conditions of growth for 14 days. Figure 1 represents the experimental set up for the experiment. Illumination was provide by two cold white fluorescent light of 1250 lux each for a light/dark period 12:12 hours. Cultures were maintained at room temperature (30 ± 2^0C).

Characterization of $ZnFe_2O_4$ nanoparticles

The structural characterization of the prepared sample was done using the X – ray diffraction (XRD) pattern recorded using BRUKER made AXS D8 ADVANCE powder X – ray Diffractometer with Cu – Kα radiation (λ = 1.5406Å) at 40kV and 35mA from 20^0 to 80^0 in steps of 0.02^0 per second. The obtained XRD pattern was refined using the GSAS program [7] developed by Larson and Van Dreele. The detail of the refinement method is explained in one of our previous work [8]. FTIR spectrometer [Thermo Nicolet Avatar 370] was used to record the Fourier Transform Infrared (FTIR) spectrum of the samples in the range 4000 cm^{-1} to 400 $cm^{-1}$with potassium bromide (KBr) as binder. The particle size of zinc ferrite nanoparticles were confirmed by Transmission Electron Microscope (Philips CM200) operated at 20 – 200kV with resolution 2.4 Å. Magnetic

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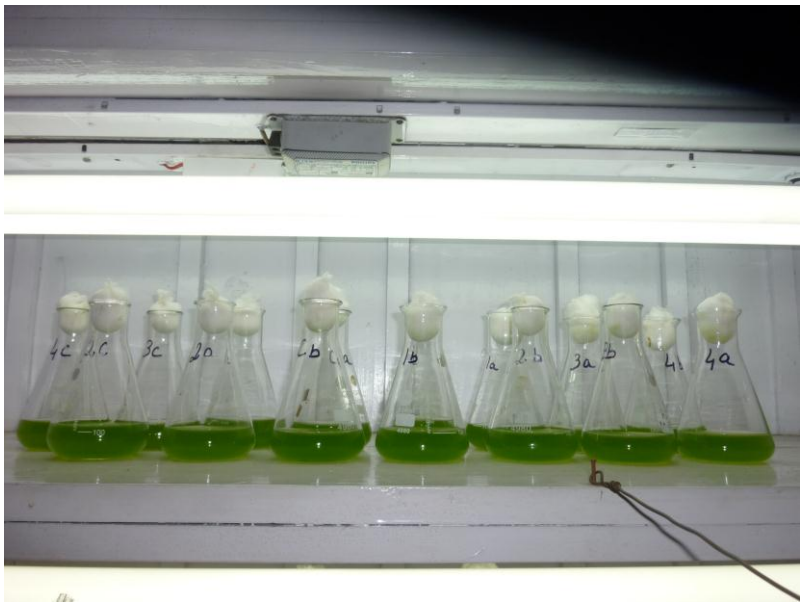
studies at room temperature was carried out using vibrating sample magnetometer (VSM Lakeshore 7410) up to a maximum field of 15kOe.

Figure 1: Experimental setup for algae cultivation.



Algal growth inhibition study

Algal growth inhibition study was performed by regular measurement of algal chlorophyll content and cell count at an interval of 48 hrs. Neubauer hemocytometer was used to measure the algal cell density in cell numbers (algal cell/ml). The specific growth rate was calculated using the equation [9].

$$\mu = \frac{Ln\, N_t - Ln\, N_0}{t_n} \quad \text{---------- (1)}$$

Where N_t is the measured final cell density, N_0 the initial cell density (6 x 10^4 cells/ml), t_n is the period of incubation (in days).

The chlorophyll content of the algal cells was measured using modified Strickland and Parson method [10], where 1ml of the culture sample was withdrawn and filtered through 45mm Whatman GF/C filter paper(gentle vacuum filtration). The filter paper containing algal cells were introduced into a screw capped test tube containing 10ml of 90% (v/v) acetone. The test tubes were covered with aluminum foil to ensure the absence of light and were incubated in a dark room for 1 hr. The clear supernatant was taken for pigment quantification. Absorbance (O.D) of the sample was measured at 750nm, 665nm, 645nm and 630nm with 90% acetone as blank using a HITACHI-U 3900 UV – Vis Spectrophotometer**.** The absorption at 750nm is subtracted from the absorption values of all other measured wavelengths to nullify the effect of turbidity produced by the filter paper in the sample. The amount of Chlorophyll **a** in the sample is calculated using the equation [11].

$$Chlorophyll\ a = 11.85(O.D\ 665) - 1.54(O.D\ 645) - 0.08\,(O.D\ 630) \quad \text{---------- (2)}$$

$$Chlorophylla\ (gm/l) = \frac{Chla \times Extract\ volume\ (ml)}{Volume\ of\ sample\ (l)} \quad \text{---------- (3)}$$

Inhibitory rate of growth was obtained by the formula [12]

$$Inhibitory\ Rate\,(IR)\% = \left(1 - {}^{N}/_{N_0}\right) \times 100\% \quad \text{---------- (4)}$$

Where N is the cell density (cell per milliliter) in the Zinc ferrite treated culture N_0 is the cell density (cell per milliliter) in the control culture.

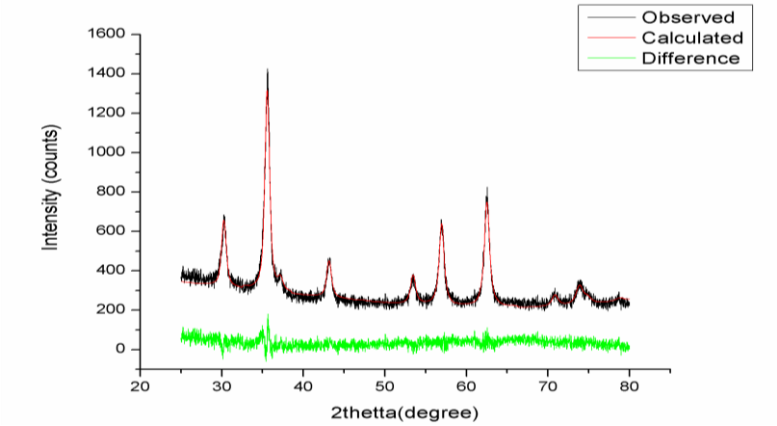
RESULT AND DISCUSSION

Structural Analysis

X - Ray diffraction Studies

Figure 2 represents the Rietveld refinement pattern of $ZnFe_2O_4$. The pattern was compared with standard data (JCPDS card No: 82 – 1042). X ray data points are shown by plus marks; the solid line is the best fit to the data and tic mark shows the positions for the allowed reflections. The lower curve represents the difference between the observed and calculated profiles. The values of goodness of fit (χ^2) reliability factor (R_p and R_{wp}) are 1.574, 7.12%, 5.72% respectively.

Figure 2: Rietveld refinement pattern for ZnFe2O4 nanoparticles.



The lattice constant calculated by Rietveld method was found to be 8.438Å and was in good agreement with the standard values. The low value of χ^2, R_p, R_{wp} confirms the goodness of refinement. The crystallite size of the sample was calculated using Scherrer formula from the most prominent peak (311) and was found to be (13±1) nm.

FTIR Spectra

FTIR spectrum is depicted in figure 3. Two main absorption bands were observed in the range (600 – 500) cm^{-1} and (450 – 385) cm^{-1}, which are attributed to the vibration of tetrahedral and octahedral metal – oxygen bands in the lattice of the nano particles. The band around 3400 cm^{-1} and 1600 cm^{-1} are contributions of O – H stretching vibrations of free and absorbed water molecules [13].

Figure 3: FTIR spectrum of zinc ferrite

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TEM Analysis

Figure 4(a) shows the representative TEM image of $ZnFe_2O_4$. From the figure it is clear that most of the nano particles are spherical in shape and agglomerated. The agglomeration of the nanoparticles may be due to the tendency of nanoparticles to achieve low surface energy state by reducing the interface with other particles [14]. Figure 4(b) shows the histogram of particle size distribution. The average particle size was found to be (14±2) nm. The value is comparable to the crystallite size obtained from XRD.

Figure 4(a): TEM photograph of ZnFe2O4

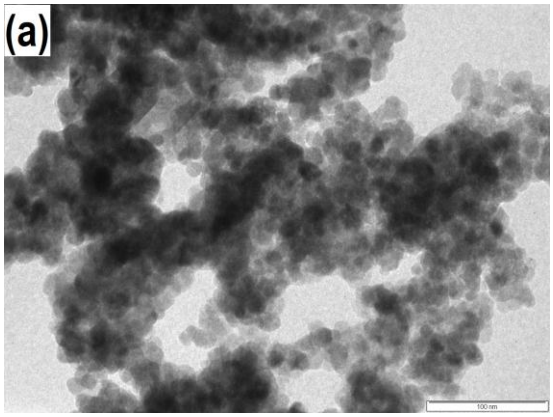
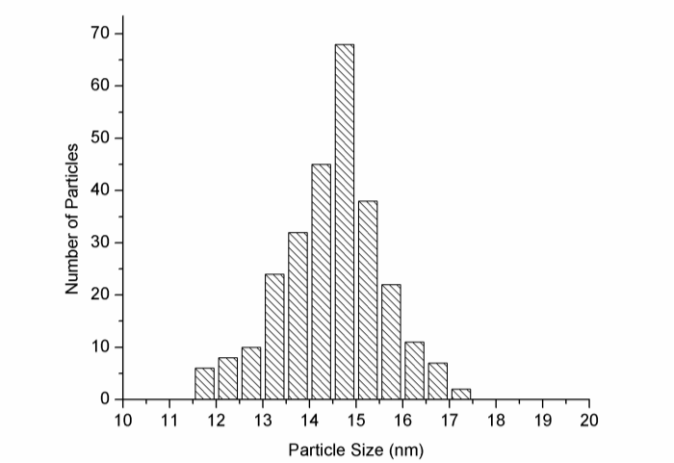


Figure 4(b): shows the size distribution histogram of ZnFe2O4.



XRF Analysis

Wavelength Dispersive X ray Fluorescence Spectrometer (WD – XRF) was used to analyze the stoichiometry of the prepared zinc ferrite sample. From the table it is obvious that sample showed expected stoichiometry. No trace of impurity elements was found which indicates the purity of the sample.

Magnetic Studies

Figure 5 shows the H – M curve of the Nanosized zinc ferrite sample. It is clearly seen that the sample exhibits very low value of coercivity and almost zero value of retentivity indicating the super paramagnetic nature. Furthermore from the hysteresis curve it is clear that saturation magnetization is not achieved even at 15kOe, which points to the presence of super paramagnetic and single domain particles [15].

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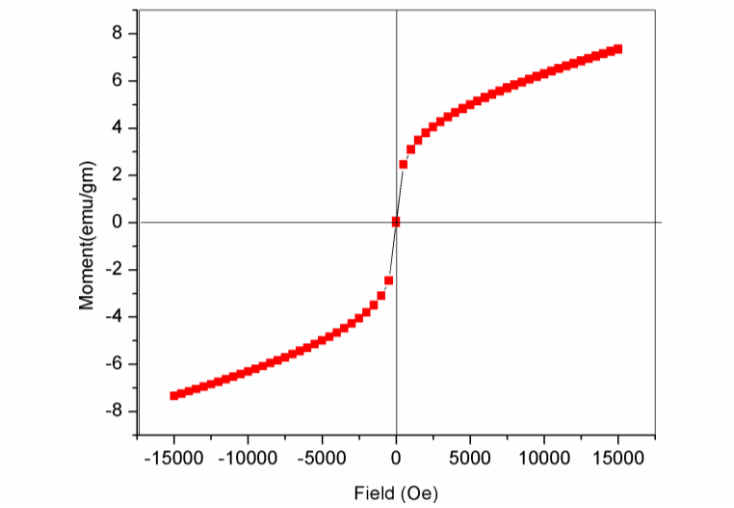
Figure 5: H – M Plot for ZnFe2O4.

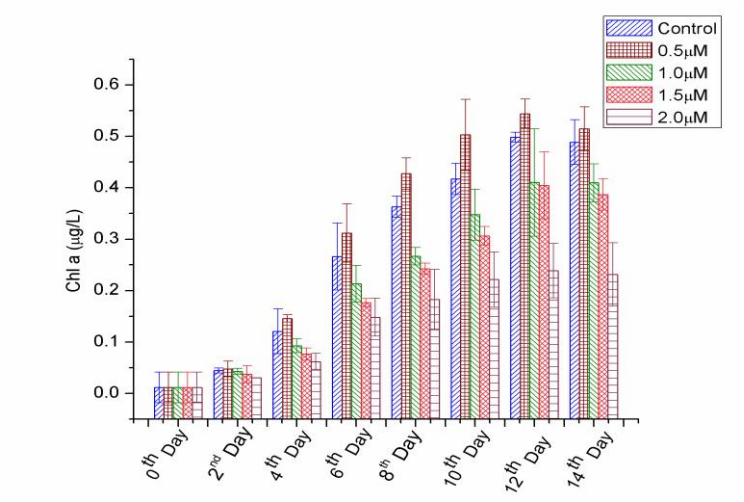


Bio toxicity of nano $ZnFe_2O_4$ to algae

Photosynthesis pigment analysis

Effect of Zinc ferrite nano particles on pigment content as a measure of algal growth and photo synthetic efficiency was investigated. Figure 6 shows the variation of Chlorophyll **a** for a time gap of 48hrs for the control (without nano particles) and for four different concentrations (0.5µM, 1.0µM, 1.5µM, 2.0µM) of $ZnFe_2O_4$.

Figure 6: Chlorophyll a for different concentrations of ZnFe2O4 at an interval of 48 hrs, values are reported as mean of three replicates ± standard deviation (SD).



Content of Chlorophyll **a** exhibited a significant reduction with exposure to $ZnFe_2O_4$ nanoparticles in contrast to the control (untreated) value except for the lowest concentration of nanoparticles (0.5µM). Similar abnormal increase of growth at low concentration of $ZnFe_2O_4$ has been reported by Oprisan et al. [16] on Helianthus annuus. This abnormal behavior of zinc ferrite on Chlorella pyrenoidosa may be due to the fact that even if zinc and iron are essential micronutrients for algal metabolism, they can also be toxic and inhibit metabolic processes when applied in amounts higher than the optimal level [17]. Several factors may be responsible for this observed variability in algal toxicity. Abiotic factors like composition of culture and test medium can significantly affect the tolerance level of the organism [18].

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Effect of $ZnFe_2O_4$nanoparticles on number and properties of algale cells.

From the figure 7 it is clear that the cell density shows an increase than the control for the lowest concentration of nanoparticles tested (0.5µM) whereas cell density shows a decrease thereafter with increase in $ZnFe_2O_4$ concentrations. This is clear indication of stimulatory effect of growth at low concentrations and inhibitory effect at high concentrations by Zinc ferrite on Chlorella pyrenoidosa. It was noted that the cell count diminished up to 48% when the concentration of the magnetic particles was 2µM. The Inhibitory rate (IR) varied from 10% to 47% with the small variation of concentration of 1µM to 2µM which is a very promising result.

Figure 7: Cell count for different concentrations of ZnFe2O4 at an interval of 48 hrs,values are reported as mean of three replicates ± standard deviation (SD).

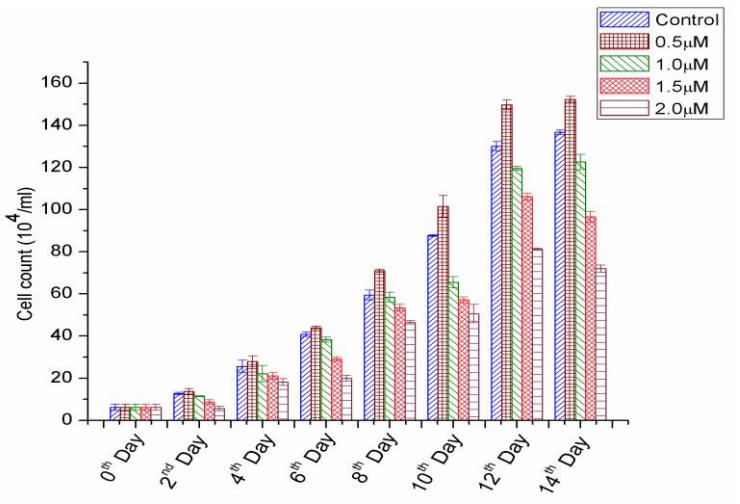

Figure 8 illustrates the relationship between overall growth rate after 14 days and the cell diameter for different concentrations of $ZnFe_2O_4$. It is evidently seen that growth rate and algal cell diameter showed an inverse relationship. The culture with nanoparticles at 0.5µM concentration showed the highest growth rate and lowest cell diameter, whereas the culture with the highest concentration (2µM) showed the lowest growth rate and cells with largest diameter. The increases in cell diameter might be due to adsorption of the nanoparticles on cell surface. In another related study an increase in cellular weight was reported due to TiO_2 nano particles absorbed into the algal cells [19].

Figure 8: Relationship between overall growth rate and cell diameter for different concentrations of Zn Fe2O4,

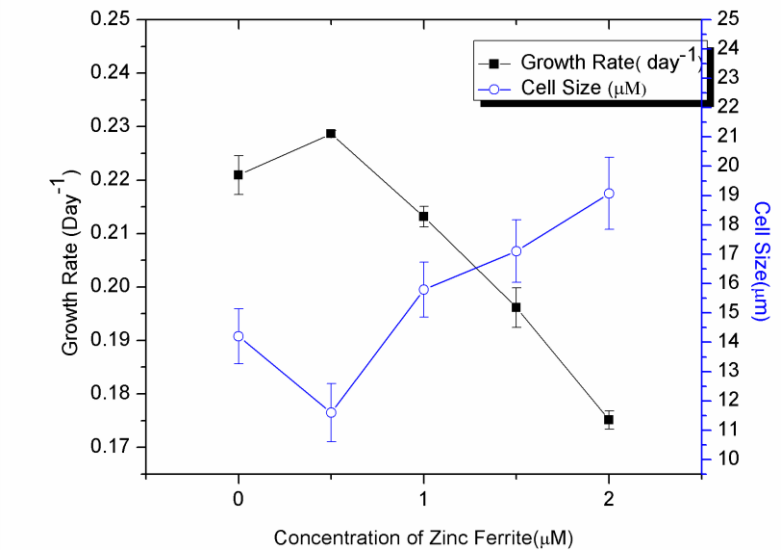

Figure 9(a) shows the interaction of algal culture with 2µM $ZnFe_2O_4$ nanoparticles, algal cells are found settled at the bottom of the flask. Figure 9(b) & 9(c) are the phase contrast microscope images showing morphology of un-interacted cells of *Chlorella pyrenoidosa* and clumped algal cells after interaction.

Figure 9 (a:) Interaction of algal culture with 2M ZnFe2O4 nanoparticles indicating the settling of algae at the bottom of the flask.

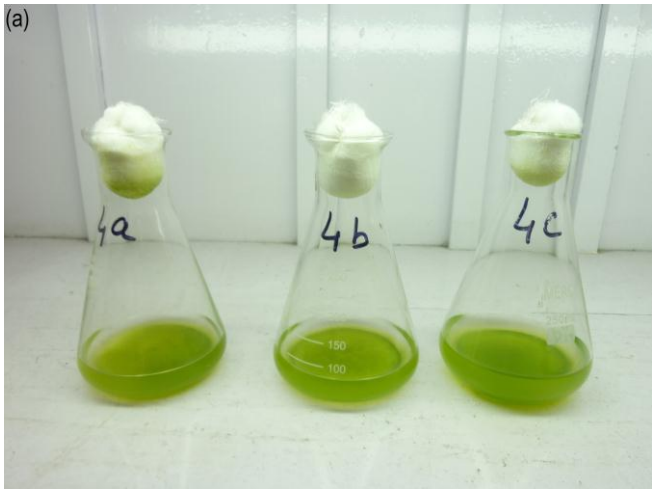

Figure 9 (b:) Phase contract microscope image showing morphology of un-interacted cells of Chlorella pyrenoidosa.

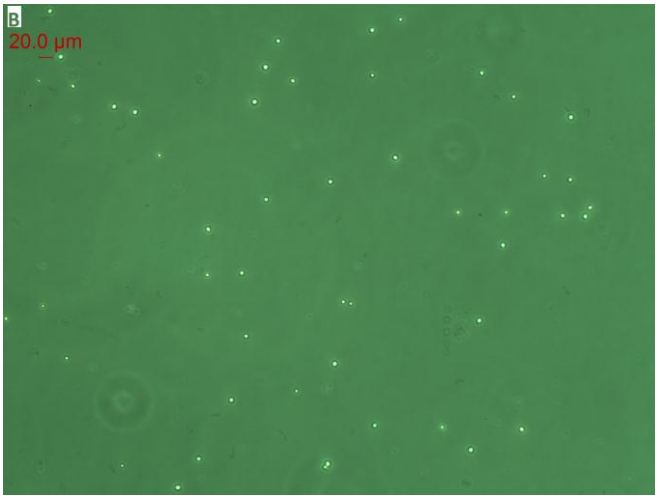

Figure 9 (c): Phase contrast microscope images showing clumped algal cells after interaction.

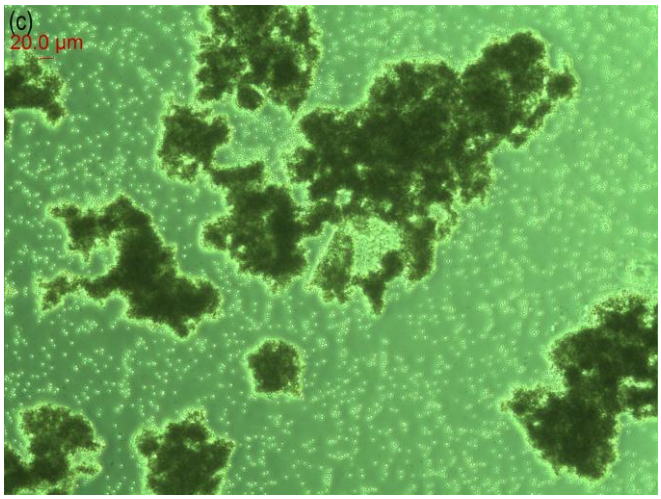

Table 1: Value of concentration of elements present in zinc ferrite compared with expected concentration

Elements present	Concentration(%) in the prepared sample	Expected concentration (%) in the sample
Fe	43.370	42.331
O	26.500	26.548
Zn	26.170	27.121

The phase contrast images of the algae after interaction with the nanoparticles showed clearly damaged cells with abnormal cells and cell debris. The agglomeration tendency of nanoparticles could be due to the absorption of nanoparticles into the algal cells. There could be several chemical and physical factors deciding the toxicity of nano particles towards algae like concentration, size, composition, ion dissolution etc. Here together with the toxicity of the trace metal zinc in $ZnFe_2O_4$ at higher concentrations, the opacity of nanoparticle suspension causing shading effect can also indirectly play a role in nano particle toxicity towards algae. Navarro *et al.,* in one of his studies have pointed out physical resistance to light as one of the indirect mechanism to decrease algal growth rate with nanoparticles [20]. However further studies are needed to obtain a mechanistic understanding of the algal toxicity effect.

CONCLUSION

In this study we focused on the effect of zinc ferrite nanoparticles towards the green algae *Chlorella pyrenoidosa* for the first time. $ZnFe_2O_4$ nanoparticles were synthesized by sol gel technique. XRD analysis revealed spherical single phased cubic spinel structure having space group Fd3m. The absorption bands in FTIR are found in the expected range of spinel ferrite. The magnetic measurements indicated superparamagnetic nature. *Chlorella pyrenoidosa* can be used as a daily dietary supplement for reducing high blood pressure, serum cholesterol limit and to enhance immune functions, whereas their dense and unwanted growth may affect irrigation, fishing, and municipal water supply hindering the flow of water. Our results indicate that $ZnFe_2O_4$ at low concentration shows a stimulatory effect on growth of *Chlorella pyrenoidosa* but at higher concentrations inhibit the growth. This dual influence of zinc ferrite on the algal growth depending upon the concentration , may be due to the fact that even if zinc and iron are essential micronutrients for algal metabolism they can be toxic and can inhibit metabolic processes when applied in amounts higher than the optimal level. It was found that the algal growth was inhibited by 47% even at a low concentration of 2µM which shows that zinc ferrite nanoparticles are promising agents for the control of undesirable algal growth. The superparamagnetic property of the synthesized zinc ferrite can be used as a quick and cheap bioengineering strategy for separation of algal cells.

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