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ARTICLE

MORPHOLOGY, PHENOTYPIC RESPONSES AND VIABILITY OF HAPTOPHYCEAE MARINE MICROALGA *PAVLOVA LUTHERI* DURING Cu (II) EXPOSURE – A LABORATORY STUDY

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ABSTRACT

Background: *Metal pollution has provoked the disorder in aquatic ecosystem; nevertheless some dissolved metals serve as nutrient in microalgal physiology. Microalgae form the base of a food chain. Hence, any impairment at the base of a food web will eventually affect the next trophic level. Copper (Cu) is an oligodynamic metal in microalgal physiology and is ubiquitously present in the aquatic environment. In this aspect the impact of Cu (II) was evaluated upon morphology and physiology of Pavlova lutheri (NIOT-3 & EMA Accession No: LT009495), as a model organism.* ***Methods:*** *An optimized monoculture of P. lutheri was utilized during the experiment based on the OECD protocol through growth inhibition, SEM and micrometric assay. During data analysis and illustrations Mean ± S. D. of triplicates were considered.* ***Results:*** *The data revealed the dose dependent decline in the phenotypic profile with increasing concentration of Cu (II) and subsequently morphological injury, alteration in cell size and viability were evidenced substantiating the cellular stress during Cu (II) exposure.* ***Conclusions:*** *The P. lutheri strain may be potential species in removing Cu (II) from the aquatic environment, if exploited efficiently during the application. However, the removal efficiency is subjected to the differential media and biotic or abiotic stress.*

KEY WORDS
Pavlova lutheri, BCF, morphology, Cu (II), phenotypic profile

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INTRODUCTION

Metal pollution has become a serious threat to the environment lately [1]. Metals reach to the environment from various industrial, anthropogenic and natural resources. Ultimately these metal ions from various alochthonus and autochthonous sources reach to the marine ecosystem via surface run-off, precipitation, etc. In marine ecosystem phytoplankton acts as a primary producer and use metal ions as nutrient. Few metal ions, for instance, zinc, molybdenum, copper, etc. at trace quantities serve as essential nutrient whereas other metals, viz. chromium, arsenic, etc. serve as nonessential metal ions in phytoplankton physiology. The presence of essential metal ion in excess or existence of nonessential metal ion in the aquatic matrix brings physiological stress in microalgal physiology. Since phytoplankton plays key role as a primary producer in the aquatic environment and forms the base of a food chain, any impairment at the base has potential to affect the next trophic level.

Copper is one such heavy metal which falls under essential nutrient category for the microalgal physiology at trace level. Though, it can be toxic and hazardous [2] at higher concentration to the aquatic biota with special reference to phytoplankton. It is reported that Cu^{+2} is the most toxic to the unicellular algae and inhibits the photosynthetic activity damaging the cell membrane. Copper is a biologically active metal and plays vital role in photosynthesis during phosphorylation and enzymatic reactions of algal cells [3]. The sensitivity or toxicity of Cu (II) is indirectly proportional to the initial cell density of the phytoplankton and also subjected to the differential efficiency of the exposed microalgal species.

Recently, marine microalgae which belong to the phytoplankton community and also the vital resource for nutrceuticals and biomedical aspects, are being explored for the removal of toxic metal xenobiotics with a new tempo. In comparison to other techniques, for instance, chemical, mechanical, etc., the biological techniques remain ahead in view of its cost effectiveness in terms of energy, economy and efficiency. Though, the ecophysiological aspects of the microalgae have been poorly explored.

In this study, we report the impact of Cu (II) exposure upon the haptophyceae marine microalga *Pavlova lutheri* (NIOT-3 & EMA Accession No: LT009495) as a model organism, during the exponential growth phase of the microalga, on its morphology, phenotypic profile and simultaneous metal uptake in controlled laboratory condition. The results may be useful during the formulation of effluent discharge criteria or strain optimization for the nutracuticals.

MATERIALS AND METHODS

Microalgal culture and growth media

The unicellular marine microalga *Pavlova lutheri* (NIOT-3 & EMA Accession No: LT009495) was received from microalgae culture collection bank of NIOT and explored in Cu (II) exposure as a model organism. The culture was maintained in seawater enriched with f/2 media (Guillard and Ryther, 1962) and was acclimatized upto 5th generation under uniform abiotic conditions [photosynthetically active radiation: 54

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µMol m^{-2} s^{-1}, photoperiod: 12 h : 12 h (light : dark), temperature: 24 ± 2°C]. Initial pH, salinity and cell count were 8.0, 35 psu and 10^6 nos. of cells/mL, respectively.

Toxicant

Cuprous chloride (Cu_2Cl_2) was procured from Merck and used as Cu (II) toxicant. A stock solution of 5000 µg/mL of Cu (II) was prepared and used for the experiments. The required lower concentrations of Cu (II) xenobiotics were prepared through proper dilution of the stock solution using sterile Milli Q water.

Experimental method

The experiment was performed in triplicate based on the protocol of the Organization for the Economic Cooperation and Development (OECD, 201) [4]. The *P. lutheri* cells were treated with various concentrations of Cu (II) in 250 mL conical flask with a culture volume of 100 mL. The concentration of Cu (II), ranging from 0.9 – 6.47 µg/mL, was determined from the initially conducted range finding test.

Followed by, the study was conducted in five groups of Cu (II) concentrations along with control culture of *P. lutheri* for 96 h. Cu (II) dose was applied in each group at appropriate concentration at the 0^{th} h and incubated upto 96 h in the experimental condition. The growth of the culture was monitored at 24 h interval upto 96 h and swirled gently thrice daily to avoid the settling of the culture.

Further, upon determining the median inhibitory concentration (IC_{50}) value of Cu (II) upon *P. lutheri*, the culture was exposed to this concentration in triplicate for 96 h along with control and samples were aliquoted for morphological, physiological, cytotoxicity and micrometric analysis.

Measurement of Growth Parameters

Algal cells were counted using haemocytometer by fixing with Lugol's iodine and visualizing under a microscope (400X, Karl Zeiss Axioscope2), whereas optical density (OD) was measured in a spectrophotometer (660 nm, Shimadzu). The counted cells were expressed as number of cells/mL. Dry weight was measured according to the protocol prescribed by Zhu and Lee [5]. Data were expressed as µg/mL of algal suspension. Chlorophyll-a was measured following the protocol as prescribed by Jeffrey and Humphrey [6] and recorded through a spectrophotometer (Shimadzhu). Trichromatic equation was applied to calculate the Chl-a content. The pigment content was calculated as µg Chla/ mL of microalgal suspension, under consideration of the dilution factors. Nichol's equation (1973): K (/day) = [3.322/ (t_2-t_1)]*(Log N_2/N_1)] was applied to calculate the growth rate; where, N_2 is the OD value at time t_2 and N_1 is the OD value at time t_1; while the percent of growth inhibition (GI) was calculated based on the equation: GI (%) = [($GR_{control}$ - $GR_{concentration}$)/($GR_{control}$)]*100 and successively doubling time (DT) was calculated as DT(h) = (ln2/K), where K is the growth rate.

Metal Analysis

To quantitate the Cu (II) uptake, microalgal biomass was harvested, lyophilized, weighed and then digested with concentrated ultra-pure HNO_3 and H_2O_2 (30%) (1:4) in 100 mL pre-cleaned teflon vessel with closure. Post digestion the samples were analyzed for metal content in ICP-OES (VARIAN 725-ES) under uniform analytical conditions (power: 1.2 KW, plasma flow 15.0 L/min, replicate: 3 and sample uptake delay 5 s). Also, the corresponding media and matrix were analyzed as prescribed by Grasshoff *et al*. [7] with necessary modification. Blanks and spikes were analyzed to validate the digestion process of the spectroscopic analysis, obtaining 95% recovery. In addition, 0.5 µg/mL of multi-elemental standard was analyzed upon every 10 samples to monitor the matrix effects of the analytes and for quality assurance and quality control.

Further, bio concentration factor (BCF) was calculated as defined by Brooks and Rumsby [8] which is the concentration ratio of an element in dry biomass and in the water (culture growth media), whereas % uptake of Cu (II) by the microalgal biomass was calculated as: % Uptake = [µg of Cu (II) in biomass/µg of dry biomass]*100.

Microscopic analysis and cell viability

An aliquot (1 mL) of cell suspension from control and treated samples were harvested to pellet through centrifugation at 6000 rpm for 15 min at 4 °C. The respective pellets were resuspended in 100 µL of 0.1% of Trypan blue (SIGMA). The mixture was incubated at room temperature for 5 min. The cells were visualized and examined through haemocytometer under light microscope (1000X, Karl Zeiss Axioskope2). The % of cell viability was calculated as the ratio of the number of viable cells to the total number of cells and multiplied by 100. Followed by, about 10 µL of the resultant sample was smeared onto a glass slide and sealed with cover slip (Blue Star, No. 0) for observation and capturing image.

Field Emission Scanning Electron Microscopy (SEM Analysis)

Both pristine and treated samples were processed following the protocol of the Huck Institute of Lifesciences [9] and Elumalai *et al*. [10]. Optimization and modifications were carried out during sample preparation to suit the best for our sample of interest. An aliquot (1 mL) of the samples were fixed in buffered glutaraldehyde for overnight at 4 ˚C. The samples were incubated in 4% of OsO_4 and subsequently kept at room temperature in dark. The fixed samples were then dehydrated with series of ascending grade (%) of alcohol and subjected to gold sputter-coating for approximately 30 min. Finally, the

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mounted samples were visualized under HR-SEM (Fei Netherlands, voltage 20 KeV, spot size 3) at high vacuum mode. The corresponding images were captured using XP Microscope Server software.

Fourier Transform Infrared (FTIR) spectroscopy

FTIR analysis was carried out to look into the metal binding functional groups located on the microalgal cell surface. The freeze-dried microalgal cells were subjected to FTIR analysis by KBr method using PerkinElmer Spectrum 1 equipped with Deuterated triglycine sulfate detector (DTGS) within the wave number range 450 – 4000 cm^{-1} (4 cm^{-1} resolution , 3 number of scans, and air as background) in transmission mode [11].

Statistics

Data were subjected to one-way Analysis of Variance (ANOVA) using Microsoft excel in Windows 7 Ultimate. ANOVA effects and treatments were considered significant at $p < 0.05$ and further post hoc analysis was carried out upon finding out the presence of significant difference between the groups. All the dataset presented over here are Mean ± S.D. of triplicates unless otherwise specified. Metal concentration values are reported based on the measured concentration values only.

RESULTS

Microalgal phenotypic characteristics get impaired due to heavy metal exposure [11,12,13,14,15]. The growth profile of *P. lutheri* in the conducted experiment revealed congruency with Fathi *et al.*[12], Namita *et al.*[13], Sucheta *et al.*[11,15] and many other research works carried out earlier. During our investigation it is observed that the duration and exposure concentration of Cu (II) leads to the retardation in growth profile of the exposed *P. lutheri*. The microalga was found to tolerate upto 5.24 µg mL^{-1} of Cu (II). The variations observed were found to be significant ($p < 0.05$) at all concentrations as compared to control. All experimental value reported over here are Mean ± S.D of triplicates. Cu (II) at lower concentrations is favored for the nutrition of microalgae, however, it is reported that in phytoplankton physiology Cu (II) is an oligodynamic metal as it is stimulatory at lower concentration whereas toxic at high levels. The growth retardation [Fig. 1] of *P. lutheri* can be attributed to the presence of the Cu (II) cations in the growth media. The Cu (II) ions create an uneven electrical charge between extracellular and intracellular matrix. Data analysis revealed that the % uptake of Cu (II) by *P. lutheri* is directly proportional to the exposure concentration of Cu (II) which is unlike to our previous investigation on Cr (VI) uptake of *Chlorella vulgaris* [15] while BCF profile was found to follow a declining trend with increasing Cu (II) concentration. Highest BCF (0.34) was found at 0.5 µg/mL of Cu (II) concentration [Fig. 2]. The biomass and chlorophyll-a were found to follow declining trend with increasing concentration of Cu (II) cation. This phenomenon substantiates the fact of potentiality of Cu (II) to affect the photosynthesis [14] of the microalga [Fig. 3].

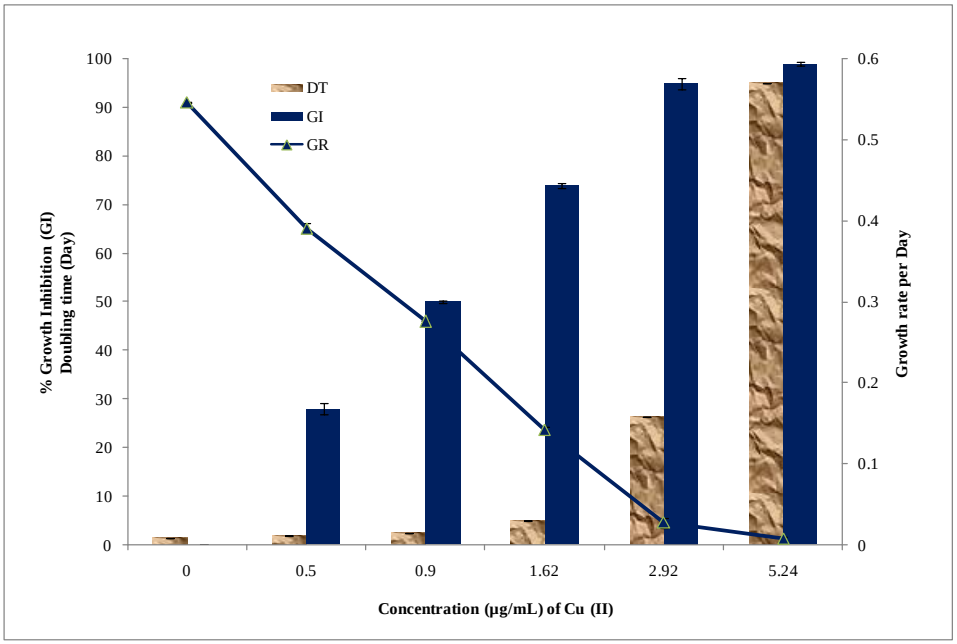

Fig. 1: Dose response curve of *P. lutheri* at various concentrations of Cu (II) after 96 h exposure.

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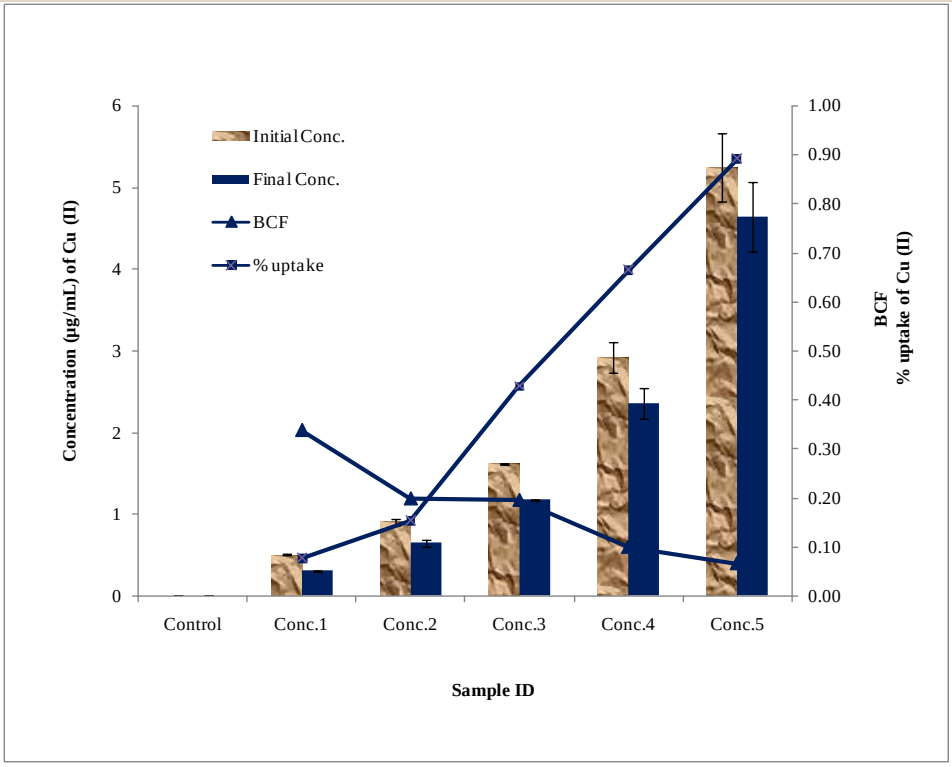

Fig. 2: Metal uptake, BCF and % removal of *P. lutheri* at various concentrations of Cu (II) after 96 h exposure.

Copper tolerant strains of microalga can tolerate exalted concentration through cellular exclusion or through formation of intracellular copper complexes during detoxification mechanism [3]. The Cu (II) removal efficiency of *P. lutheri* from the culture media was documented as 40.0%, 28.0%, 27.8%, 19.3% and 11.4% at the exposure concentration of 0.5 µg/mL, 0.9 µg/mL, 1.62 µg/mL, 2.92 µg/mL, 5.24 µg/mL, respectively [Fig. 2]. Hence, the *P. lutheri* strain may be potential species in removing Cu (II) from the aquatic environment [16].

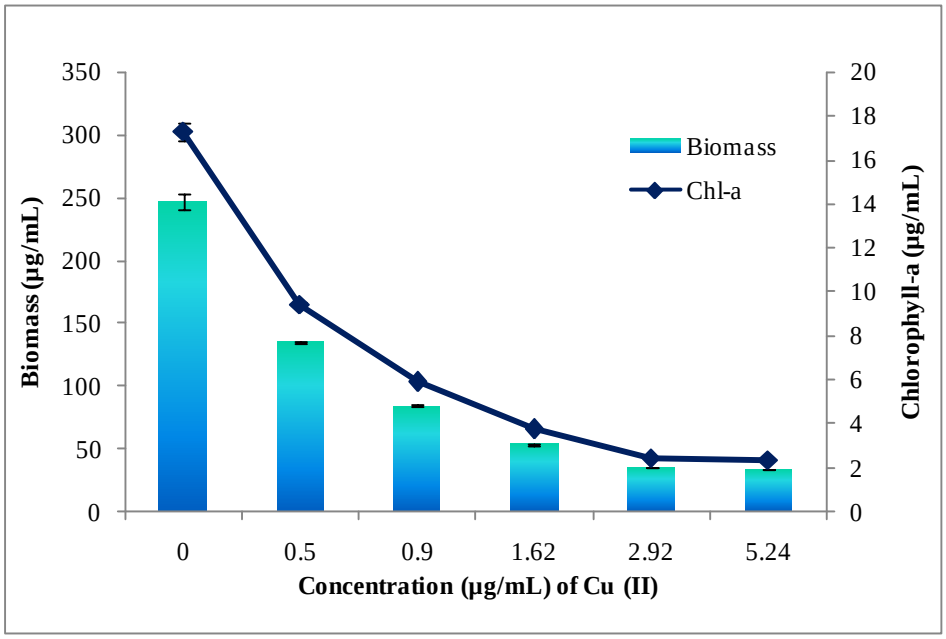

Fig. 3: Chl-a and biomass of *P. lutheri* at various concentrations of Cu (II) after 96 h exposure

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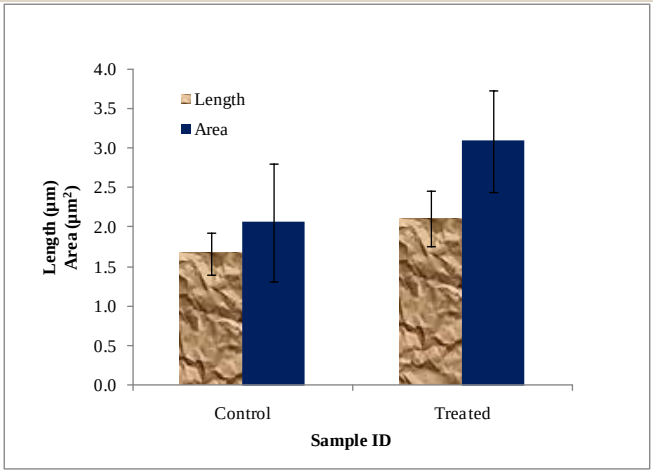

Fig. 4: Cell size of *P. lutheri* at IC_{50} of Cu (II) after 96 h exposure

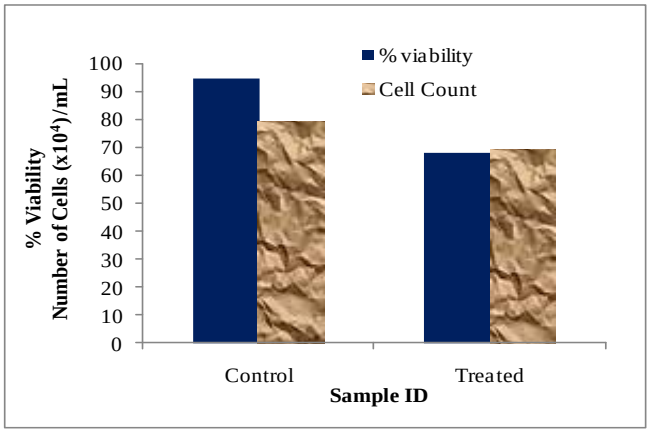

Fig. 5: Cell viability and cell count of *P. lutheri* at IC_{50} of Cu (II) after 96 h exposure

Light microscopic (LM) observations revealed the spherical healthy cells in control culture whereas swollen cells are seen in the Cu (II) treated culture of *P. lutheri* [Fig. 6]. The viability assay disclosed the reduction of cell number due to Cu (II) exposure. At IC_{50} of Cu (II), it is estimated that there was 12.7% reduction in cell count [Fig. 5].

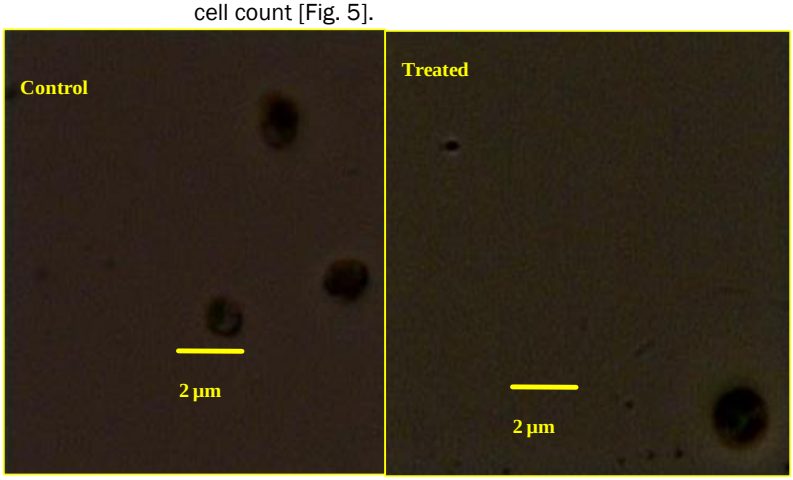

Fig. 6: LM (400X) photomicrograph of *P. lutheri* at IC_{50} of Cu (II) after 96 h exposure

Upon calculation the cell viability was found to be 95% in case of control, whereas 68% for the Cu (II) treated (IC_{50}) microalga at the end of 96 h [Fig. 4]. The amplification of cell size substantiates the fact that the cell expansion occurs during exposure of Cu (II) as compared to that of control. During micrometric analysis of *P. lutheri* at IC_{50} of Cu (II) exposure, it is found that there is an increase in cell length (27%) and in cell area (50%) as compared to the respective control [Fig. 4].

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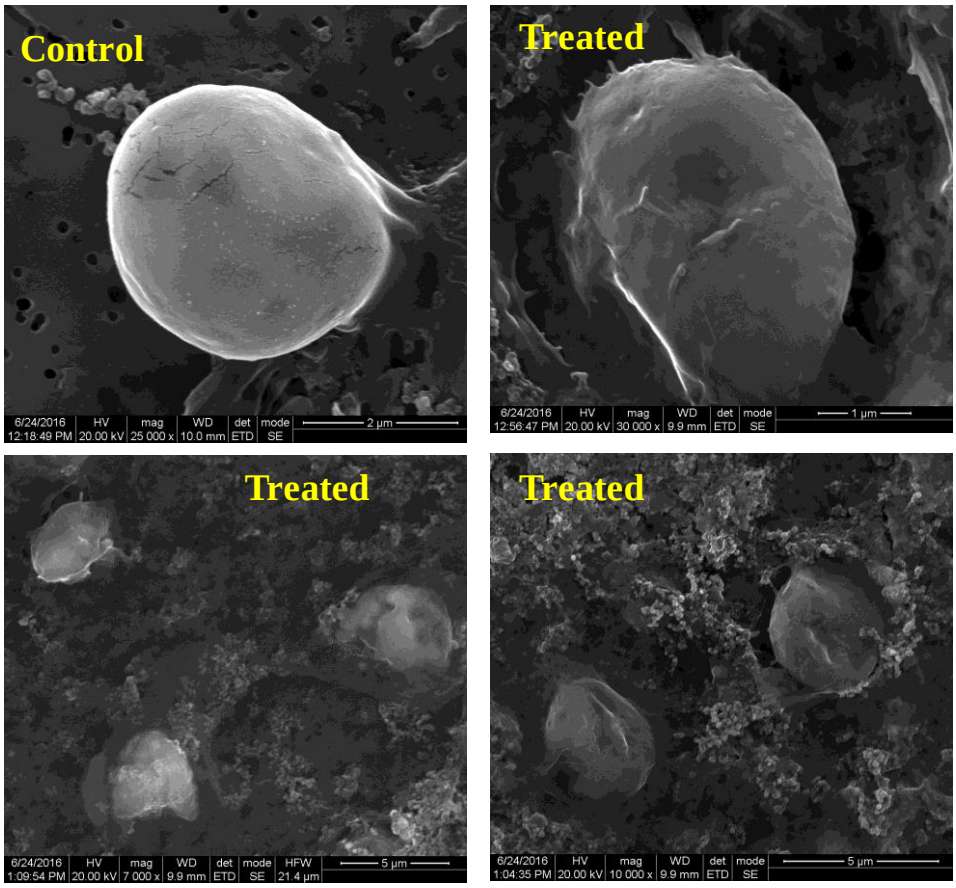

Fig. 7: SEM photomicrograph of *P. lutheri* after 96 h Cu (II) exposure at IC_{50}.

The integrity of the cell membrane of *P. lutheri* was studied through photomicrograph of the microalga using Scanning Electron Microscopy. The cell wall of *P. lutheri* is composed of largely hemicelluloses fibrils and saccharides for instance manose, ramnose, xylose, galactose, glucose, etc. During control condition an irregular smooth surface topology of *P. lutheri* cell was observed [Fig. 7]. The corresponding Cu (II) exposed cells [Fig. 7] were seen with ruptured membrane integrity. The heavy metals are adsorbed passively and assimilated actively from their surrounding niche as during the stress, release of organic ligands takes place into the media [17, 18]. The released ligands efficiently bind metal ions. Also, owing to heterogeneous cell surface, microalgae bind metal ions through various functional groups, for instance, carboxyl, amino, carbonyl, hydroxyl, etc. Though there are a variety of functional groups but the total number of functional groups present on the biosurface is constant leading to a maximum proton adsorption capacity. The efficiency of metal binding property with various functional groups was also revealed through SEM surface micrograph as compared to its respective control [Fig. 7]. During the exposure into the metal solution either heavy metal ions get attached on to the surface primarily or they enter into the cell through cell wall during passive transport of the metal ions. The adherence of metals on the surface of the microalgal cell is devoid of cellular metabolism whereas the ingress of metal into the cell and subsequently accumulation into the cell is dependent on cellular metabolism as reported by Sen and Ghosh Dastidar [22]. From the SEM photomicrograph formation of nanoparticles is also detected. Similar evidence was recorded by Radhika *et al.* [19] in *Chlorella vulgaris* upon treatment with ZnO nanoparticles and Sucheta *et al.* [11] in *P. lutheri* during Cr (VI) exposure.

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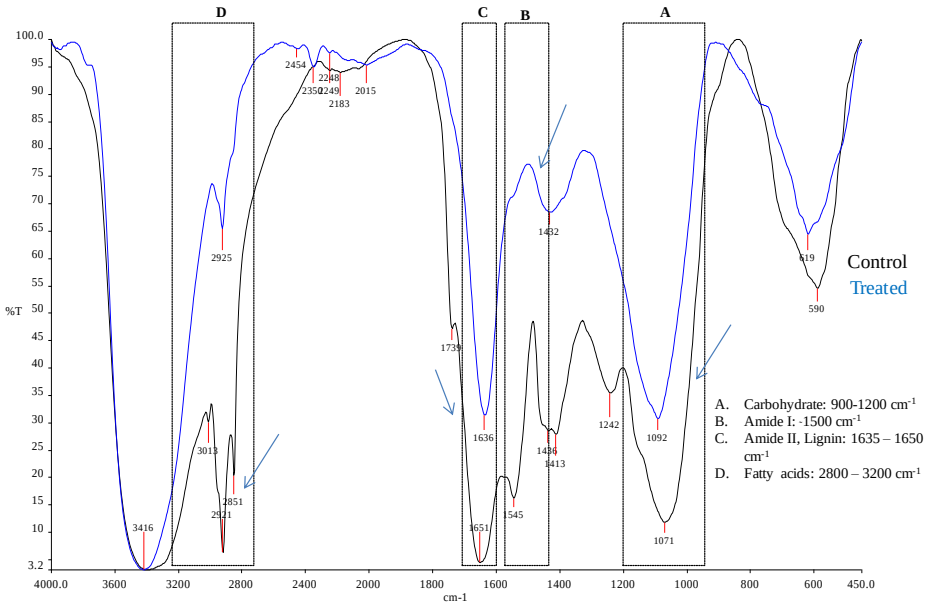

Fig. 8: FTIR spectrum of the control and Cu (II) treated *P. lutheri* at harvest (96 h)

To study the biotic and abiotic stress induced response in microalgae, FTIR spectroscopy was carried out. FTIR spectroscopy is an easy, non-destructive and entrenched technique [20]. The peak intensities in spectrum of the treated one were found to be different as compared to that of the control. These peak modulations substantiated the involvement of the bio molecules located on the cell wall to interact with the Cu (II) ions. These bio molecules exert a whole negative charge and bind the metal ions through their binding sites [21]. Distinct changes were observed in the wave number regions, for instance 900-1200 cm^{-1}, 1500 cm^{-1}, 1635-1650 cm^{-1}, 2800-3200 cm^{-1} in the spectrum of the treated one as compared to that of the control [Fig. 8], which can be attributed to the involvement of the carbohydrate, amide I, amide II and fatty acid moiety, respectively. The surface of the microalgae contains a number of electron dense functional group, for instance, carboxyl (–COOH), hydroxyl (-O-H), carbonyl (-C=O), etc. with high affinity towards positively charged metal ions and act as the binding sites of the metal ion/s to transport the metal ions across the cell wall. Ingestion of metal ions into the cells takes place through two distinct phases, viz. rapid assimilation when equilibrium is established between the intracellular and extracellular metal ion pool and a slower facilitated ion transport into the cytoplasm [22]. An extensive variation of peaks in the fingerprint region (1500 cm^{-1} to 500 cm^{-1}) of the spectra was also observed. The adsorption of metal ion on the microalgal cell surface provides an insight about the initial toxicant loading of the cells which is also a function of the microalgal cell surface area and the nature of the binding sites [23].

CONCLUSION

The present study revealed that the cellular density and growth rate is a function of the exposure concentration of copper and time of exposure as well. Doubling time and growth inhibition was found to be directly proportional with the increasing Cu (II) concentration. *P. lutheri* was found to tolerate up to 5.24 µg/mL of Cu (II), however depending on initial cell density, exposure duration and concentration of copper, the results are likely to vary. The microalga was found to remove 40 % of Cu (II) from the growth media during 0.5 µg/mL of Cu (II) exposure for 96 h, which is recorded as the highest removal efficiency during the experiment. The cell wall of the microalga *P. lutheri* gets damaged during the Cu (II) exposure and subsequently inhibits the photosynthesis process. The microalga can be beneficial for detoxification of Cu (II) while exposed to lower concentration (0.5 -0.9 µg/mL).

CONFLICT OF INTEREST

Conflict of interest is declared none.

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