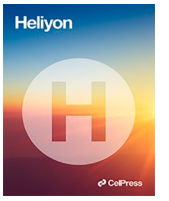
Heliyon 10 (2024) e29131

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Copper removal efficacy and stress tolerance potential of *Leptolyngbya* sp. GUEco1015

Nilamjyoti Kalita *, Partha Pratim Baruah

Plant Ecology Laboratory, Dept. of Botany, Gauhati University, Gopinath Bordoloi Nagar, Jalukbari, Guwahati, Assam 781014, India

A R T I C L E I N F O

Keywords:
Cyanobacterium
Leptolyngbya sp. GUEco 1015
Copper
Antioxidants
Removal efficacy

A B S T R A C T

Cyanobacteria, a group of microalgae are the potent organism having the ability to survive in the copper rich environment and recently gained too much attention for their profuse proliferation in such water bodies. Amongst the members of cyanobacteria, the current study was conducted on *Leptolyngbya* sp. GUEco1015, collected from hydrocarbon rich water bodies of Assam, India. Morphological images of treated samples showed a remarkable damage in the cell surface as well as the organelles over the control. Biochemical results revealed a significant increase of enzymatic and non-enzymatic antioxidants during oxidative damage of Cu^{2+}. But, ascorbate in 1.2 ppm ($p < 0.01$), 1.5 ppm ($p < 0.001$) and catalase content 1.5 ppm ($p < 0.05$) showed a significant reduction after a certain level. The cells were optimized to evaluate the maximum Cu^{2+} removal potential by the cells related to growth. Initial metal concentration 0.1 ppm, pH 7.5, temperature 25 °C and shaking rate 100 rpm are the optimized abiotic parameters which showed maximum 83% of Cu^{2+} removal. FTIR spectroscopy and EDX data has identified a number of notable functional groups that were involved in Cu^{2+} binding mechanism and revealed a distinctive peak of Cu with 0.41 wt % which makes the species as one of the competent copper adsorbents.

1. Introduction

Heavy Metals (HM) are noteworthy widespread pollutants that have negative impacts on environment. Various anthropogenic practices such as open cast mining, smelting industries, petroleum refineries and atomic power plants are some of the major sources for different HMs accumulation. Among those HMs, majority are hazardous viz. cadmium (Cd), lead (Pb), chromium (Cr), nickel (Ni), arsenic (As) etc. and a few like, copper (Cu), zinc (Zn), iron (Fe), manganese (Mn) etc. are essential to living organisms in small quantities for growth and development. But, considerably high percentage of those essential HMs may cause substantial damage to the ecosystem in general and biota in particular. Therefore, several traditional industrial methods such as industrial ion exchange, chemical precipitation, electro dialysis, ultrafiltration, and reverse osmosis technique are recently being used to remove HMs contaminants from the substratum [1,2], however, enormous drawbacks [2] make all these processes unsuccessful in case of few HMs which includes Copper.

Copper (Cu) is one of the toxic heavy metals belonging to the micronutrient group which are generally exaggerated due to both natural (volcanic eruptions, soil erosions, weathering of rocks) and anthropogenic activities (mining, agriculture, pesticides, electrochemical industries). Cu generally has three known varied oxidation states viz. Cu^{0}, Cu (I) and Cu (II), and reports claimed that Cu (II)/Cu^{2+} is the most toxic and powerful complexing agent having a strong affinity towards hydrolysis in its free cationic state [3].

* Corresponding author.
E-mail addresses: nilam2021@gauhati.ac.in, nilamjyotikalita2014@gmail.com (N. Kalita).

https://doi.org/10.1016/j.heliyon.2024.e29131
Received 4 February 2024; Received in revised form 18 March 2024; Accepted 1 April 2024
Available online 6 April 2024

Table 1
LC50 value of copper (Cu^{2+}) and treatments based on it.

Test organism	Metal precursor	Ionic form	LC_{50} Value (ppm)	Treatment based on LC_{50} Value (ppm)
Leptolyngbya sp. GUEco1015	$CuSo4.5H_2O$	Cu^{2+}	0.9	0.3 0.6 0.9 1.2 1.5

Acquaintance of this HMs can be a reason of serious health issues like brain damage, altering blood chemistry, enzymatic activities and metabolic pathways [4].Further, catalytic copper are known to generate reactive oxygen species (ROS) and may lead to carcinogenesis [5].

Considering the hazardous impact of Cu^{2+}, many scientists have hitherto engaged themselves in a challenge to remove Cu^{2+} from the substratum with the intervention of biological organisms throughout the globe. Cyanobacteria mediated removal of metal contaminants has now been socially accepted for their eco-friendly, cost-effective, energy-saving, and environmentally sustainable [6] processes. As Cyanobacteria are the most diverse, photosynthetic planktonic group of algae found in almost every aquatic ecosystem in unicellular to filamentous forms with having myriad functional groups (FG) in their cell walls like, amino ($-NH_2$), carboxyl (-COOH), phosphate ($-PO_3$), hydroxyl (-OH), sulfhydryl (-SH) etc., the members of the group can be assessed for their efficiency in metal binding. The presence of elevated mucilage makes the members of this group a suitable absorbing agent [7,8]. A few can accumulate HMs using metabolism dependent pathways via cell wall and exopolysaccharidic (EPS) layers [9,10]. Further, synthesis of metal-binding peptides, metallothioneins is one of a kind absorption mechanism in cyanobacteria [8]. These peptides can sequester ions inside cells due to their strong affinity for the metal ions which is reflected in the changes of biochemical parameters including enzymatic and non-enzymatic antioxidants.

Considering the aforementioned fact, this communication attempts to regulate the consequence of Cu^{2+} on *Leptolyngbya* sp. GUEco1015, a cyanobacterial species isolated from Polyaromatic hydrocarbon (PAH) contaminated sites of upper Assam, India. The cell surface of the test organism was also characterized to gauge the functional groups involved for Cu^{2+} binding. Furthermore, Cu^{2+} removal efficacy of the isolated strains was then assessed by optimizing the abiotic parameters related to sorption.

2. Materials and methods

2.1. Isolation and maintenance of test organism

Heterocystous cyanobacterial strains, *Leptolyngbya* sp. GUEco1015 was confirmed as a bio-accumulator of copper (minimum of 0.035 mg/gm to a maximum of 2.73 mg/gm) in the form of cyanobacterial mat based on random sampling from a PAH contaminated site (27°23′10"& 95° 36′56″) of Assam, India. Physicochemical parameters like pH and temperature of the sampling sites were recorded to be 7.1 (Minimum) to 8.3 (maximum) and 25 ± 2 °C respectively. Monoculture of *Leptolyngbya* sp. GUEco1015 was done through repeated streaking and serial dilution process. Then pure sample was cultured in Erlenmeyer flask in BG-11 media [11] without nitrogen source at 25 ± 2 °C, pH 7.5 and 3600 lux intensity of light for 7–10 days. The harvested biomass of *Leptolyngbya* sp. GUEco1015 was centrifuged followed by cleansed with deionized water and prepared for testing.

2.2. Cu^{2+} treatment

$CuSo_4.5H_2O$ (EMPLURA^r) was used for preparation of stock solutions by calculating the value of metal ions separately. The amount of metal conferred in the stock was ratified by operating atomic absorption spectrophotometer. For experimental setup stock solution of metal ions were subjected to a series of concentration viz., 0.1, 0.3, 0.6, 0.9, 1.2, 1.5 ppm respectively based on the lethal concentration (LC50) of *Leptolyngbya* sp. GUEco1015 (Table 1).

2.3. Morphological observations

Copper treated *Leptolyngbya* sp. GUEco1015 were harvested at 7th days after incubation and washed several times to avoid ionic contaminants and used for microscopic study. For light microscopic images cells were visualized under Euromax Delphi X observer series microscope following the protocol of Baruah et al. [12,13].

Scanning electron microscopic (SEM) images were taken following the standard protocol of Sadiq et al. [14]. The cultivated samples (treated and non-treated) were repeatedly centrifuged and then rinsed vigorously with deionized water. The samples were plummeted onto a glass cover slip, allowed spreading out uniformly, and air dried at ambient temperature. It is followed by fixation with 3% glutaryldehyde solution for 2 h at 4 °C and washing with 0.1 M phosphate buffer. Subsequently, cultivated organisms were dried in varying percentages of absolute alcohol previously being sheathed with gold and photographed using SEM-Zeiss Sigma 300.

2.4. Growth estimation

2.4.1. Chlorophyll-a

Estimation of Chlorophyll-a was done by following the protocol of Mackinney et al. [15]. Copper treated samples were repetitively washed with double distilled water to get the pellet which is then homogenized in a heated water bath at 65 °C for 30 min after being mixed with 95% methanol. The resulting suspension was then subsequently centrifuged at 3000 rpm for 10 min and the supernatant's absorbance was measured at 650 nm and using 95% methanol as a blank in an ultraviolet–visible spectrophotometer.

2.4.2. Carotenoid

The treated cyanobacterial pellet was collected through centrifugation and washed with double-distilled water to estimate the amount of carotenoid. 3 ml of 80% acetone was added to the pellet before it began to undergo numerous cycles of freezing and thawing until the pellets became colorless. The extract volume was then measured and the volume is adjusted up to 10 ml using 80% acetone. The total amount of carotenoids in mg/ml was then calculated using the optical density or absorbance measured at 450 nm with 80% acetone as the blank. The computed value was done in accordance with Wellburn [16].

2.5. Determination of oxidative injury

2.5.1. Hydrogen peroxide (H_2O_2) content

The amount H_2O_2 was measured using the standard protocol of Sagisaka's [17]. 200 mg of fresh *Leptolyngbya* sp. GUEco1015 cells were blended with 5 percent of TCA (trichloroacetic acid) and at 4 °C centrifugation was performed in 17000×*g* for 10 min. A total of 1.6 mL supernatant was collected and mixed thoroughly with 0.2 mL Potassium thiocyanate (KSCN), 400 μL of 10 mM ferrous ammonium sulphate and then 400 μL of 50% (w/v) TCA. Finally the absorbance was measured with test mixture at 480 nm to get the H_2O_2 content.

2.5.2. Lipid peroxidation activity

Using the approach of Nahar et al. [18], the amount peroxidation of lipid was estimated from the quantity of MDA (malondialdehyde). 200 mg of fresh tissue was homogenized in 5 mL of 0.25% (w/v) TBA (thiobarbituric acid) having 10% (w/v) TCA. The subsequent extract was warmed for 30 min at 95 °C in hot water bath (HWB) previously being permitted to chill in ice. The subsequent composition of the mixture was centrifuged up to 10 min at 4 °C at 10,000g. The supernatant was collected, and the absorbance at 532 nm was measured. Nonspecific turbidity was reduced by subtracting the absorbance value at 600 nm.

2.6. Determination of enzymatic and non-enzymatic antioxidants

2.6.1. Proline content

Proline content of the test organism was extracted following the protocol of Bates et al. [19]. Primarily 3% sulphosalicylic acid was used to disrupt the *Leptolyngbya* sp. GUEco1015 cell using ultrasonicator. 1 mL of the homogenized cell extract was allowed to suspend in the mixture solution of 2 mL acid ninhydrin solution with 2 mL glacial acetic acid and then gestated at HWB for 1 h followed by immediate cooling using ice flakes for 5 min to terminate the reaction. Later, 4 mL of toluene was supplemented to the concoction and allowed for rigorous shaking for 1 min which formed two separate layers. The upper pinkish layer containing proline was then taken and absorbance was quantified at 520 nm.

2.6.2. Total phenol content (TPC)

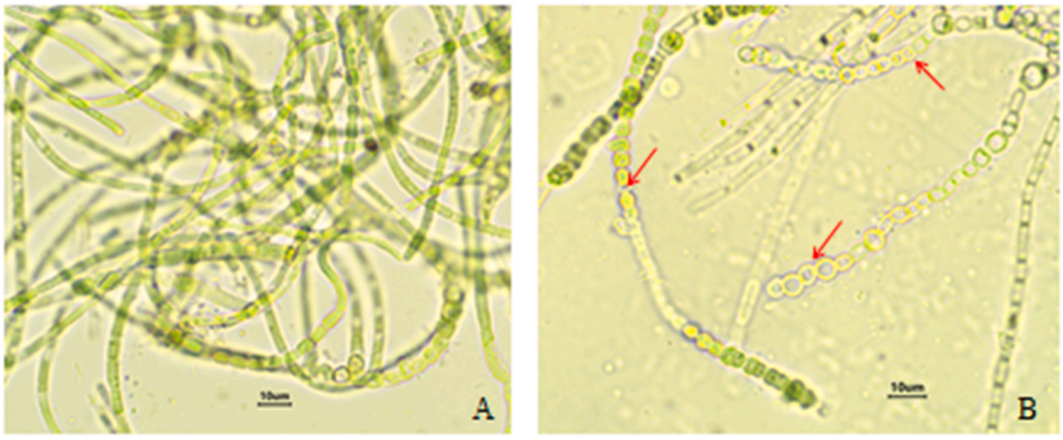
The Folin-Ciocalteu technique was employed to estimate the total phenol content in the *Leptolyngbya* sp. GUEco1015 biomass [20]. 5 mL of 80% ethanol was used for homogenization of the organism and followed by centrifugation at 10000×*g* for 10 min. The resultant supernatant was combined with 5 mL of distilled water and 1 mL of Folin-Ciocalteu's reagent before being incubated at ambient temperature in the absence of light for 5 min. After that, mixture was added with 2 mL of sodium carbonate, and then allowed to store at a comfortable moderate temperature for 1 h in the dark. At the wavelength of 650 nm, the solution's absorbance was calculated in comparison to a blank sample. Further, the TPC of the samples was measured in microgram/mg using gallic acid as standard.

2.6.3. Ascorbate content (AC)

500 mg of fresh *Leptolyngbya* sp. GUEco1015 cells were homogenized in 5% phosphoric acid solution and centrifuged the mixture at 4 °C in 17000 g for 10 min. Immediately after centrifugation 1 mL of supernatant was vortexes with 1 mL Na_2HPO_4, 2 mL 0.15 N H_2SO_4 and 2 mL 2% sodium molybdate. The composition was incubated in a hot water bath at 60 °C up to 40 min and instantly allowed to cool in ice. To obtain the supernatant, the mixture was re-centrifuged at 3000g up to 10 min. Absorbance of the subsequent supernatant mixture was finally measured at 660 nm and AC content was measured using the formula given by Panda et al. [21].

2.6.4. Catalase activity

Catalase (CAT) activity of the test organism was apparently determined following the protocol of Aebi [22]. 100 mL enzyme extract of *Leptolyngbya* sp. GUEco1015 was considered for the study where 100 μL of 100 mM H_2O_2 and 1.8 mL phosphate buffer (50 mM) having pH-7 was added. Absorbance of the solution was then recorded at an intermission of 15 s up to 2 min in 280 nm to calculate the



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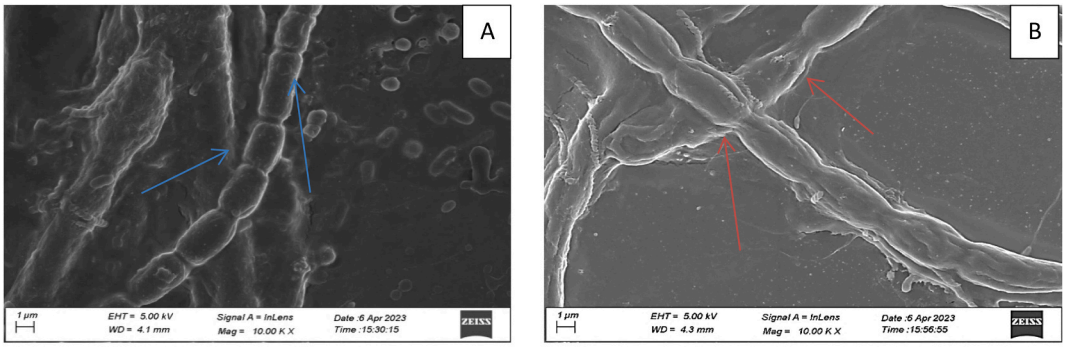

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Fig. 1. a: Light microscopic images of *Leptolyngbya* sp. GUEco1015 (A) Control and (B) Copper treated & b: Scanning electron microscopic images of *Leptolyngbya* sp. GUEco1015 (A) Control and (B) Copper treated.

activity of CAT.

2.6.5. Guaicol peroxidase activity

The Guaicol peroxidase (GPx) activity activity in *Leptolyngbya* sp. GUEco1015 was estimated following the standard protocol of Rao et al. [23]. 100 μL of enzyme extract was initially added with 1.5 mL phosphate buffer (100 mM) having Ethylenediaminetetraacetic acid (EDTA). After maintaining the pH 7.0, mixture solution of 200 μL of 10 mM guaicol and 200 μL of 10 mM H_2O_2 were then added to the enzyme extract and keep it stand for few minutes at ambient temperature prior to record the absorbance at 470 nm at the interval of 15 s for 2 min. Activity of GPx was then calculated according to the standard formula of Rao et al. [23].

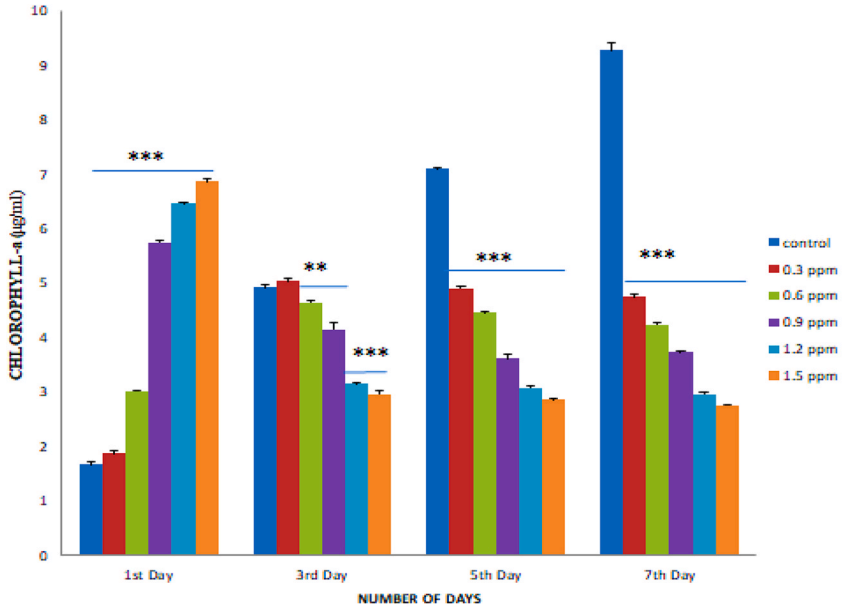
2.7. Characterization of adsorbent

2.7.1. Fourier-transform infrared (FT-IR) spectroscopy analysis

FT-IR spectroscopy analysis was achieved using the protocols of Ahad and Syiem [24] and Choi et al. [25]. After 24 h of treatment, 10 ml of cultivated samples were placed onto petri plates and dried in oven at 40–45 °C for 2 h. Spectroscopic-grade KBr was blended 1:10 (w/w) with desiccated samples to generate pellets. FTIR spectroscopy analysis was then finally accomplished using an FT-IR spectrophotometer in the 400–4500 cm^{-1} range. To examine the involvement of different chemical groups in metal binding, shifts in the spectrum locations of bands produced after treatment were compared to the control.

2.7.2. Energy Dispersive X ray (EDX) analysis

The EDX analysis was performed in accordance with the Ahad and Syiem [24] procedure. The algal samples were pre-treated using the Scanning Electron Microscopy technique followed by Sadiq et al. [14] with prior washing. The samples were gold-coated before taking photographs using a SEM-Zeiss Sigma 300.



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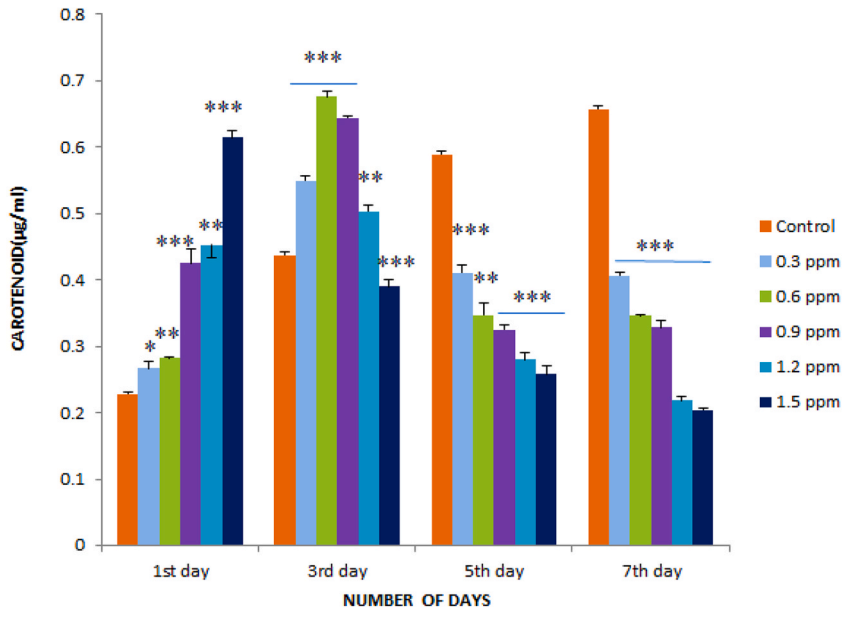

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Fig. 2. Effect of Cu^{2+} on Chl-a content (a) and Carotenoid content (b) in *Leptolyngbya* sp. GUEco1015. 'Error bars' indicates SD of three independent replicates and 'asterisks' represents significance level of treated cells over the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

2.8. Copper removal study

Studies using the batch technique were carried out to achieve the impact of various abiotic parameters on the mechanisms of biosorption [24]. The test organisms showed maximum significant growth (Fig. 2a) up to 3rd day of copper-rich culture as a result the selected study periods were 72 h. As the test organism was collected from the study sites having pH of 7.1–8.3 and temperature 25 ± 2 °C, the Cu^{2+} removal efficiency of biosorbents was therefore studied consecutively for 72 h in variable mode under a range of initial metal concentration (IMC) (0.1–0.3 ppm), pH (6.5–8.5), temperature (20–30 °C), and shaking rate (50–150 rpm). All the experiments

were carry through 100 ml Erlenmeyer flasks with 0.1 gm of *Leptolyngbya* sp. GUEco1015 strains. For optimization of pH, standard solutions of 0.1 M NaOH and 0.1 M HNO_3 were used to maintain the desired concentration. Further, optimized abiotic conditions were engaged for estimating maximum removal efficacy of Cu^{2+} by the test organism.

Acid digestion techniques were used [26] to achieve the removal efficacy of the test organism. The initial ion concentration (Co) and final concentration (Ce) of the growth media were recorded to measure the value in percentage (%). In the *Leptolyngbya* sp. GUEco1015 growing media (Cu^{2+} added) a mixture of 12 mL of $HClO_4$ and HNO_3 in a ratio of 1:3 were added for complete digestion. The reaction was carried out in a hot plate for approximately 30 min till the solution became 1 mL. The residual solution was then added with 24 mL of double distilled water and a volume set up to 25 mL. Using a Whatman no 1 filter paper with 0.45 μm pore size the solutions were filtered and the resultant solutions were analyzed by employing PerkinElmer atomic absorption spectrophotometer PinAAcle 900F (AAS). Finally, the removal efficacy was calculated by using the following formula:

$$\%\ \text{Metal removal} = \frac{Co - Ce}{Co} x100$$

2.9. Statistical analysis

Each of the investigations was conducted in triplicate (N = 3) and presented as mean ± standard deviation. The pooled results were subjected to student-t test to evaluate the significance level (*$p < 0.05$) over the control.

3. Results and discussion

3.1. Morphological observations

3.1.1. Light microscopic (LM) observation

LM observation was performed to investigate any morphological alterations in the filaments under copper treatments. The LM images of the filaments in control and copper treatment are shown in Fig. 1a. Images from the present investigation revealed that the copper treatment resulted in differential changes in morphology of the cell. The cell in control set shows a typical filament with bluish green appearance in structure having proper growth. Contrarily copper treated cells are seen to be slightly deformed and the cell wall damage can be seen clearly over the control. The chloroplast pigments are seen to be broken in the treated cells and an average decrease in the cell size and shape was also observed for the copper treated *Leptolyngbya* sp. GUEco1015 cells. A similar result were reported in *Nostoc muscorum* Meg1when treated with differential doses of copper and cadmium [24] and in *Ulva lactuca* (a green alga) with copper, lead, zinc and cadmium ions [27].

3.1.2. Scanning electron microscopic (SEM) observation

Besides LM observation, the SEM images were then ascertained to visualize the impact of copper ions on the filaments surface. SEM images of the filaments in control and in treatment with copper were shown in Fig. 1b, which revealed that the copper treated filaments were seen to be slightly deformed, shrunk. The cell wall damages are more prominent in treated filaments. Further, deformed cells were also recognized to be inflated under Cu^{2+} treatment. The results could be demonstrated by the fact that the production of EPS molecules during biosorption causes morphological changes [28]. Similar results were also detected in the filaments of Cd treated *Nostoc muscorum* Meg1 [29] and Cr treated cyanobacterial mat [30].

3.2. Effect of Cu^{2+} on growth parameters

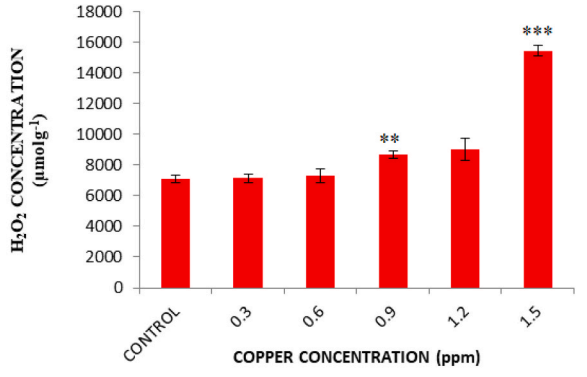
3.2.1. Chlorophyll-a

Chlorophyll-a(Chl-a), is a key light harvesting (LH) pigment in cyanobacteria which leads to trapping of sunlight and facilitation of photosynthesis. Chl-a level in the test organism were reliant on the time and concentration of Cu^{2+} (Fig. 2a). A sharp increment in Chlorophyll-content was observed on 1st day of incubation in all the grades of concentration and a non-significant increment was recorded at 0.3 ppm dose of 3rd day. The observed phenomenon can be attributed to the essential role played by copper as a microelement for the test organism, acting as a key cofactor for a range of metabolic pathways that are closely associated with growth. However, a significant decline were also marked at 1.5 ppm doses and was up to 39.7% ($p < 0.001$), 59.8% ($p < 0.001$) and 70.33% ($p < 0.001$) on 3rd, 5th and 7th day respectively based on control. Remarkably, 0.9 ppm doses on the 5th day revealed a ~50% decrease in Chl-content, signifying the LC50 value of the test organism relevant to copper.

The study found that increasing metal dosage led to a drop in chlorophyll-levels due to interference of metal in the production of porphyrin rings, which uptakes light energy [31]. The current study lines up with the work of Singh et al. [32], who had worked on the variations in Chl-a of *Anabaena* sp. upon treated with Cd. Likewise, a severe decrease in chl-content was also recorded in Cu treated *Anabaena variabilis* [33] and *Spirulina* sp. [34], Cu and Pb treated *Spirulina platensis* [35] and Cd treated *Nostoc muscorum* meg1 [36].

3.2.2. Carotenoid

In the current study carotenoids content in the cyanobacterial filaments found to be decreased with increasing Cu^{2+} stress and time from 1st to the 7th days of incubation. A gradual significant ($p < 0.001$) increase in the carotenoids content were observed on 1st day up to 1.5 ppm concentration whereas on the 3rd day a significant increase of 53.2% ($p < 0.001$) were recorded at 0.6 ppm and then it



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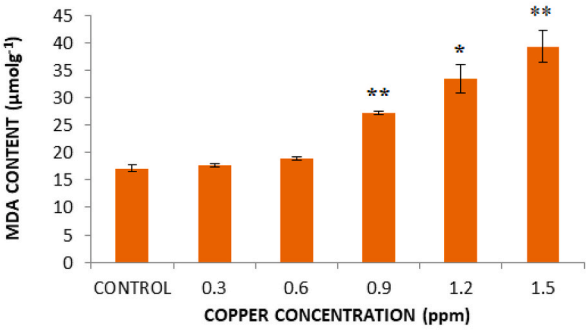

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Fig. 3. Effect of Cu^{2+} on H_2O_2 content (a) & MDA content (b) in *Leptolyngbya* sp. GUEco1015. 'Error bars' indicates SD of three independent replicates and 'asterisks' represents significance level of treated cells over the control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$)

declines gradually towards the concentration of toxicity. A significant decrease up to 57.55% ($p < 0.001$) and 69.2% ($p < 0.001$) were recorded at 1.5 ppm concentration of treatment on 5th and 7th days respectively (Fig. 2b).

Carotenoids are one of the vital pigments in cyanobacterial cells that contribute to the photosynthetic activity. They primarily serve as the light harvesting molecules in photosynthesis followed by transport of that energy to Chl-a, which plays an important role during photo oxidation. The current study reflects that cyanobacteria could accelerate amount of carotenoid at minimal doses of copper. The result may indicate that carotenoids act as antioxidants [31] and primary growth parameters where minimal harmful elements may be shows inclination of growth [8]. In contrast, towering of toxicity of copper leads to the declination of overall growth including carotenoids. A similar declining trend was observed in copper treated *Anabaena doliolum* [37], *Spirulina* sp. [34], and *Nostoc muscorum* [38]. It may be due to the generation of ROS due to high copper concentration which may leads to the disruption of membrane permeability and carotenoid biosynthesis pathway [31,38].

3.3. Determination of oxidative injury

3.3.1. Effect of Cu^{2+} on H_2O_2 content

Measurement of hydrogen peroxide is most commonly known as oxidative damage marker in any stress [39]. Higher levels of heavy metals may result in the generation of harmful oxygen species called ROS, which include H_2O_2, superoxide (O^{-2}), and hydroxyl radical (–OH). These highly reactive compounds negatively regulate the cellular activity through interacting with proteins, lipids, and nucleic acids [38]. Fig. 3a shows that copper at tested doses of 0.3, 0.6, 0.9, 1.2, 1.5 ppm significantly increases the H_2O_2 content i.e. 0.1%, 2.6%, 21.7%, 26.56%, 116% respectively on 7th days of treatment. A significant increase of H_2O_2 content at 0.9 ppm ($p < 0.01$) and 1.5 ppm ($p < 0.001$) were noticed during the study. It can be attributed that copper is generally involved in producing ROS by Haber-Weiss reaction [8] and Fenton-like phenomenon [40,41] thereby enhancing oxidative stress. Similar results were also noted by Singh et al. [42], that H_2O_2 content rose significantly in *Nostoc muscorum* and *Phormidium faveolarum* under 2 and 5 µm concentration of copper stress. Recent studies on green algae also indicated the accumulation of H_2O_2 in *Scenedesmus acuminatus* and *Chlorella sorkiniana* under Cu^{2+} stress condition [39].

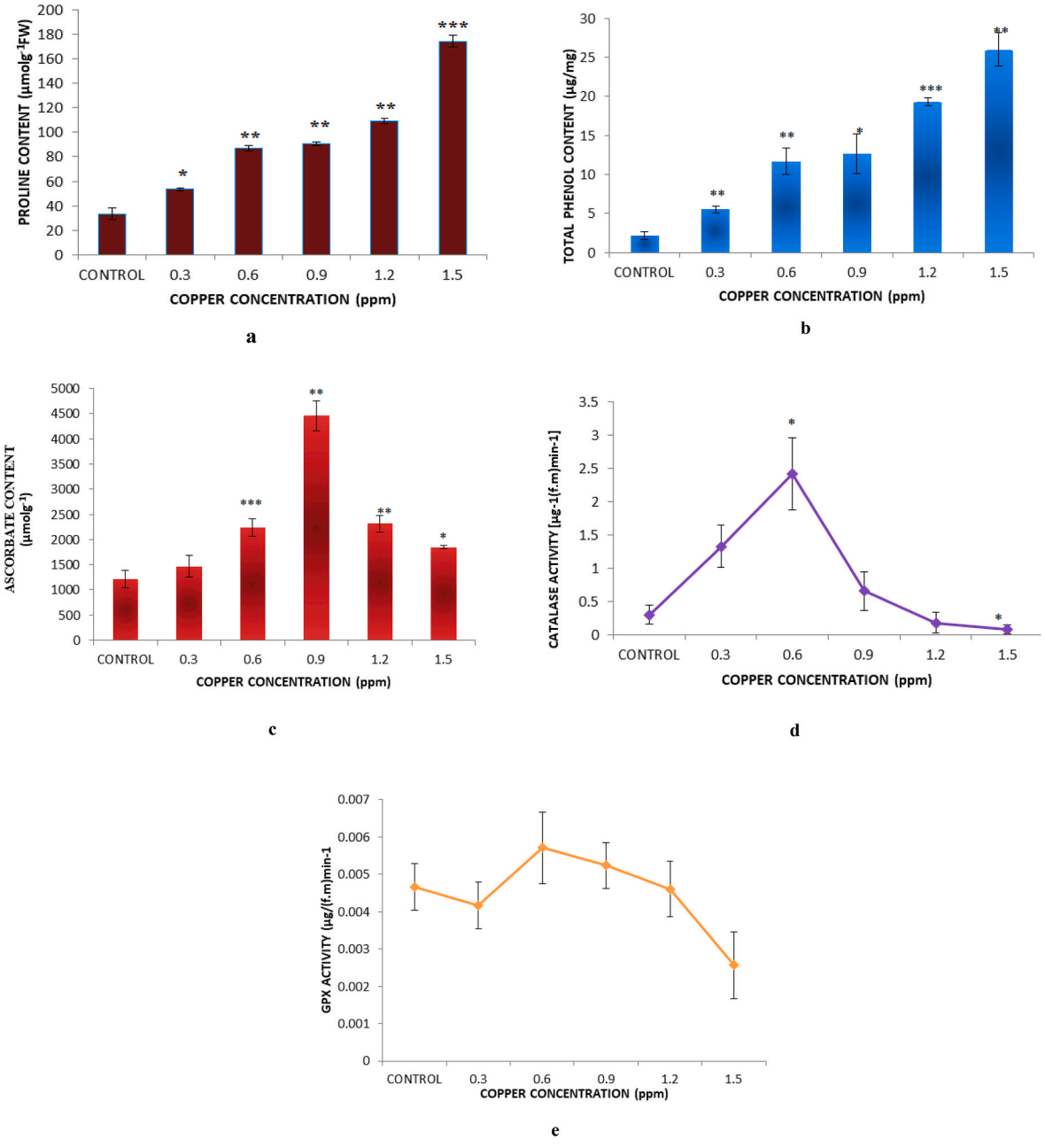


Fig. 4. Effect of Cu^{2+} on proline content(a) , phenol content (b), ascorbate content (c), catalase activity (d) and GPx activity (e)in *Leptolyngbya* sp. GUEco1015. 'Error bars' indicates SD of three independent replicates and 'asterisks' represents significance level of treated cells over the control (*p < 0.05, **p < 0.01, ***p < 0.001).

3.3.2. *Effect of Cu^{2+} on lipid peroxidation (LP)*

LP process is the result of increase of ROS in the cellular environment [21,43] due to any type of stress including accumulation of HMs inside the cells which generates few harmful cytotoxic byproduct like, 4-hydroxynonenal (HNE) and malondialdehyde (MDA) disintegrating PUFA (poly unsaturated fatty acid) precursors. As a cytotoxic byproduct of lipid peroxidation and a sign of generation of free radicals the MDA particularly ensure tissue damage [44] under stress which has been reflected in the present study. The result revealed that with the increase of Cu^{2+} concentration, MDA contents were proportionately increased by 3.2%, 10.4%, 59%, 94.9%, 129% respectively when *Leptolyngbya* sp. GUEco1015 cells were treated up to 7th day with 0.3, 0.6, 0.9, 1.2, 1.5 ppm of Cu^{2+} (Fig. 3b) which could be contributed to the generation of ROS and free radicals during the LP process. Similar results were noted in case of *Spirulina platensis*-S5 under Cu^{2+} rich culture and indicated that copper react with O^{2-} and forms HO^{-} through Fenton reaction [45] and in *Nostoc muscorum* Meg1 and *Anabaena doliolum* when the cells were exposed to Cd^{2+} [46,47].

3.4. Determination of enzymatic and non-enzymatic antioxidants

3.4.1. Effect of Cu^{2+} on proline content

The non-essential amino acid proline plays a vital function in cyanobacteria under any stresses. The intracellular proline content helps in the stabilizing and protecting protein synthesis mechanism. After 7th days of incubation under Cu^{2+} stress (0.3, 0.6, 0.9, 1.2, 1.5 ppm) a significant increase of proline content from 60% to 423% was noted under study (Fig. 4a) over the control was monitored. It could be attributed to the chelating mechanism of proline towards copper and the formation of metal-proline chelates [48] and performs the defending mechanism by the test organism against ROS damage and acting as an osmoregulator under Cu^{2+} stress [8]. Similar result was observed in *Spirulina platensis*-S5 [45], *Westiellopsis prolifica* [49], *Nostoc muscorum* meg1 [47] when treated with differential concentration of copper, lead, cadmium and zinc.

3.4.2. Effect of Cu^{2+} on phenol content (PhC)

Though copper is an essential element [8], high concentrations of it may become hazardous for entire phytoplankton community including cyanobacteria [50]. Many cyanobacterial species have phenols that stimulate to combat ROS and operate as an organism's defense system for the purpose of counteract harmful effects of copper [45]. Similar to the work of López et al. [51] and Ramadan et al. [52] the current study revealed the significant increase of phenol contents in *Leptolyngbya* sp. GUEco1015 under Cu^{2+} stress from control (2.22 μg/mg) to 1.5 ppm (26.0 μg/mg) under *in-vitro* condition (Fig. 4b). These findings may be contributed to the influence of copper metal concentrations on *Leptolyngbya* cell gene expression, which thereby regulate the synthesis of several metabolites like phenolic compounds [53] which is also in concomitant with the result of Hamed et al. [39] who worked on mitigation of copper stress through polyphenol mediated antioxidant system.

3.4.3. Effect of Cu^{2+} on ascorbate content

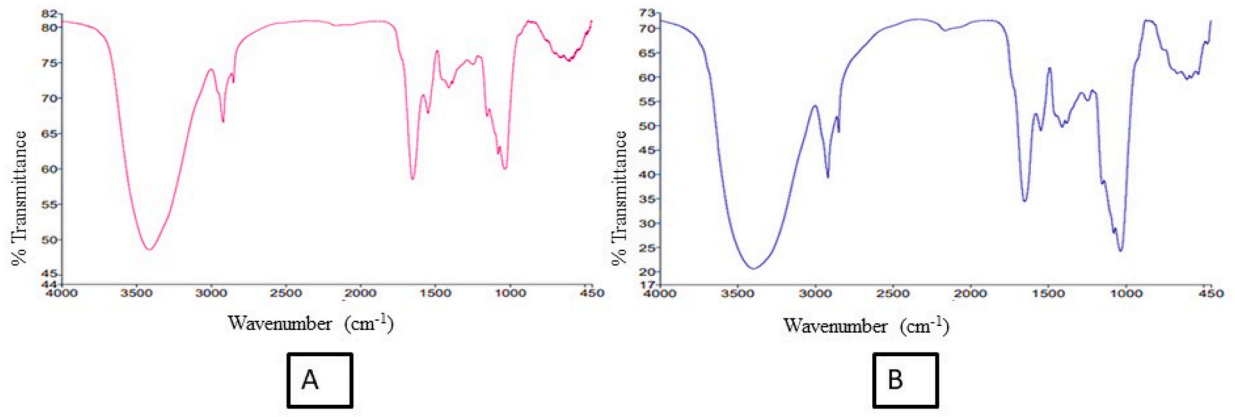
Ascorbate is an important and greatly abundant primary metabolites present in many plants including cyanobacteria under stress to cope up with the environment. Such metabolites involve in plant growth and reacts with the ROS to break it into non-toxic forms via multiple enzymatic system [54]. Ascorbate content in *Leptolyngbya* sp. GUEco1015 displayed a different result than other antioxidant systems under copper stress condition. The antioxidant activities of *Leptolyngbya* sp. GUEco1015 are not directly proportional to the metal stress (Fig. 4c). The highest significant increase ($p < 0.01$) of ascorbate content at 0.9 ppm was noted and was around 267% in comparison with the control. However, at 1.2 and 1.5 ppm concentrations of copper, a clear indication of gradual decrease of ascorbate was noticed as compared to 0.9 ppm concentration. *Chorella vulgaris,* a green alga, under copper treatment also depicted the similar results [55]. *Anabaena doliolum* also partially matches with the current study where it was detected that the ascorbate content gradually increases with the doses of copper up to a certain extent. Similar bell-shaped results of ascorbate were also noted in *Chlorella vulgaris* under Cr treatment [56].The study may gather the idea that cyanobacterium species can mitigate in the metal-rich environment via the ascorbate system present in chloroplast and cytosols, thereby proceeding to remove the ROS (H_2O_2) through the ascorbate-glutathione cycle. A declining trend of ascorbate content at higher doses of copper may indicate the involvement of associated reactions which may interfere with the accumulation behavior of the ascorbate cycle [57]. However, the bell shaped formation of ascorbate content beyond a limit in cyanobacterial cells/filaments in HM treated cell was quite unclear for the researchers till date.

3.4.4. Effect of Cu^{2+} on catalase activity

With the ability to degrade H_2O_2, the enzymatic antioxidant catalase performs an important role during abiotic stress. The high level of CAT content during Cu stress is a positively regulated mechanism performed by the cells of cyanobacteria [37]. Fig. 4d, indicates a gradual increase in catalase activity of the test organism under elevated copper concentration on 7th day of the treatment which gradually get reduced in concomitant with the increase of dose concentration. The Catalase content of the test organism were found to be 0.296 ± 0.1376 μg^{-1}(f.m) min^{-1} for control, 1.324 ± 0.3203 μg^{-1}(f.m) min^{-1} for 0.3 ppm, 2.42 ± 0.5378 μg^{-1}(f.m) min^{-1} for 0.6 ppm, 0.653 ± 0.2948 μg^{-1}(f.m) min^{-1} for 0.9 ppm, 0.176 ± 0.1575 μg^{-1}(f.m) min^{-1} for 1.2 ppm and 0.0726 ± 0.0669 μg^{-1}(f.m) min^{-1} for 1.5 ppm concentration. The highest catalase content was observed in 0.6 ppm ($p < 0.05$) and the lowest catalase content was observed in 1.5 ppm ($p < 0.05$). It may be due to the energy stress developed from copper's ionic form; eventually, CAT supplies an energy-efficient method for degrading H2O2 [58]. Similarly, *Nostoc linckia* cells also showed that metal concentration is directly related to the % of catalase activity, but after a certain concentration it gradually decreased [52]. Similar results were also noted in the CAT activity of *Anabaena doliolum* under metal stress, which follows the increasing trend at lower concentrations and subsequently declines at higher doses [46]. Whereas, increased activity of CAT was also observed in the cells of *Anabaena doliolum* under Cu treatment [37] with no declining trend.

3.4.5. Effect of Cu^{2+} on GPx activity

Guaicol peroxidase (GPx) is a heme containing peroxidase located in the cytoplasm and appoplasm part of microalgae including cyanobacteria. Guaicol peroxidase is involved in a number of critical metabolic activities and defense against abiotic stressors. Reports are available that under HMs stresses the GPx activity increases up to a certain level in cyanobacterium. The current study showed the substantial elevation in the GPx activity of *Leptolyngbya* sp. GUEco1015 when treated with differential grades of Cu^{2+}. But, no significant indication in elevation in the activity of GPx was observed (Fig. 4e). The GPx activity of the test organism showed a gradual increment of 22.1% i.e., 0.0057 ± 0.00396 μg^{-1}(f.m) min^{-1} on 0.6 ppm over the control set (0.004667 ± 0.000624 μg^{-1}(f.m) min^{-1}) which may hinder the accumulation of ROS in the cell system [59]. Similar results also observed in the *Geitlerinema amphibium* and *Anabaena* PCC 7120 when treated with differential increasing concentration of Al and As respectively. A notable elevation in the GPx



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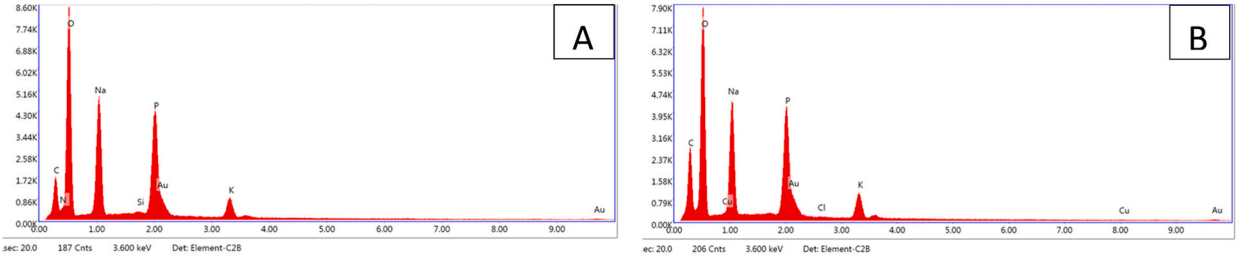

eZAF Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
C K	12.69	20.20	503.45	10.80	0.0300	1.1144	0.2121	1.0000
N K	2.12	2.90	85.59	16.22	0.0047	1.0864	0.2047	1.0000
O K	39.48	47.17	3727.83	8.15	0.1479	1.0621	0.3527	1.0000
NaK	20.20	16.79	2437.46	6.31	0.1079	0.9602	0.5558	1.0005
SiK	0.38	0.26	74.01	15.07	0.0029	0.9576	0.7986	1.0036
P K	15.99	9.87	2521.61	3.25	0.1285	0.9188	0.8727	1.0023
AuM	4.22	0.41	272.88	11.67	0.0317	0.6024	1.2203	1.0224
K K	4.91	2.40	513.39	4.09	0.0409	0.8832	0.9439	0.9991

eZAF Smart Quant Results

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	A	F
C K	18.73	28.99	817.97	10.11	0.0481	1.1092	0.2316	1.0000
O K	38.57	44.81	3477.72	8.36	0.1362	1.0572	0.3340	1.0000
NaK	17.59	14.22	2115.36	6.47	0.0924	0.9558	0.5493	1.0004
P K	14.50	8.70	2330.89	3.18	0.1172	0.9146	0.8819	1.0025
AuM	4.44	0.42	293.57	8.95	0.0337	0.5997	1.2343	1.0260
ClK	0.05	0.03	7.12	98.80	0.0004	0.8853	0.8893	1.0001
K K	5.71	2.71	604.86	3.95	0.0475	0.8791	0.9486	0.9990
CuK	0.41	0.12	7.82	62.39	0.0032	0.7471	1.0044	1.0386

b

Fig. 5. a: FTIR analysis of *Leptolyngbya* sp. GUEco1015 exposed to Copper treatment: (A) Control and (B) Copper treated.b: EDX analysis of the cell surface of *Leptolyngbya* sp. GUEco1015 exposed to different concentration of Copper: (A) Control and (B) Copper treated.

Table 2
Comprehensive report on shifting of resultant functional groups involved in *Leptolyngbya* sp. GUEco1015 before and after copper adsorption.

Detected peaks		Wavenumber (cm^{-1})	Resultant Functional group
Control (cm^{-1})	Copper treated (cm^{-1})		
3400	3425	3500–3200	O–H (alcohol) – NH (amine stretch)
2925	2950	2950–2850	Phenolic group
1630	1650	1690–1630	C═O(amide stretch)
1535	1562	1535–1640	Diketones
1050	1057	1050–1645	C–O (alcohol) stretching

activity was also observed in *Nannochloropsis oculata* under Cd rich condition. Substantial gradual decreases in the GPx activity were also visible in the current study from 0.9 to 1.5 ppm concentrations (Fig. 4e), though any report supports the negative regulation with the increase of concentration of HMs with cyanobacteria and algae. It is to be noted that similar decreasing trend in activity of GPx has previously been reported in wheat leaves under Zn and Cr stress (Panda et al., 2003) and in *Bruguiera gymnorrhiza* and *Kandelia candel* under differential concentration of Pb, Cd and Hg [60].

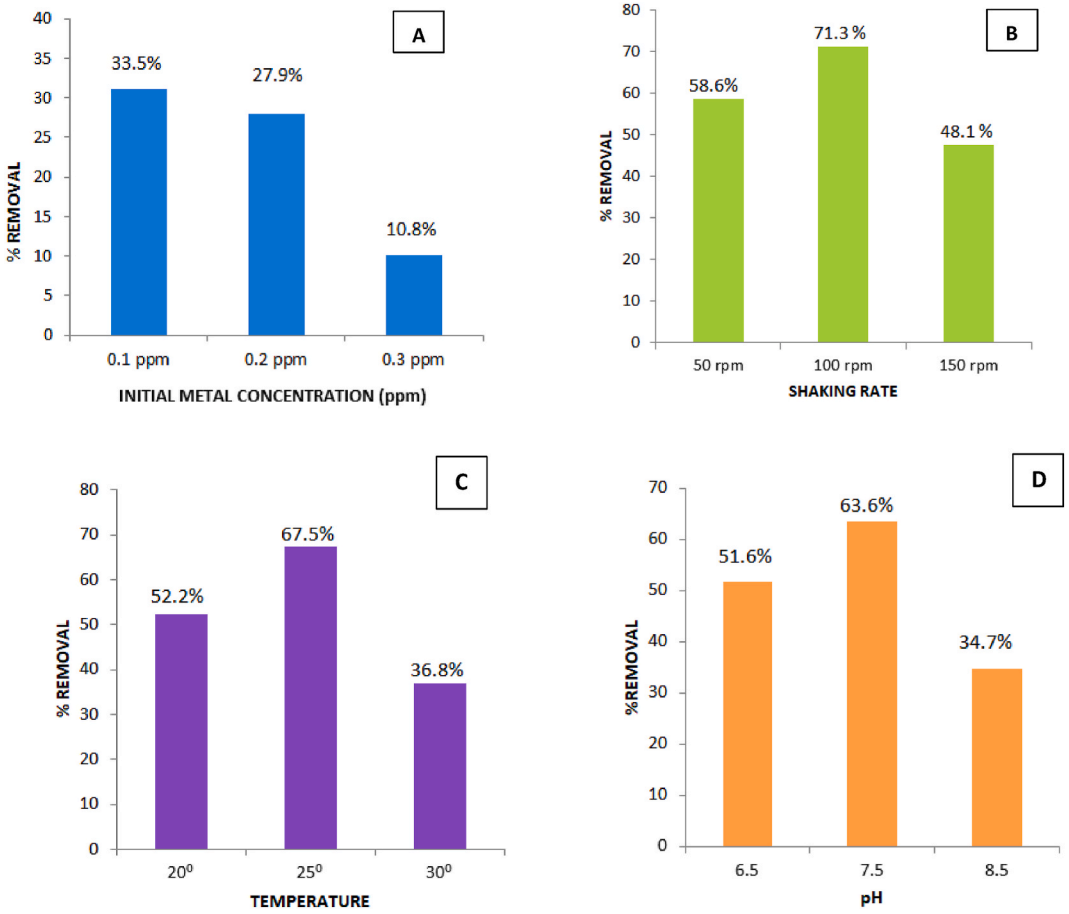

Fig. 6. Optimization of initial metal concentration (A), shaking rate (B), temperature (C) and pH (D) for Cu^{2+} removal study.

3.5. Characterization of adsorbent surface

3.5.1. FTIR analysis

To determine the major FG involved in the Cu^{2+} biosorption, FTIR spectra of Cu^{2+} treated and non-treated *Leptolyngbya* sp. GUEco1015 cells were recorded during the study. Infra-red analysis of metal treated and non-treated cells provide enormous information regarding the site of metal binding and its nature [32,61]. The shift of wave frequencies in several FTIR bands of established functional groups upon copper treatment is recorded in Fig. 5a and analyzed in Table 2. A shift of wide wavenumbers from 3400 cm^{-1} – 3425 cm^{-1} was an indication of involvement of amine and hydroxyl group [62].A wave shift in the area of 2925 cm^{-1} – 2950 cm^{-1} could be accorded to the involvement of an aromatic phenolic group [63] likely to be present on the cell surface. Indication of amide and carbonyl group was marked from the shift of wavenumber in between 1630 cm^{-1} – 1650 cm^{-1} [64]. Some shift in the frequency from 1535 cm^{-1} – 1562 cm^{-1} specified the involvement of diketone groups and a shift from 1050 cm^{-1} – 1057 cm^{-1} specified participation of C–O groups in metal binding [61].

3.5.2. EDX analysis

EDX analysis of the Cu treated cells of *Leptolyngbya* sp. GUEco1015 established a clear occurrence of the metal bound to the cell surfaces when compared to the control cells (Fig. 5b A). A distinctive peak for copper was recorded in the treated cells (Fig. 5b B) with weight % of 0.41 and atomic % of 0.12, suggesting adsorption of the element onto the cell surface. It might be associated with the fact and figures that the presence of functional groups in cyanobacterial filaments acted as an adsorbent for the Cu adsorbate.

3.6. Optimization for copper removal

3.6.1. Influence of IMC

IMC is the highly notable factors to study the HM removal efficiency in microalgae and cyanobacteria which is generally linked with the functional groups and binding sites of the cell wall of the organisms, and are directly associated with the adsorption processes [65]. The preliminary part of this study with *Leptolyngbya* sp. GUEco1015 was conducted in between the 0.1–0.3 ppm concentration of

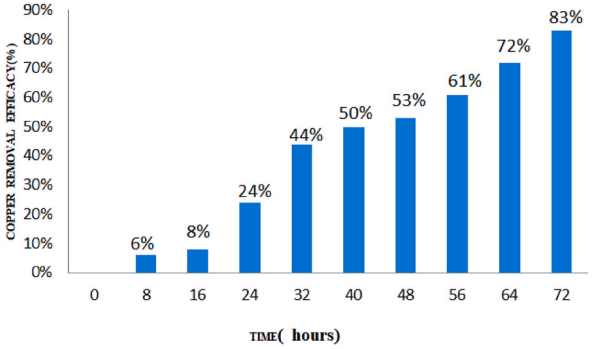

Fig. 7. Total Cu^{2+} removed by *Leptolyngbya* sp. GUEco 1015 at optimized condition in 72 h..

copper for 72 h which revealed that initial ion concentration of 0.1 ppm showed highest metal uptake and removal (33.5%) than that of 0.2 and 0.3 ppm treatment (Fig. 6A). It was also noted that the test organism treated with 0.3 ppm of ion concentration showed the lowest removal efficacy. The minimal metal ion concentration in the substratum could therefore be considered as suitable during the removal process. The results may indicate that lowering ion concentrations provides the highest binding sites and does not lead to saturation, thereby allowing metal ions to bind more efficiently. However, the rise of initial ion concentration resulted in the metal ions competing for the available binding sites, and eventually reaching saturation [8,66]. Similar results were also previously represented by Mehta and Gaur [67] during *Chlorella vulgaris* incubated with differential concentrations of copper. They reported that 2.5 mg/L ion concentration could remove 80% of copper in contrast to 10 mg/L (42%). Further, consortia of *Limnococcus limneticus* and *Leptolyngbya subtilis* also showed a similar result while treated with the different chromium ion concentration [68].

3.6.2. Influence of shaking rate

Innumerable studies have revealed that shaking rate (SR) is one of the limiting factors in metal removal mechanism [36,47,69]. Shaking maintains the homogeneity of the metal ions and makes it available for the respective functional groups used for adsorption [69]. The variability in SR in the current study showed a remarkable result in copper removal process. During optimization process for the current study a shaking value of 50 rpm, 100 rpm and 150 rpm was taken and showed removal of 58.6 %, 71.3 %, and 48.1% copper respectively (Fig. 6B). The study revealed that for 0.1 ppm concentration of copper, 100 rpm shaking is suitable to maintain the homogeneity. It can be attributed to the fact that the shaking leads to agitation of cyanobacterial cells to prevent the biomass from settling down, allowing uniform availability to the cells [70,71]. Though a very less investigation has been done on SR mediated metal bio sorption but concomitantly a few available results grabbed attention towards this factor [36]. The results could indicate that an optimum shaking is required for proper metal binding during adsorption process. A parallel trend was also noted in *Nostoc muscorum* cells during zinc (Zn) absorption which showed that 150 rpm is more suitable for adsorption of Zn than that of 50–100 rpm and 200–250 rpm [72], in contrast to Ahad et al. [73] who stated 50 rpm is the most favorable SR condition for cadmium biosorption by *Nostoc* sp. The present study on *Leptolyngbya* mediated copper removal proposed a moderate SR for maximum removal Cu^{2+}.

3.6.3. Influence of temperature

Temperature plays a vital role on metal biosorption and removal with microorganisms including cyanobacteria in any type of substratum [66,74]. *Leptolyngbya* sp. GUEco 1015 was treated at 20°, 25°, and 30 °C to establish a favorable temperature for the study. Results indicated that 25 °C is the most favorable (67.5%) condition for copper removal than to 30 °C (36.8%) and 20 °C (52.2%) (Fig. 6C). Similar result was found in *Anabaena cylindrica, Anabaena spiroides, Microcystis aeroginosa* [75], *Aulosira fertilissima* [76], *Nostoc* sp. [7], *Nostoc sphaeroides* [77] etc., where maximum removal and absorption of copper was observed at 25 °C. Further, other cyanobacterial members like *Anabaena sphaerica* [78], *Anabaena variabilis* [79], *Calothrix paritiana* [80], *Gloeocapsa calcarea* HH-1 [81], *Hapalosiphon schmidlei* [80] could also remove some other heavy metals at 25 °C. This may be explained by the fact that moderate ambient temperatures are often required for metal ions to function properly as high temperature may cause substantial damage to the cell wall and binding sites [74] for metal sorption.

3.6.4. Influence of pH

Metal bio-sorption can be varied at different pH condition for a particular species. Hence it is very necessary to select the optimum pH for highest metal removal efficacy under *in vitro* conditions. Current study was conducted in three pH ranges of 6.5, 7.5 and 8.5. With efficient removals of 63.6%, pH 7.5 was indicated as most effective pH state for removal of Cu^{2+} using *Leptolyngbya* sp. GUEco1015 (Fig. 6D) Many results demonstrated that at a range of 7–7.5 is suitable for maximum metal absorption [80,82–84], however, Kalita and Baruah [8], stated that pH 7.5 is suitable for maximum metal removal and absorption. The results may attribute that the presence of hydronium ions at low pH may hamper the activity of cell surface that would be due to the repulsive force between the metal ion and hydronium ion. Interestingly considerable reports are there that shows the maximum removal at low pH ranges i.e. from 2.5 to 6 [64,85–87]. Besides, myriad reports stated that the high pH is favorable for the involvement of FGs and association of metal ions profoundly [66,88].Contrarily the current endeavor may summarize that beyond the optimum level of pH, precipitation of

Table 3
Comparative copper removal (%) analysis by various cyanobacterial members.

Organism	Optimum condition				Removal efficiency (%)	References
	ion conc^{n}	pH	Temp^{r} (°C)	Shaking rate		
Anabaena oryzae	–	7.2	25–35	–	90.64	[90]
Anabaena variabilis	–	7.2	25–35	–	90.99	[90]
Anabaena fertilissima	-	5	25	–	80	[76]
Synechocystis sp.	–	7.1	25	–	3.54	[89]
Tolypothrix ceytonica	–	7.2	25–35	–	94.63	[90]
Leptolyngbya sp.	0.1 ppm	7.5	25	100	83	Current study

the metal ions put hindrance in removal process [8].

3.7. Copper removal efficacy of Leptolyngbya sp. GUEco1015

The amount of copper ion (%) removed by the cells of *Leptolyngbya* sp. GUEco1015 at optimized abiotic factors (0.1 ppm IMC, 100 rpm SR, 25 °C at pH 7.5) was estimated at 8 hourly intervals. The maximum growth of *Leptolyngbya* sp. GUEco1015 was noted to be on 3rd day of the incubation period from the days of inoculation and the present experiment was therefore conducted up to 72 h (Fig. 2a). It has been observed that a substantial increase of removal of copper from the substratum from 6% (8hrs) to 83% (72 h) in a gradient order (Fig. 7).This occurrence might be attributed to a wide range of factors, including the growth periods of cyanobacteria or modifications in the regulation of genes. As the *Leptolyngbya* sp. GUEco1015 shows its maximum significant growth up to 72 h, they may enhance their copper accumulation possibly to maintain important cellular processes or adapt to shifting environmental circumstances. The current outcomes are in accordance with the copper removal efficacy (Table 3) of *Anabaena oryzae* [79], *Anabaena variabilis* [79], *Anabaena fertilissima* [76], *Synechocystis* sp. [89], *Tolypothrix ceytonica* [79]. The findings may point out that the optimized abiotic parameters may involve in the activation of functional groups and the ion transporters (CTR transporter for Cu^{2+}) that take part during absorption of ions.(Kumar et al., 2015). The removal of Cu^{2+} from the substrata may be through the surface phenomenon or internalization of the ions. As Cu^{2+} acts as a micronutrient for the whole biota which is significantly used for the activation of various enzymes, it is therefore supposed to be an internalization or accumulation phenomena.

4. Conclusion

From the present study *Leptolyngbya* sp. GUEco1015 can be ascertained as an extremely suitable candidate for the removal of HMs like Cu^{2+}. Though towering up of Cu^{2+} enhances the morphological as well as oxidative damage to the cells, it simultaneously triggers itself to mitigate via enzymatic and non-enzymatic antioxidants. The shifting of many proton active FGs on the surface of the Cu^{2+} treated cells indicates the involvement of the adsorption event in the Cu^{2+} removal mechanism. The atomic % value observed during the cell surface screening through the EDX pattern showed proficient strain for Cu^{2+} removal. The findings also revealed with facts and figures that up to 83% removal of Cu^{2+} can be possible within 72 h. Thus, *Leptolyngbya* sp. GUEco1015, a potent cyanobacterial strain for removal of Cu^{2+} from any aqueous substratum subjected to set an optimized condition of IMC of 0.1 ppm, SR of 100 rpm, the temperature of 25 °C at 7.5 pH would act as a better bio remediator in future.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Nilamjyoti Kalita: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Partha Pratim Baruah:** Writing – review & editing, Supervision, Resources, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are grateful to Head, Dept. of Botany, Gauhati University for providing laboratory facilities developed under DST-FIST, UGC-SAP, OIL, MoEF & CC to carry out the research work.

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