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INDUCTION OF ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS IN RESPONSE TO Cd^{2+} STRESS IN A CYANOBACTERIUM

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ABSTRACT: The present study was aimed at analyzing the oxidant-antioxidant homeostasis under Cd^{2+} exposure in cyanobacterium *Nostoc sp.* (KX 814344). Exposure to variable doses of Cd^{2+} (10 and 20 µM) showed an increase in the ROS content by 65 % and 107 % as compared to control. An increase in protein oxidation and lipid peroxidation indicated cellular damage with increasing Cd^{2+} concentrations. There was a consequential increase in the activities of various oxidant mitigating enzymes such as CAT, SOD, GR and GPx at exposure to 10 µM Cd^{2+} by 46 %, 32 %, 48 % and 36 % respectively. The contents of non-enzymatic antioxidants (proline, ascorbic acid, GSH, phytochelatin, cysteine, total thiol content, phenol and flavonoid content) also registered a significant rise in their contents at 10 µM indicating that the cells were prepared to mount a multi-pronged attack on the increased ROS at all levels. However, the entire antioxidant defense mechanism was highly compromised in presence of 20 µM Cd^{2+} which increased the ROS content within cells to more than 100 %. The activities of all enzymes working in the management of oxidative stress declined by 20 % (CAT), 46 % (SOD), and 48 % (GR and GPx). Similarly, the cells' ability to produce non-enzymatic antioxidants was also severely compromised. Among these, the most affected were phytochelatin and the thiol content. Regardless, this study throws light on how the microbe garnered an array of various cellular metabolites to combat oxidative stress caused by exposure to heavy metals such as Cd^{2+}.

Keywords: Cd^{2+}, ROS, Antioxidants, *Nostoc sp.* (KX 814344).

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1. INTRODUCTION

Cyanobacteria constitute a group of gram-negative, photoautotrophic and nitrogen-fixing microbes that are believed to be one of the oldest organisms on Earth which evolved during the protozoic era, with fossil records dating back to 3.5 billion years ago [1,2]. They form a well-diversified bacterial phylum, with unique complex morphologies and cellular differentiation [3]. They exist in a wide range of habitats including terrestrial, marine, fresh and brackish water environments. They are highly versatile forming symbiotic, commensal, or parasitic associations with other organisms [4]. Certain cyanobacterial species can survive in very harsh conditions, in extreme temperatures and under different stress conditions [5]. Cyanobacteria play a key role in the food webs of many ecosystems where they are primary producers carrying out oxygenic photosynthesis and releasing oxygen as a by-product into the atmosphere [3]. Due to their photosynthetic activities, these organisms have been estimated to contribute ~ 20 - 30 % of global oxygen production annually [6]. In recent times, research into various aspects of the cyanobacterial metabolic profile indicated that they are potential sources of diverse compounds with bioactivities such as antibacterial, antifungal, anticancer, immunosuppressive, anti-inflammatory, anti-tuberculosis and many more. Such findings have raised the importance of these organisms in various fields like pharmacology, cosmetology, agriculture, food industry, or biofuel [7]. The alarming rise of pollutants in the environment with increasing modern agricultural practices, fast industrialization and urbanization have caused concerns over environmental degradation in recent times [8–10]. The resulting large-scale discharge of wastewater into freshwater sources is causing water pollution rendering the water unsuitable for drinking, irrigation and for aquatic life [11]. Among the various soil and water pollutants, heavy metal contamination is currently of great concern [8]. Accumulation of heavy metals not only affects aquatic animals and aquatic plants but are also known to enter the food chain and put human health in danger. Cyanobacteria being the primary producers, are also deeply affected by the presence of such pollutants in the environment and the resulting deterioration of their health and number is therefore affecting the entire ecosystem through the food chain [12,13]. Research into another aspect of cyanobacteria has shown that cyanobacterial cell surfaces are rich in negatively charged functional groups that can be exploited in binding and immobilizing positively charged metal ions and thereby these organisms can be potentially used in cleaning water contaminated with heavy metals [14–16]. Already, the use of cyanobacterial species as an adsorbent agent in the removal of heavy metals such as cadmium, lead and chromium has been reported [17,18]. Many heavy metals such as copper, zinc, magnesium, manganese and iron, when present at low concentrations, act as essential micronutrients which play important biological roles. However, when present in excess, these metals can be toxic affecting various physiological characteristics of microbes [19]. On the other hand, many other metals such as cadmium, lead, nickel and chromium have no known biological function and are found to be increasing in the environment due to various anthropogenic

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activities such as mining of coal and uranium, agricultural runoffs and discharge of sewage from domestic and industries [20]. It is known that different species of cyanobacteria can take up different heavy metals from the surroundings and accumulate these inside their cells [21,22]. One of the most common and toxic metals found to be polluting the earth's surface is Cadmium (Cd^{2+}) because of its high toxicity and low redox potential [23]. Several investigations have found that Cd^{2+} affects the biological systems by down-regulating pathways such as the Calvin cycle, proteins and fatty acids biosynthesis and by affecting glutathione synthesis, ATP metabolism and protein folding [24–26]. Cd^{2+} has also been shown to interfere with essential metals that are cofactors, affecting the functions of many proteins and enzymes [27]. That the effects of most heavy metals including Cd^{2+} are mediated via the generation of reactive oxygen species (ROS) is now a well-established fact. It has been known that under normal metabolic conditions, oxidant-antioxidant homeostasis in living cells is maintained through the participation of enzymatic and non-enzymatic antioxidant protection systems. However, environmental stresses such as strong light [28], ultraviolet radiation [29] and heavy metal exposure [30] are known to generate ROS such as oxygen radical ($^1O_2^{\bullet}$), superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) in the cells (Table 1) [31]. In cyanobacteria, ROS have been reported to have metabolic significance as they can act as a signal or secondary messengers influencing gene expression, activating cell differentiation and promoting apoptosis, etc [32–34]. However, when present at higher concentrations, being potent oxidants, ROS can be extremely destructive affecting all forms of biomolecules such as proteins, nucleic acids and membrane lipids [31,35]. As a countermeasure to mitigate excess ROS produced, cyanobacteria have been shown to enhance the activities of various enzymes such as catalases, superoxide dismutases, and peroxidases. Many non-enzymatic molecules (glutathione, Vitamin A, C, E, carotenoids, etc.) have also been implicated in neutralizing ROS. It has been noted that changes in the oxidant-antioxidant homeostasis produces oxidative stress affecting the physiological system of an organism and may lead to enhanced cell death [36–38]. Among the enzymatic antioxidants, SOD converts superoxide radical ($O_2^{\bullet-}$) to H_2O_2 and O_2 [39]. Enhanced enzyme activities of ascorbate peroxidase (APx) and CAT have been shown to lessen the damage caused by the ROS under Cd^{2+} stress [40]. CAT and GPx convert H_2O_2 to H_2O using glutathione (GSH). GSH being the redox pool in cells, cells stringently try to maintain the balance between the production and utilization of GSH at all times. GSH is regenerated from GSSG by GR. Production of GSH is accelerated in organisms exposed to toxic compounds in preparation to fight against oxidative stress [41]. Also, an increase in the contents of GSH in the cell can be credited to the fact that GSH is the precursor of phytochelatins that are involved in the removal of heavy metals [42–44]. The role of phytochelatins in the detoxification of cadmium in *Anabaena doliolum* has been earlier shown by Mallick et al. (1994) [45]. On the other hand, non-enzymatic antioxidants are as crucial as enzymatic antioxidants to mitigate ROS. The most dangerous and reactive ROS such as ·OH radical, known to interact with

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organic molecules, are being neutralized only by non-enzymatic antioxidants [46–48]. Also, cyanobacteria and algae produce excess flavonoids, ascorbate, sugars that fight ROS such as ·OH radicals under metal stress [49–51]. This study was undertaken to understand the effect of ROS caused by cadmium toxicity on a cyanobacterium *Nostoc* sp. (KX 814344) that was isolated from highly polluted coal mine waters that had high concentrations of many metal ions including Cd^{2+} [17]. The presence of the organism in such contaminated waters was intriguing and an attempt was made to study the biomolecular contributions in terms of both enzymatic and non-enzymatic antioxidants in protecting against oxidative stress generated by the metal ions for the organism to thrive under such stressful conditions.

Table 1

ROS mechanism of action and their scavenging systems. GPx: Glutathione peroxidase; GR: Glutathione reductase; CAT: Catalase; SOD: Superoxide dismutase; $O._2^-$: superoxide anions; .OH: Hydroxyl radicals; $^1O_2$·:singlet oxygen; $O_2^{\cdot-}$: superoxide anion radical; H_20_2: hydrogen peroxide [52].

Type of ROS	Biomolecules effected	Antioxidants involved
H_2O_2	▪ Protein as H_2O_2 reacts to cysteine and methionine residues ▪ Heme ▪ DNA molecules	GPx, CAT, GR, GSH, Ascorbate, etc.
.OH	▪ Extremely reactive with DNA, RNA, proteins and lipids.	Proline, Flavonoids, Ascorbate, etc.
$^1O_2$	▪ Lipids, protein and DNA	Phytochelatin, Cystein, Thiol, Ascorbate, etc.
O_2^-	▪ Fe–S proteins. ▪ Dismutates to H_2O_2.	Ascorbate, SOD, flavonoids.

2. MATERIALS AND METHODS

Maintenance of cultures and growth conditions

The cyanobacterium used in the present study was isolated from a coal mining site in West Khasi Hills, Meghalaya and identified as *Nostoc* sp. (KX 814344) using 16S RNA gene sequencing by Warjri et al. (2018). The isolate is maintained in BG-11_0 medium at pH 7.5, under continuous light (photon fluence rate of 50 µmol photon m^{-2} s^{-1}) and a temperature of 25 ± 2 °C [53].

Cadmium (Cd^{2+}) treatment

$3CdSO_4$. $8H_2O$ was the source of Cd^{2+}. Two different concentrations, i.e., 10 µM and 20 µM Cd^{2+} were used for the study. Ten days old cyanobacterial culture (3 µg mL^{-1}) from the exponential phase was taken as initial inoculum for all experiments.

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Assessment of reactive oxygen species (ROS)

The quantity of ROS produced was assessed using 2, 7-dichlorofluorescein di-acetate (DCFH-DA) as the fluorimeter indicator. For this, 5 mL cell culture along with 5 µM DCFH-DA was incubated with constant shaking (50 rpm) in the dark for 1 h at room temperature. The solution was then sonicated and centrifuged. The fluorescence of the supernatant was immediately read in a fluorescence spectrophotometer (Cary Eclipse). The excitation wavelength was kept at 485 nm while the emission wavelength was kept between 400 nm and 600 nm [54].

Lipid peroxidation

The extent of lipid peroxidation was estimated by measuring the content of 2-thiobarbituric malondialdehyde (TBA-MDA) adduct formed during Cd^{2+} treatment [55]. Pellet obtained after centrifugation (3000 x g for 3 min) of 5 mL cyanobacterial culture was mixed with 5 mL 10 % TCA, sonicated and further centrifuged at 10000 x g for 10 min. 1 mL of the supernatant was mixed with 2 mL of 0.5 % TBA in 10 % TCA solution, incubated at 95 ˚C for 45 min and was allowed to cool down before centrifuging at 10000 x g for 10 min. The absorbance of the resulting supernatant was read at 532 nm and 600 nm. For the determination of MDA concentration, the molar extinction coefficient of 155 mM^{-1} cm^{-1} was used.

Estimation of protein oxidation

For protein oxidation, 5 mL cell culture was mixed with an equal volume of 50 mM potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 2.5 µg mL^{-1} streptomycin sulfate and sonicated. To 1 mL of the supernatant obtained after the solution was centrifuged at 15000 x g for 25 min at 4 ˚C, 4 mL of 10 mM DNPH in 2 N HCL was added and kept on a shaker for 1 h. At the end of incubation, proteins were precipitated by adding 2 mL of 20 % TCA. The precipitated proteins were washed thrice with ethanol: ethyl acetate in a ratio of 1:1 (w/v) followed by solubilization in 6 M guanidine hydrochloride prepared in 2 mM potassium phosphate buffer (pH 2.3). The absorbance of the reactive carbonyl groups (RCG) product formed was read at 375 nm and its concentration was calculated using a molar absorption coefficient of 22000 M^{-1} cm^{-1} [56].

Enzymatic antioxidants

Catalase (CAT) activity

5 mL cell culture was sonicated after washing in 50 mM Tris- NaOH buffer (pH 8.0) containing 0.5 mM EDTA, centrifuged at 3000 x g for 3 min and the resultant supernatant was dialyzed in cellophane membrane tubings for 6 h with 4 changes of the cold extraction buffer. 0.1 mL of the dialyzed supernatant was added in a reaction mixture containing 100 mM KH_2PO_4 buffer (pH 7.0), 20 mM H_2O_2 in a final volume of 3 mL in this order. A UV- Vis spectrophotometer set at 240 nm (extinction coefficient = 0.036 mM^{-1} cm^{-1}) was used to measure the decline in H_2O_2 concentration for 5 min at an interval of 30 s. The catalase activity was expressed in terms of unit mg^{-1} protein [57].

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Superoxide dismutase (SOD) activity

The pellet obtained after centrifugation of 5 mL cell culture at 3000 x g for 3 min was washed twice in 100 mM K-phosphate buffer (pH 7.8) and resuspended in an equal volume of the same buffer. The solution was centrifuged at 3000 x g for 5 min after sonication. The supernatant obtained was dialyzed in cellophane membrane tubings against 4 changes of cold extraction buffer in a cold room for 6 h and was used for measuring SOD activity. The assay mixture contains 50 mM sodium carbonate-bicarbonate buffer (pH 9.8), 0.1 mM EDTA, 0.6 mM epinephrine and 0.1 mL of the cell extract. The development of adrenochrome was read at 470 nm for 5 min at an interval of 30 s spectrophotometrically. The SOD activity was expressed in terms of unit mg^{-1} protein [58].

Glutathione reductase activity

5 mL cell culture was washed with double distilled water, resuspended in extraction buffer [50 mM K-phosphate, 0.1 mM EDTA, 0.025 % (v/v) Triton-X-100 and 1 % (w/v) polyvinylpyrrolidone] and sonicated. 0.1 mL of this solution was added in an assay mixture (3 mL) containing 100 mM K-phosphate buffer, 4 mM EDTA, 2.4 mM GSSG (oxidized glutathione) and 0.6 mM NADPH. Absorbance was read at 340 nm for 5 min at an interval of 30 s to measure the concentration of NADPH (extinction coefficient = 6.22 x 10^{-3} mM^{-1} cm^{-1}). The enzyme activity was expressed as unit mg^{-1} protein [58].

Glutathione peroxidase activity

5 mL culture was centrifuged at 3000 x g for 3 min and sonicated in an extraction buffer for 1 min. 0.15 mL of the enzyme extract was added to an assay mixture containing 2 mL Milli-Q water, 0.35 mL of 100 mM phosphate buffer (pH 6.8), 0.2 mL of 0.5 % H_2O_2, and 0.3 mL of 5 % pyrogallol. The solution was mixed thoroughly and absorbance was read at 420 nm at an interval of 30 s for 5 min. The enzyme activity was expressed as µmol H_2O_2 oxidized min^{-1} mg^{-1} protein [59].

Contents of non-enzymatic antioxidants

Proline

3 % sulphosalicylic acid was added to the culture and sonicated for 2 min. 1 mL of the cell extract was mixed with 2 mL of acid ninhydrin reagent (3.75 g ninhydrin in 90 mL glacial acetic acid, 24 mL of ortho-phosphoric acid and 36 mL of Milli-Q water) and 2 mL glacial acetic acid. After incubation in a boiling water bath for 1 h, the reaction was terminated by placing the tubes in an ice bath for a few minutes. To this, 4 mL of toluene was added and vortexed thoroughly to extract proline. The development of pink color in the upper layer containing proline was read at 520 nm using a UV-Vis spectrophotometer and expressed in terms of ng mg^{-1} dry weight [60].

Ascorbic acid

4 % TCA was added to the cells, sonicated and centrifuged at 2000 x g for 10 min. To the supernatant, a pinch of activated charcoal was added and mixed vigorously before centrifuging at 2000 x g to remove the charcoal. 1 mL of supernatant was mixed with 1 mL of 4 % TCA and 0.5 mL of 2 %

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DNPH reagent (2,4-dinitrophenyl hydrazine in 9 N H_2SO_4). Following this, 2 drops of 10 % thiourea was added and incubated at 37 °C for 3 h. To the cooled tubes, 2.5 mL of 85 % H_2SO_4 was added before measuring absorbance at 540 nm. The ascorbic concentration was calculated using a calibration curve and expressed in terms of ng mg^{-1} dry weight [61].

Reduced glutathione (GSH)

10 mL of cell culture was centrifuged at 6000 x g for 10 min at 28 °C, washed twice with distilled water and sonicated in 5 mL of 0.2 M phosphate buffer (pH 8) for 3 min. After centrifugation at 20000 x g for 20 min at 4 °C, 0.2 mL of supernatant was added to 3 mL of phosphate buffer (pH 8) and 0.5 mL of DTNB. The absorbance of the colored solution was read at 412 nm and the GSH content was expressed as μmol GSH mg^{-1} protein [62].

Cysteine

Cell culture was homogenized in 5 % of chilled perchloric acid and was centrifuged at 8000 x g for 10 min. To each sample, an acid ninhydrin reagent (0.5 g ninhydrin, 12 mL glacial acetic acid and 8 mL of concentrated HCl) was added and incubated at 95 °C for 30 min. The mixture was cooled and absorbance was read at 560 nm. Cysteine content was measured using a calibration curve and was expressed as ng mg^{-1} dry weight [63].

Phenols and Flavonoids

Cell culture was washed thrice with Milli-Q water and was homogenized in methanol: water solvent in the ratio of 80:20. The cell extract was incubated for 6 h at room temperature with constant shaking and filtered using the filter paper having the pore size of 0.7 μm. The filtrate was dried at 37 °C. 5 mg dry residue mL^{-1} of methanol-water solvent was made. This extract was used for the determination of phenol and flavonoids contents.

0.1 mL of the methanolic extract was mixed with 0.8 mL of 10 % Folin-Ciocalteau reagent and kept for 5 min incubation. Next, 3.1 mL of 10 % Na_2CO_3 solution was added, mixed vigorously with intermittent shaking and were allowed to stand for 2 h. The absorbance was measured at 760 nm using a spectrophotometer. The standard graph of gallic acid was calculated and the concentration of phenol content was measured and expressed as ng mg^{-1} dry weight [64].

For estimation of flavonoid content, a methanolic solution of 1 mL of 2 % aluminum chloride ($AlCl_3$) was added with an equal volume of the extract solution. After 10 min, the absorbance of the mixtures was taken at 415 nm spectrophotometrically. The standard graph of quercetin was prepared and the concentrations of flavonoids content was measured and expressed as quercetin equivalents in ng mg^{-1} dry weight [65].

Total thiol (T-SH)

3 mL of cell cultures were centrifuged at 3000 x g for 3 min. To the pellet, 3 mL of 0.02 M EDTA was added and sonicated. 0.5 mL of the cell extract was mixed with 1.5 mL of 0.2 M Tris buffer (pH 8.2) and 0.1 mL of 0.01 M DTNB. The final volume of the mixture was made to 10 mL by adding

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absolute methanol. A blank (without sample) and a sample blank (without DTNB) were prepared. After 30 min incubation at room temperature, the mixtures were filtered twice and then centrifuged at 3000 x g for 15 min. The absorbance measurements were read at 412 nm spectrophotometrically (molar extinction coefficient is 13100 mM^{-1} cm^{-1}) and expressed as µmol mg^{-1} protein [66].

Phytochelatin (PC)

The phytochelatin content was estimated according to the method described by Hartley-Whitaker et al. (2001) [67]. This is done by subtracting the GSH content from the T-SH content and the formula is expressed as:

PC (µmol mg^{-1} protein) = T-SH (µmol mg^{-1} protein) – GSH (µmol GSH mg^{-1} protein).

Scanning electron microscopic (SEM) analysis

Cell culture was centrifuged and treated with 4 % glutaraldehyde in phosphate buffer. After 24 h incubation at 4 °C, the sample was washed thrice in 0.1 M sodium cacodylate buffer at an interval of 15 min each. Dehydration of the samples was done at 20 %, 40 %, 60 %, 80 %, 85 %, 90 %, 95 % and 100 % of acetone at 4 °C at a time period of 15 min each. The cells were then mounted on brass stubs and coated with gold before viewing under SEM (JSM, 6360, JEOL, Japan) to check the morphological changes in the organism.

Transmission electron microscopic (TEM) analysis

Changes in the ultrastructure were studied using TEM (JEM-2100, JEOL, Japan). The samples were fixed in 4 % glutaraldehyde (in phosphate buffer) for 24 h at 4 °C and were washed thrice in sodium cacodylate buffer (0.1 M) at intervals of 15 min each. 2 % of OsO_4 (prepared in 0.2 M sodium cacodylate buffer) was used for post-fixation of the samples for 1 h followed by washing with the same buffer for 15 min with three times changes. The samples were dehydrated with 20 %, 40 %, 60 %, 80 %, 85 %, 90 %, 95 % and 100 % of acetone at 4 °C and then treated with propylene oxide for 30 min. These were transferred to embedding molds, embedding medium Araldite CY 212 was added and these molds were incubated for 24 h at 50 °C for polymerization. Further incubation for 48 h was done at 60 °C. The samples were then cut into thin sections (60 - 90 nm) using the Ultramicrotome TMX (Boeckeler Instruments, Tucson, Arizona, USA) and were stained with uranyl acetate and lead acetate before viewing under TEM.

Data analysis

All experiments were carried out in triplicate and data were presented in means ± standard deviation (SD). One-way analysis of variance (ANOVA) was performed to check the significant difference between control and different treatments (Dunnett test) using GraphPad Prism 5 software.

3. RESULTS AND DISCUSSION

Effect of Cd^{2+} exposure on ROS generation, lipid peroxidation and protein oxidation

The level of ROS showed a significant increase (↑65 % in presence of 10 µM and ↑107 % in presence of 20 µM Cd^{2+}) in the organism *Nostoc* sp. (KX 814344) compared to control [Fig. 1(a)].

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The effect of ROS produced was further determined by estimating the extent of lipid peroxidation and protein oxidation. Both these parameters registered significant changes in the treated cultures. Lipid peroxidation went up by 131 % and 233 % while protein oxidation registered an increase by 25 % and 125 % in cultures treated with 10 and 20 µM Cd^{2+} respectively [Fig. 1(b)].

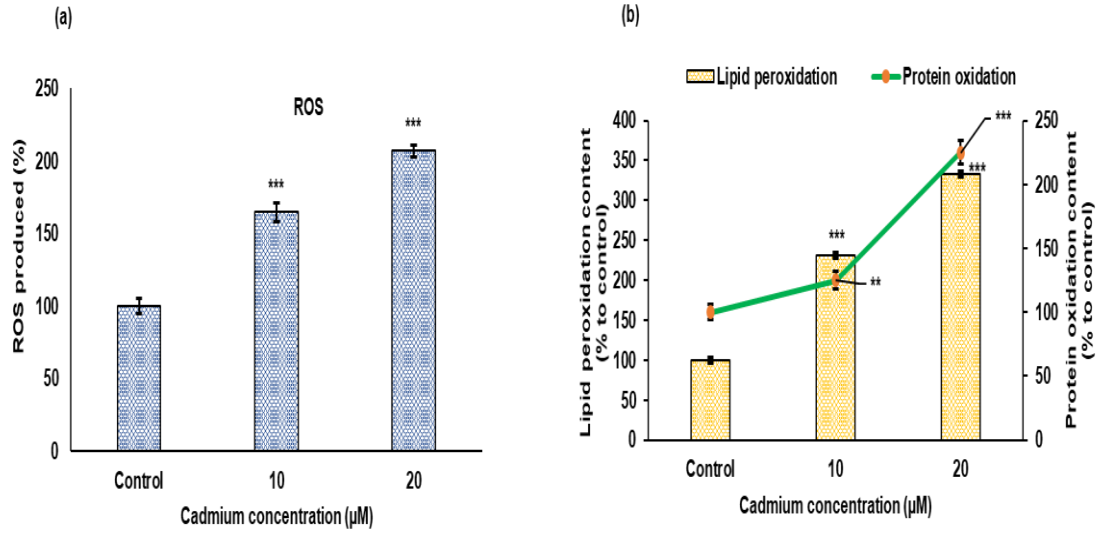

Fig. 1 (a) ROS content; **(b)** Lipid peroxidation and Protein oxidation content; All the values are in Mean ± SD (N = 3) and asterisks (*$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$) above the histogram bars specify significance in differences between control and Cd^{2+} treated cells.

Effect of Cd^{2+} exposure on enzymatic antioxidants: CAT, SOD, GR and GPx

Under exposure to 10 µM Cd^{2+}, the enzyme activities of CAT, SOD, GR and GPx were significantly increased by 46 %, 32 %, 48 % and 36 % respectively compared to control. However, a drop in the activities was recorded in 20 µM Cd^{2+} treated cultures by 20 % in CAT, 44 % in SOD, 38 % in both GR and GPx [Fig. 2 (a) & (b)].

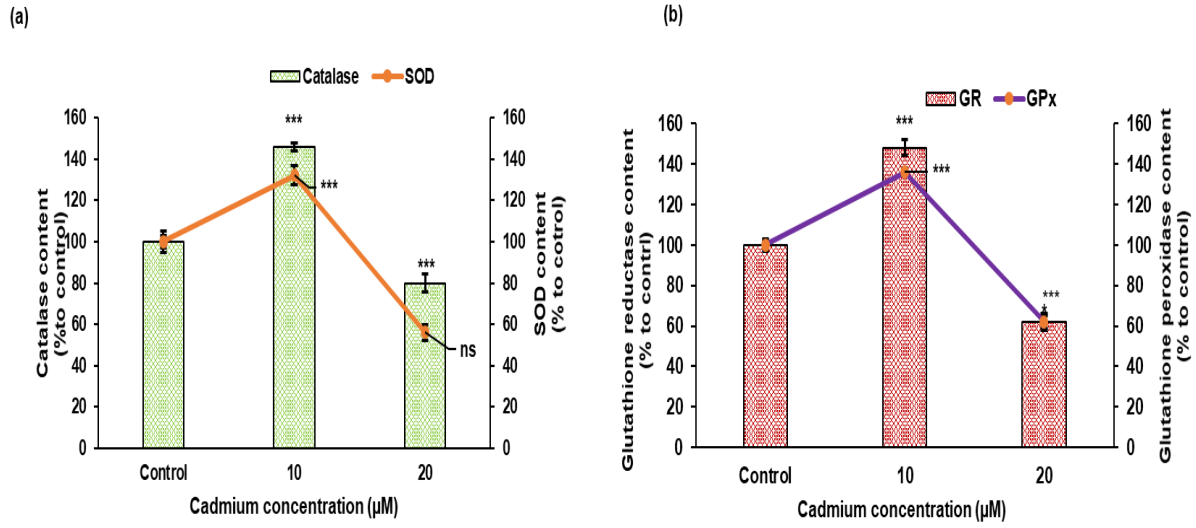

Fig. 2 (a) CAT and SOD content; **(b)** GR and GPx content; All the values are in Mean ± SD (N = 3) and asterisks (*$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) above the histogram bars specify

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significance in differences between control and Cd^{2+} treated cells.

Effect of Cd^{2+} stress on non-enzymatic antioxidants

In comparison to control, the proline content of the treated cultures (10 and 20 µM Cd^{2+}) increased by 28 % and 44 % respectively. Similarly, an increase in ascorbic acid content by 15 % in 10 µM and 7 % in 20 µM Cd^{2+} was also noted [Fig. 3 (a)]. GSH content showed a rise by 39 % in 10 µM Cd^{2+} while its content was compromised by 8.6 % in presence of 20 µM Cd^{2+} ($p < 0.001$) [Fig. 3 (b)]. Phytochelatin content too was enhanced by 30 % in presence of the 10 µM while a 13 % reduction in the phytochelatin content was seen in cultures treated with 20 µM Cd^{2+} [Fig. 3 (b)]. Although an upsurge of 20.4 % in cysteine content was registered in 10 µM Cd^{2+}, a drop by 8.2 % in its content was seen in 20 µM Cd^{2+} as compared to control ($p < 0.001$). The thiol content went up by 22 % at 10 µM but a considerable decrease of 11.9 % was recorded for 20 µM treated culture [Fig. 3 (c)]. Phenol content, however, showed a 26 % and 40 % increase in 10 and 20 µM Cd^{2+} treated culture respectively. In contrast, flavonoid content registered a 27 % increase under the influence of 10 µM Cd^{2+} but its content declined by 9 % in presence of 20 µM Cd^{2+} [Fig. 3(d)].

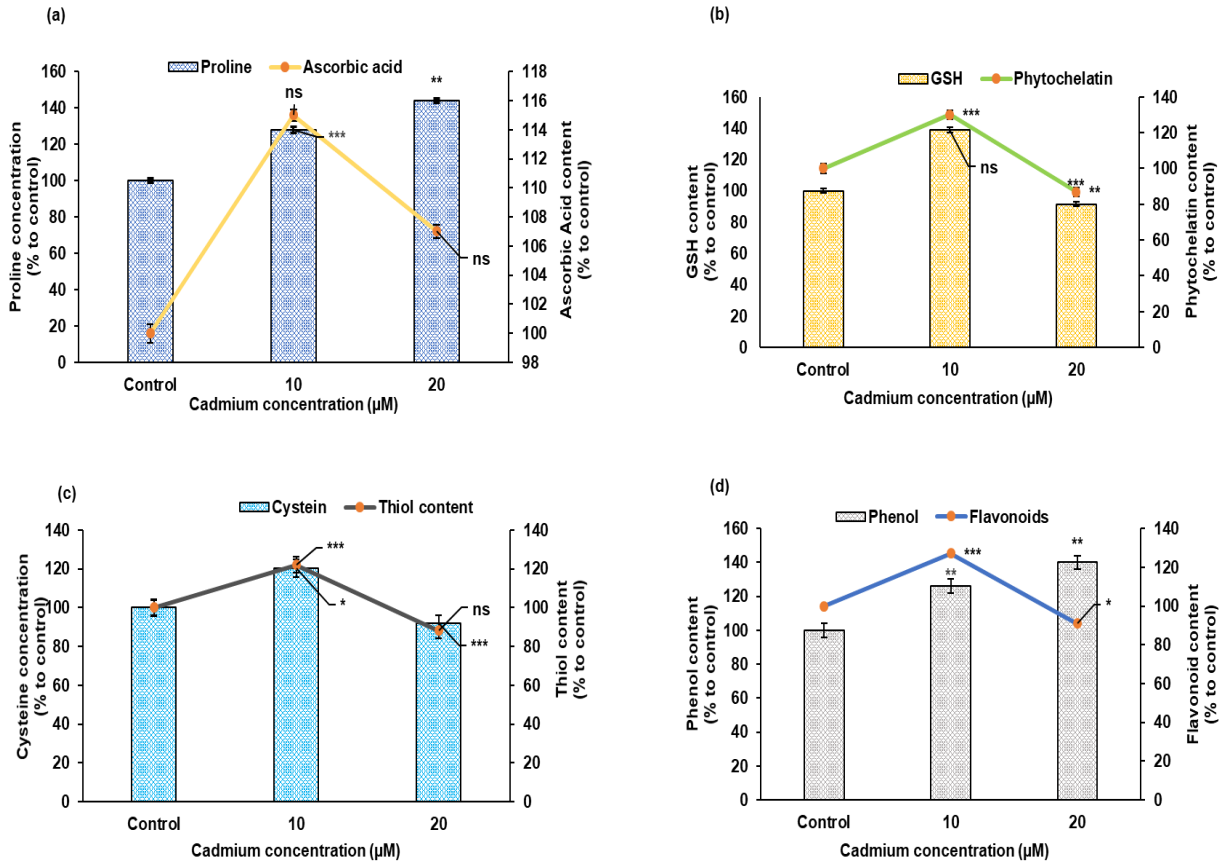

Fig. 3 (a) Proline and Ascorbic acid content; **(b)** GSH and Phytochelatin content; **(c)** Cysteine and total thiol content; **(d)** Phenol and Flavonoids content. All the values are in Mean ± SD (N = 3) and asterisks (*$p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$) above the histogram bars specify significance in differences between control and Cd^{2+} treated cells.

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Effect of Cd^{2+} stress on morphology and ultrastructure of *Nostoc* sp. (KX 814344)

Fig. 4 (a) shows healthy cells of control culture of *Nostoc* sp. (KX 814344) under SEM. At 10 µM Cd^{2+} exposure, cell shrinkage and pore formation begin to appear [Fig. 4 (b)]. Filament breakage, distortion, cell shrinkage and perforations in the cell walls are highly visible in presence of 20 µM Cd^{2+} [Fig. 4 (c)].

The ultrastructural changes in the organism were revealed under TEM. In comparison to control cells [Fig. 4 (d)], under the exposure to 10 µM Cd^{2+}, there was distortion in the thylakoid membranes and also the appearance of gas vacuoles that were distinctly visible [Fig. 4 (e)]. In cultures treated with 20 µM Cd^{2+}, there was cell membrane distortion, enlargement of gas vacuoles leading to alterations in the organization of thylakoid membrane structure [Fig. 4 (f)].

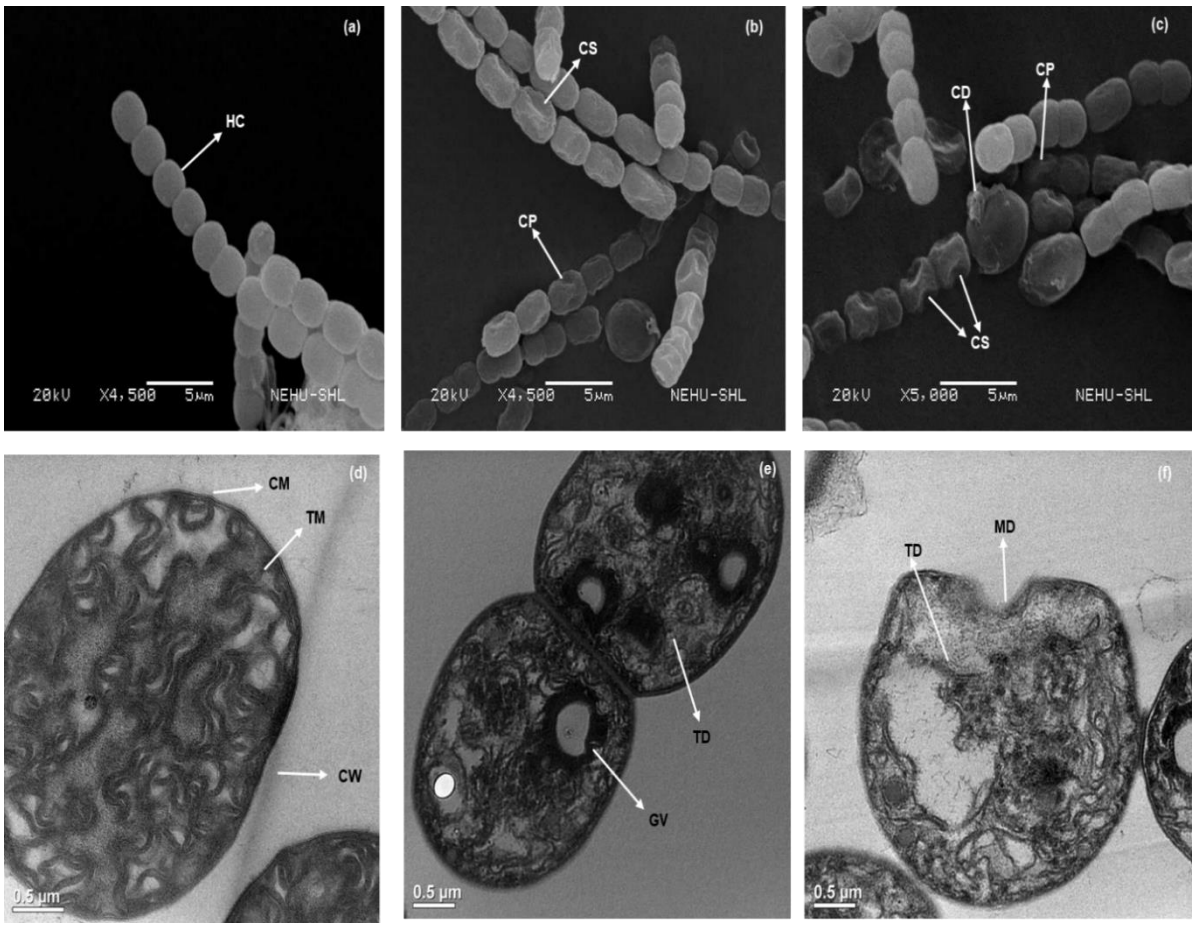

Fig. 4 Scanning and transmission electron microscopy: SEM study of the morphology of *Nostoc* sp. (KX 814344) treated with Cd^{2+}: **(a)** Control; **(b)** 10 µM exposure; **(c)** 20 µM exposure. TEM study of the ultrastructure: **(d)** Control; **(e)** 10 µM exposure; **(f)** at 20 µM exposure. HC: healthy cell; CP: perforations of cell; CF: cell fragmentation; CS: cell shrinkage; DC: cell distortion; TM: Thylakoid membrane; CW: cell wall; CM: cell membrane; TD: Thylakoid membrane disintegration; MD: membrane distortion.

ROS are the by-products of metabolic pathways located in various cellular compartments such as chloroplast, mitochondria and peroxisomes [68]. Under physiological conditions, ROS may act as

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secondary messengers regulating gene expression resulting in protection from various environmental stresses [32,34,54]. However, excess ROS production becomes toxic to the cells affecting the physiological function of the organism. Thus, it becomes necessary to check the oxidant-antioxidant homeostasis via the inbuilt enzymatic and non-enzymatic protection system [69]. This equilibrium is stringently maintained among various oxidants produced and antioxidants generated in response that keep checks on the amount of oxidants present in the intracellular environment. The antioxidant defense mechanism present in the cellular compartment regularly scavenges excess ROS and re-equilibrates the oxidant-antioxidant balance within the cells [70]. However, many environmental factors such as heavy metals, UV radiation, extremes of temperatures, high salt concentration, etc. can affect this delicate oxidant-antioxidant homeostasis [71]. Coming to the present study, it was deduced from the fact that as the cyanobacterium *Nostoc* sp. (KX 814344) taken for this study was thriving in mine water samples containing many heavy metal ions, it must have developed strategies to mitigate excess ROS generated in presence of high concentrations of heavy metals. So, how far this capability to alleviate ROS could be stretched? To understand this, we exposed the organisms to two concentrations of Cd^{2+} **(**10 µM and 20 µM) and checked the amount of ROS generated and how the contents of both enzymatic and non-enzymatic antioxidants were modulated. That Cd^{2+} is highly toxic is evident from the fact the ROS content has considerably increased in its presence (↑65 % in presence of 10 µM and ↑107 % in presence of 20 µM Cd^{2+}). One must understand that the overall increase that was registered was after the cellular anti-oxidant system has already scavenged to its maximum capability. Therefore, the surplus ROS recorded indicated that many cellular anti-oxidant molecules working in tandem for ROS mitigation, could not completely remove the excess ROS from the intracellular environment. The result of excess ROS presence was seen on lipid peroxidation and protein oxidation, both of which increased considerably upon Cd^{2+} exposure in comparison to control. Lipid peroxidation indicated damages in the cell walls and cell membranes resulting in the release of the cellular contents which was seen in SEM and TEM micrographs with the appearance of pores, shrinkage of cells and destruction of thylakoid membranes (Fig. 4). Various other researchers have also reported ROS-induced lipid peroxidation leading to cell membrane damages [72,73]. A similar effect on protein oxidation (25 % and 125 % in cultures treated with 10 and 20 µM Cd^{2+}) reiterated the toxic nature of Cd^{2+}. Although percent lipid peroxidation was much higher (131 % and 233 % 10 and 20 µM Cd^{2+} in treated cultures) than percent protein oxidation, it can find its reason in the fact that cell membranes were in direct contact with surrounding Cd^{2+}, whereas only a certain amount of Cd^{2+} entered the cells to affect the proteins. However, an increase in protein oxidation means that many enzymes and key proteins were affected resulting in compromised physiological functions. Earlier, Pinto et al. (2003) [33] has linked ROS generation to damages in protein molecules when cyanobacteria and algae were exposed to heavy metal stress. Cuypers et al. (2010) [74] and Nemmiche (2017) [76] also indicated

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that interference of cadmium by the involvement of H_2O_2 in oxidizing thiol groups not only affects the functions of many proteins but also the redox state in the cell. As stated earlier that ROS have metabolic significance as signal or secondary messengers, generated as a result of metabolic processes in cells. But, when present in excess, the cells' in-built antioxidant system neutralizes the excess ROS via enzymatic and non-enzymatic antioxidant defense mechanisms [69]. At exposure to 10 µM cadmium, the increase in the activities of the enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase indicated that the cell's physiology was directed towards the production of antioxidant defense components to scavenge the ROS generated [77]. Even then it was seen that the cells were not completely successful in removing the entire amount of excess ROS (the residual ROS was 65 % higher than the control value). 20 µM cadmium presence proved to be too toxic, where the activities of all these enzymes were considerably compromised and the cellular ROS content remained significantly (~ 107 %) higher than that of control cultures. Other researchers have also shown that increasing the concentration of cadmium inside the cell beyond the cell's ability to remove these ions, interfere with the interaction of essential metal ions such as Zn^{2+}/Cu^{2+} with enzyme active sites disrupting their functions [52,78]. In a cellular defense system, non-enzymatic antioxidants are equally important as they increase the defense system's efficiency to include ROS not being taken care of by the enzymatic components. Reactive oxygen species such as hydroxyl radical, considered to be dangerous and highly reactive are only scavenged by non-enzymatic oxidants such as proline, cysteine, glutathione, ascorbic acid, phenol, and flavonoids [47,48,79]. During oxidative stress, proline plays key roles in osmoregulation, stabilization of the machinery involved in protein synthesis, protection of the integrity of enzymes and many other antioxidative processes including inhibition of lipid peroxidation. Proline has been implicated as an effective scavenger of ·OH and $^1O_2$ and provides a defense mechanism to tolerate the heavy metal stress by binding with metal ions due to its chelating ability [71]. Proline content in this study was noted to increase right after exposure to Cd^{2+} and continued to elevate in a higher concentration of Cd^{2+} (28 % and 44 % in cultures treated with 10 and 20 µM Cd^{2+}). It can be safely assumed that with an increase in the ROS content, the proline synthesis was accelerated not only as an amino acid needed for protein synthesis to replace protein loss due to protein oxidation but also as a component of non-enzymatic antioxidant system. The excess proline is used up to scavenge the ROS in the cells. Choudhary et al. (2007) [80] and Fatma et al. (2007) [81] have also demonstrated similar results indicating enhanced production of proline in cyanobacteria *Spirulina platensis*-S5 and *Westiellopsis prolifica*-Janet strain-NCCU331 upon exposure to lower doses of Cu^{2+}, Pb^{2+} and Zn^{2+}. A similar increase in the ascorbic acid (15 % and 7 % in cultures treated with 10 and 20 µM Cd^{2+}) content was also seen in the study. Ascorbic acid is an effective water-soluble vitamin that scavenges H_2O_2 and ·OH radicals by taking part in the ascorbate-glutathione cycle [52]. Abd El-Baky et al. (2009) [82] have also reported an elevation in

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cellular ascorbic acid content in *Spirulina platensis* in response to increasing H_2O_2 concentration indicating the contribution of ascorbic acid in H_2O_2 scavenging. Furthermore, ascorbate is vital in the restoration of α-tocopherol from α-chromanoxyl radical generated during lipid peroxidation [71]. Another amino acid cysteine gives an indication of oxidative damage to a cell. Cysteine serves as a precursor molecule for the synthesis of glutathione, metallothioneins, phytochelatins and some other sulfur-containing moieties [38]. The content of cysteine was higher by ~ 21 % in cultures exposed to 10 μM Cd^{2+} reflecting that with demand to enhance defense against ROS, various soldiers of the defense mechanism were fortified with an enhanced number. The decrease in its content by ~ 8.6 % in cultures exposed to 20 μM Cd^{2+} however is indicative of severe toxicity of Cd^{2+}. The reduction could also be attributed to reduced N_2-fixation and assimilation under Cd^{2+} exposure [83] where there could be a shortage of available nitrogen for the synthesis of nitrogen-containing molecules. GSH (γ-Glu-Cys-Gly) is the most abundant non-protein thiol which serves as a redox buffer, maintaining a reduced intracellular environment. It plays a key role in the removal of H_2O_2 and in addition, further helps in the regeneration of ascorbate. The resultant oxidized GSSG is reduced to GSH by the enzyme GR immediately to maintain the reducing equivalents for reductive reactions in the cells [84]. There was a significant rise (39 %) in the GSH content under the influence of 10 μM Cd^{2+}. This showed that cells were preparing for a reducing environment for mitigation of the excess ROS generated due to cadmium exposure. However, a decrease of 8.6 % in GSH level from its control value in 20 μM Cd^{2+} had shown how stressful was the intracellular condition at higher cadmium exposure. Also, reduction in one of the key ingredients- cysteine, whose production declined in the 20 μM Cd^{2+} exposure aided in reduced synthesis of GSH. This further indicated that with an increase in protein oxidation, lipid peroxidation the general health of the organism was already so compromised that it was not able to function to its full capacity to produce all essential molecules at the required speed. Further, [85] showed that GSH participation in the antioxidant defense mechanism is substantial to combat the oxidative stress produced under Cd^{2+} exposure. One of the molecules of importance in metal ion chelation is phytochelatins (PC). They are polymers of GSH [$(\gamma\text{-Glu-Cys})_n$-Gly] synthesized by the enzyme phytochelatin synthase [86]. They serve as detoxifying agents in response to heavy metal stress as they act as biological chelators of incoming metal ions [87]. PC concentration was escalated by 30 % in cultures treated with 10 μM Cd^{2+}. This is understandable as when there was an influx of cadmium ions into the cells, cells immediately responded by synthesizing a large number of phytochelatin molecules in its haste to arrest the metal ions and make them inaccessible to any cellular metabolism. The increase in phytochelatin content can be attributed to elevating the synthesis of GSH by the exposed cells [52]. However, with a reduction in GSH content in the cells in presence of 20 μM Cd^{2+}, the synthesis of phytochelatin was also negatively affected. There was a significant 13 % reduction in the PC content under these conditions. These results find further support in the experiments conducted by Kawakami et al.

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(2006) [88] who reported that enhanced production of PCs was noted in *P. tricornutum* upon exposure to a low dose of Cd^{2+}. Several researchers have reported in cyanobacteria that phenolic compounds are important as antioxidants and as metal chelators [10,89,90]. Exposure to 10 and 20 μM Cd^{2+} saw a 26 % and 40 % increase in total phenol indicating that the synthesis of these groups of molecules was not so negatively affected and the organism was able to increase their number under stress conditions. Orabi and Abouhussein in 2019 [91] reported that the antioxidant properties of phenolic compounds are mediated by such mechanisms as scavenging of free radical species like ROS, suppressing ROS formation by inhibiting certain enzymes and chelating trace metals involved in free radical production. A similar result has also been reported by [92] whereby the cyanobacterium *P. boryanum* showed a dose-dependent increase in total phenolic content after exposure to Ni^{2+} (2 - 4 μM). Another set of molecules important as antioxidants are thiols that are a group of organosulfur compounds which includes cysteine, GSH, coenzyme A, phytochelatins, metallothioneins, etc. These biological compounds enhance various processes like protein folding, enzymatic reactions and also serve as metal chelators. Thiols play a key role in the regulation of the redox balance [93]. The 22 % increase in thiol content in 10 μM Cd^{2+} exposed culture indicated that cells synthesized them to chelate the incoming cadmium ions as well as enhance processes like protein folding etc. required to manage the oxidative stress. However, the toxicity of cadmium became evident from the fact that the total thiol content was compromised by ~ 12 % at 20 μM Cd^{2+}. A similar finding of increased thiol contents has been reported in the cyanobacterium *Nostoc spongiaeforme* under exposure to low concentrations of Ni^{2+} [94]. Flavonoids are known antioxidants that lessen the damage caused by oxidative stress inside the cells [95,96]. The presence of flavonoids in cyanobacteria plays a key role in the protection of the organisms against environmental stress [97,98]. The antiradical efficacy of flavonoids is dependent on their structure and on the position and number of phenolic hydrogens available for rapid donation to free radicals to form less reactive flavonoid phenoxyl radicals [99]. In the present study, flavonoids content showed an increase by 27 % in cells exposed to 10 μM Cd^{2+}, which decreased by 9 % under the influence of 20 μM, as was the case with many molecules studied so far. The overall findings of this work have been presented in Fig. 5 for a quick understanding.

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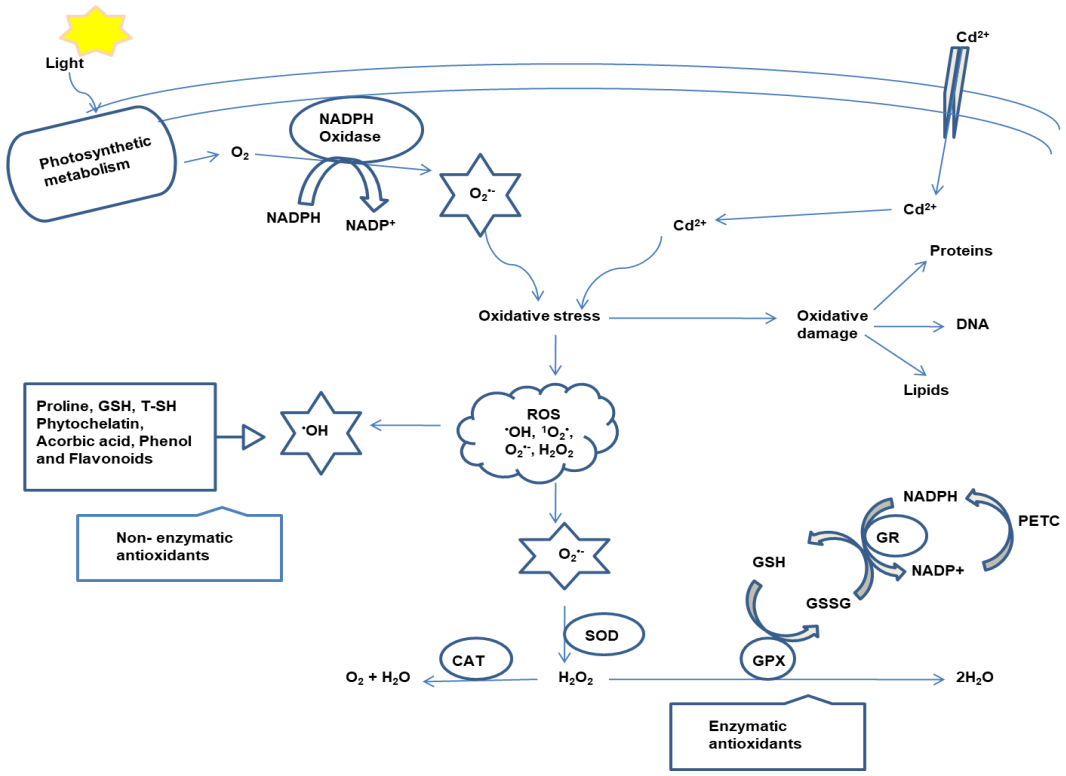

Fig. 5 Relationship among the various by-products induced upon Cd^{2+} exposure in *Nostoc* sp. (KX 814344). ROS: reactive oxygen species; SOD: superoxide dismutase; CAT: catalase; GR: glutathione reductase; GPx: glutathione peroxidase; GSH: reduced glutathione; GSSG: oxidized glutathione; T-SH: total thiols; PETC: photosynthetic electron transport chain; $O_2^{\cdot-}$: superoxide anions; .OH: Hydroxyl radicals; $^1O_2^{\cdot}$:singlet oxygen; $O_2^{\cdot-}$: superoxide anion radical; H_20_2: hydrogen peroxide.

4. CONCLUSION

As reported by our group (Warjri and Syiem, 2018) [17], the concentration of Cd^{2+} in the spring water contaminated with mine effluent from which the organism *Nostoc* sp. (KX 814344) in West Khasi Hills, Meghalaya, India was isolated, was 0.41 µM. Looking at the tolerance of the organism to a much higher Cd^{2+} concentration (10 µM), it can be argued that by being constantly present in such a contaminated environment the organism had developed strategies to overcome acute cadmium toxicity by eliciting the synthesis of an array of enzymatic and non-enzymatic antioxidant molecules to counteract against a wide variety of derogatory ROS molecules generated by metal exposure. This characteristic of the organism thus establishes the potential of this organism in the bioremediation of heavy metals, particularly cadmium from wastewaters. The biosorption parameters, equilibrium isotherms and thermodynamic studies of metal removal potential of the organism have already been published (Warjri and Syiem, 2018) [17]. This study further provides information about how the organism mitigates cadmium-induced toxicity when present in the cadmium-rich environment.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The author confirms that the data supporting the findings of this research are available within the article.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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