ROLE OF SULPHUR IN MODULATING CHROMIUM (CrVI) TOXICITY IN THE CYANOBACTERIUM *ANABAENA* SP.PCC 7120

Dissertation submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

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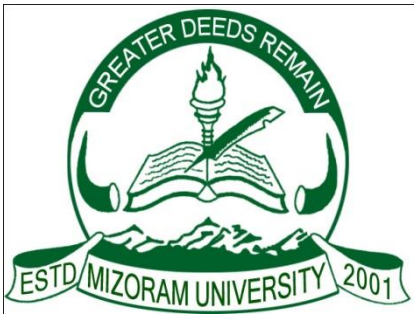
MZU Registration no: 1505352

M.Phil RegnNo: MZU/M.Phil./534 of 17.05.2019



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January, 2021

Role of sulphur in modulating chromium (Cr VI) toxicity in the cyanobacterium *Anabaena* sp.PCC 7120

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Submitted

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CERTIFICATE

This is to certify that Thokchom Thajamanbi chanu, Department of Biotechnology, Mizoram University, Aizawl has completed her dissertation work entitled **"Role of sulphur in modulating chromium (CrVI) toxicity in the cyanobacterium Anabaena sp. PCC 7120"** in partially fulfillment of M.phil degree in Biotechnology from August 2018 to January 2021 under the guidance and supervision.

No part of this dissertation has been submitted for any other degree or diploma

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DECLARATION

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January, 2021

I, Thokchom Thajamanbi Chanu hereby declare that the subject matter is the work done by me, that the contents of this thesis did not form basis of the award of any previous degree to me or to do the best of my knowledge to anybody else, and that the thesis has not been submitted by me for any research degree in any other University/institute.

This is being submitted to the Mizoram University for the degree of Master of Philosophy in Biotechnology.

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ACKNOWLEDGEMENT

First and foremost, I profoundly thank my supervisor, **Dr. Jyotirmoy Bhattacharya**, Department of Biotechnology, Mizoram University, Aizawl, for his excellent guidance, assistance and for sharing his precious treasure of knowledge in caring out the experimental work.

I wish to express my heartiest gratitude to my lab mates, who spared their valuable time and gave me constant support and necessary help.

I would like to thank to the entire concerned person, teachers, friends, and all the faculties of Department of Biotechnology who have shown great cooperation during the course of my M.phil work.

I thank the Department of Biotechnology (DBT), New Delhi for the Bioinformatics Infrastructure, State Biotech Hub which help me throughout my research work.

I would also like to thank University Grant Commission, Govt. of India, for University Non-NET fellowship.

And above all, I thank Almighty God, my family and friends for their love and support in my studies.

(Thokchom Thajamanbi Chanu)

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CHAPTER 1

Introduction

Metals are ubiquitous in nature. Many of them, for example, iron, manganese, copper, magnesium, nickel, cobalt and zinc serve as enzyme cofactors, and play essential biological roles (Baptista and Vasconcelos, 2006). On the other hand, there are many naturally occurring metals which have no known biological function. Chromium (Cr) is one such biologically non-essential heavy metal abundant in earth's crust. Cr is a member of group VI-B of periodic table with atomic number 24, and exists in several oxidation states ranging from +2 to +6. The trivalent Cr(III) and hexavalent Cr(VI) are the most stable and abundant forms in nature (Viti et al., 2013). Whereas, Cr(III) is relatively insoluble in water, Cr(VI) is highly soluble and more mobile within the environment. Cr(VI) is also more toxic to microorganisms and plants than Cr(III) (Cervantes et al., 2001). Cr(VI) enters into the environment through both natural and anthropogenic activities. The natural sources of Cr include volcanic eruptions, forest fires, and soil weathering. The anthropogenic sources are primarily the industrial wastes discharged into water bodies from Cr-using factories such as electroplating, steel and automobile manufacturing, wood treatment, leather tanning, textile dyeing etc. (Viti et al., 2013). Due mainly to such industrial activities, Cr has become a pollutant of major concern in many aquatic and terrestrial ecosystems with potential implications for growth and survival of resident biota, and ecosystem functionality.

Photoautotrophic cyanobacteria are an eco-physiologically important group of Gram-negative ubiquitous prokaryotes. They are major primary producers in many natural ecosystems and play critical roles in sustenance of food web (Ramakrishnan et al., 2010). For optimum performance, they must be able to adapt to various stresses, including that imposed by elevated levels of Cr(VI). In cyanobacteria, Cr(VI) exposure have been shown to result in loss of growth, cell-structure integrity, photosynthesis and

several other vital metabolic activities (Gupta and Ballal, 2015; Khattar et al., 2013; Kumar et al., 2013; Tiwari et al., 2018). Such effects of Cr(VI) have been attributed mainly to enhanced production of reactive oxygen species (ROS) such as superoxide radical anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) (Gupta and Ballal, 2015). High concentrations of ROS are extremely harmful to cells, and cause oxidative damage to virtually all biomolecules (nucleic acids, proteins, lipids) and accelerate cell death (Latifi et al., 2009). Thus, one can assume that organisms with higher ability to detoxify ROS may tolerate Cr(VI) stress better than those having poor ROS detoxification capacity. This has been demonstrated in the unicellular non-diazotrophic cyanobacterium *Synechococcus* sp. PCC 7942 with inherently higher antioxidative activities (ROS-detoxification) of superoxide dismutase, catalase, peroxiredoxin, and non-enzymatic antioxidant molecule glutathione (GSH), which can tolerate 12-fold higher concentration of Cr(VI) than phylogenetically closely related *Synechocystis* sp. PCC 6803 with lower ability to detoxify ROS (Gupta et al., 2013; Gupta and Ballal, 2015). The importance of antioxidative systems in preventing Cr(VI) toxicity is also evident in many other cyanobacteria (Bano et al., 2012; Kumar et al., 2012; Tiwari et al., 2018).

It is well established that Cr(VI) enter into cells through sulphate transporters due to structural similarity between Cr(VI) and sulphate (Viti et al., 2013). Cr(VI) have been shown to competitively inhibit sulphur/sulphate uptake in various organisms, including in cyanobacteria (Aguilar-Barajas et al., 2011; Gupta et al., 2013). Cr(VI) have also been shown to affect assimilation of sulphate in yeast leading to reduced synthesis of many sulphur-requiring structural proteins and enzymes, and metal chelating molecules such as phytochelatins, metallothioneins, and antioxidant GSH (Pereira et al., 2008). Given the importance of sulphur in cellular physiology and its interaction with Cr(VI) at transport and assimilation level, it is expected that Cr(VI) sensitivity would increase in organisms when availability of sulphate is poor. The effects of sulphate starvation on Cr(VI) toxicity has been studied in plants and algae. Whereas, sulphate deficiency has been shown to aggravate Cr(VI) toxicity in some plants, a transient

increase in Cr(VI) tolerance was noted after sulphate starvation in algae (Gorbi et al., 2006, 2007; Schiavon et al., 2007, 2008). It was hypothesized that enhanced sensitivity in plants was due to increase in the number of sulphate transporters following sulphate starvation leading to enhanced uptake of Cr(VI) (Appenroth et al., 2008). In algae, however, low uptake of Cr(VI) through high affinity sulphate transporters (induced by sulphate deficiency) was suggested as the basis for enhanced Cr(VI) tolerance (Marieschi et al., 2015). As sulphate deficiency and elevated levels of Cr(VI) can co-occur in various natural ecosystems, an attempt was made in this study to unearth the relationship between sulphate availability, ROS metabolism, and Cr(VI) tolerance in a filamentous heterocystous diazotrophic cyanobacterium *Anabaena* sp. PCC 7120.

CHAPTER 2

Review of literature

Cyanobacteria are an ancient group of Gram-negative prokaryotes. Their origin dates back to approximately 3 billion years ago. They were the first organisms to pioneer oxygen evolving photosynthesis on earth, and are responsible for the “great oxidation event” that gradually changed the earth’s atmosphere from anaerobic to aerobic facilitating the emergence of aerobic life on earth. They are also believed to be the progenitors of modern-day chloroplasts in plants. Due to their photoautotrophic life style, cyanobacteria have occupied diverse ecological habitats ranging from terrestrial to fresh water and oceans, hot spring to arctic environments, where they are the major drivers of carbon cycle (Bryant, 1994). Many cyanobacteria are also endowed with the ability to fix atmospheric dinitrogen and significantly contribute to global nitrogen cycles. However, their ability to perform such ecological important roles is often hampered by various stress conditions that prevail in their natural habitats. Heavy metals introduced into the environment by industrial activities, constitutes one such stress.

The physiological and biochemical responses to heavy metals have been studied in many cyanobacteria. In general, heavy metals affect growth, photosynthetic pigments, photosynthetic activity, membrane structure and function, and also disturb redox homeostasis (Arora et al., 2006; Babu et al., 2010; Gupta and Ballal, 2015; Khattar et al., 2004; Kumar et al., 2012, 2013; Tiwari et al., 2018). Given the toxic effects, cyanobacteria have evolved several mechanisms to combat heavy metal toxicity (Cassier-Chauvat and Chauvat F, 2015). One such mechanism is increased production of uronic acid rich negatively charged extracellular polymeric substances (EPS). These EPS protect cells by sequestering heavy metals. Indeed, EPS confer significant protection to *Synechocystis* sp. PCC 6803 against cadmium (Cd) and cobalt (Co) toxicity. In addition to extracellular chelation activity, heavy metals are also chelated within cells by some cyanobacteria possessing metallothioneins (MTs), cysteine rich

proteins that binds a large number of metals non-specifically through SH-group of cysteine. In some cyanobacteria, such as *Synechocystis* PCC 6803, MTs are absent, and instead metal efflux pumps are present. In others like *Anabaena* and *Oscillatoria*, MTs and efflux pumps both contribute to heavy metal tolerance. The metal-reductases in conjunction with metal efflux proteins have also been shown to play critical role in protection against heavy metals such as arsenic (As), mercury (Hg), uranium (U) etc. in cyanobacteria (Cassier-Chauvat and Chauvat F, 2015).

Previous studies in various cyanobacteria indicate that metal-induced oxidative stress is one of the critical factors contributing to heavy metal toxicity (Babu et al., 2010; Bano et al., 2012; Gupta and Ballal, 2015; Kumar et al., 2013; Tiwari et al., 2018). For instance, Cr(VI) metabolism [reduced to Cr(III) by ascorbic acid, glutathione, and/or flavoenzymes] generates a variety of ROS, as described earlier in chapter 1. Thus, protection against heavy metal stress must involve mechanisms pertaining to scavenging of ROS. Cyanobacteria are equipped with a variety of antioxidants (enzymes and non-enzymes) that may protect them from ROS. SOD is one such ubiquitous enzyme, which detoxifies ROS superoxide radical into less toxic ROS hydrogen peroxide (H_2O_2) and oxygen. Although H_2O_2 detoxifying catalses are not ubiquitous, cyanobacteria which possess them are able to manage heavy metal-induced H_2O_2 better than those lacking this enzyme. Since H_2O_2 detoxification in cyanobacteria is also catalyzed by multiple peroxiredoxins, these proteins could also play a critical role in heavy metal tolerance (Banerjee et al., 2012; Latifi et al., 2009). Indeed, the importance of such antioxidative enzymes in tolerance against Cr has been reported (Babu et al., 2010; Bano et al., 2012; Gupta and Ballal, 2015; Kumar et al., 2012, 2013; Tiwari et al., 2018). Similarly, the non-enzymatic molecule such as glutathione with dual roles (antioxidant and metal chelation) has also been shown to provide protection against Cr(VI) stress in cyanobacteria (Gupta and Ballal, 2015). As sulphur is an essential component of many proteins and non-proteins like glutathione, sulphate deficiency may impact Cr tolerance. The relationship between sulphate deficiency and Cr(VI) tolerance has not been reported

in cyanobacteria earlier. This knowledge may be helpful in understanding the consequences for cyanobacteria when it faces Cr(VI) stress and sulphur limitation at the same time, a situation that may occur even in natural ecosystems. The present study was initiated to understand the response of the cyanobacterium *Anabaena* sp. PCC 7120 to Cr(VI) stress in sulphate varying growth conditions. The specific aims were to assess the tolerance ability and response of antioxidative enzymes (superoxide dismutase and catalase) to Cr(VI) in sulphate-sufficient and sulphate-deficient conditions in *Anabaena* sp. PCC 7120, an ecologically important diazotrophic cyanobacterium.

CHAPTER 3

Materials and Methods

3.1 Organism: *Anabaena* sp. PCC 7120 (hereafter *Anabaena*), a filamentous, heterocystous, diazotrophic cyanobacterium, was used as an experimental organism in the present study.

3.2 Culture methods:

3.2.1 Preparation of culture medium: $BG11_0$-medium (N_2-medium; Rippka et al., 1979) pH 7.5 was used for growing *Anabaena*. $BG11_0$-medium was prepared in Millipore water, and composed of the following macronutrients (mM): Dipotassium hydrogen phosphate, 0.18; Sodium carbonate, 0.19; Magnesium sulphate, 0.30; Calcium chloride, 0.25; Ethylenediaminetetraacetic acid disodium salt), 0.003; Citric acid, 0.029; Ferric ammonium citrate, 0.030 and micronutrients (µM): Boric acid, 46; Manganese chloride, 9.2; Zinc sulphate, 0.77; Sodium molybdate, 1.6; Copper sulphate, 0.32; Cobalt nitrate, 0.17.

3.2.2 Preparation of sulphate-limited $BG11_0$-medium: Sulphate-limited $BG11_0$-medium was prepared by adding 15 µM Magnesium sulphate, 185 µM less than the sulphate concentration used in the standard $BG11_0$-medium. The concentration of Magnesium was adjusted by adding 185 µM Magnesium chloride.

3.2.3 Sterilization: $BG11_0$-medium (sulphate-sufficient and sulphate-limited), culture glass vessels and plasticwares were autoclaved at 121 °C (15 pounds per square inch) for 15 min before use.

3.2.4 Preparation of $K_2Cr_2O_7$ solution for Chromium/Cr(VI) tolerance studies: A 50 mM $K_2Cr_2O_7$ stock solution was prepared in 1 ml Millipore water, and sterilized through nitrocellulose membrane filter (0.2 μm pore size). The sterilized $K_2Cr_2O_7$ solution was then added to sulphate-sufficient and sulphate-limited $BG11_0$-medium at concentrations ranging from 0 to 150 μM.

3.2.5 Culture conditions: *Anabaena* was maintained on agar (1.5 % w/v) slants and grown in liquid $BG11_0$-medium at a room temperature of 25 °C under continuous white light illumination (photon fluence rate 20-23 μmol m^{-2} s^{-1}).

3.3 Growth estimation: Growth of *Anabaena* was measured by estimating the concentration of chlorophyll *a* (Chl *a*) in cultures grown in the presence or absence of Cr(VI) in sulphate-sufficient and sulphate-limited $BG11_0$-medium. To 1 ml culture, an equal volume of 100 % methanol was added and incubated for 10 min to extract Chl *a* (Mackinney, 1941). Chl *a* absorbance was measured at 663 nm using a Cary 60 spectrophotometer (Varian, USA), and its concentration was calculated using the following formula: Chl *a* (μg ml^{-1}) = Absorbance at 663 nmx13.43.

3.4 Microscopic examination of cultures: The cultures of *Anabaena* were routinely examined under a light microscope (Olympus, Japan) at 400x magnification. Approximately 10 filaments were counted to determine the frequency of heterocyst in *Anabaena*. The frequency of heterocyst was calculated as a percentage of total cells (vegetative cells and heterocysts) in the filaments.

3.5 Estimation of accessory photosynthetic pigments: The concentrations of phycocyanin (PC, λmax) and allophycocyanin (APC, λmax 652 nm) were determined from the optical density (OD) of cell free extracts of *Anabaena* at 615 and 652 nm, respectively, using equation [PC] = OD_{615}-0.474xOD_{652}/5.34 and [APC] = OD_{652}-0.208x OD_{651}/5.09, as described by Bennett and Bogorad (1971).

3.6 Determination of intracellular hydroperoxide levels: The cultures of *Anabaena* treated and untreated with Cr(VI) in sulphate-sufficient and sulphate-limited $BG11_0$-medium were centrifuged at 2300x*g* for 5 min at room temperature to harvest the cells. Cells equivalent to Chl *a* concentration of 1.5 µg ml^{-1} were washed twice with sterile Millipore water. The cell pellets obtained after centrifugation (2300x*g* for 5 min) were mixed with a methanolic solution (0.8 ml) containing 0.01 % butylated hydroxytoluene (BHT). To this reaction mixture, 0.1 ml of reagent A (2.5 mM ammonium ferrous sulphate, 0.25 mM sulphuric acid) and 0.1 ml of reagent B (40 mM BHT, 1.25 mM xylenol orange in methanol) was added. The mixture was centrifuged at 10000x*g* following incubation at room temperature for 30 min. Absorbance of the supernatant was measured at 560 nm,

and the concentration of hydroperoxides was calculated using an extinction coefficient of $4.3x10^4$ M^{-1} cm^{-1}, as described by Wolf (1994).

3.7 Extraction of proteins: *Anabaena* cultures treated and untreated with Cr(VI) in sulphate-sufficient and sulphate-limited $BG11_0$-medium were centrifuged at 2300x*g* for 5 min at room temperature to harvest the cells. The cell pellet was suspended in 36 mM potassium phosphate buffer (pH 7.4), and washed twice with the same buffer. Then, the cell pellet was resuspended in the same buffer with protease inhibitor (phenylmethylsulphonyl fluoride, 1 mM) and broken at 4 °C by ultrasonication using a Soniprep 150 (MSE). The cell extracts were then centrifuged at 10000x*g* for 10 min at 4 °C, and the supernatant containing soluble proteins was used for estimation of total protein.

3.8 Estimation of total protein: The total protein concentration in *Anabaena* cultures treated and untreated with Cr(VI) in sulphate-sufficient and sulphate-limited $BG11_0$-medium was measured by Bradford method (I976) using BioRad kit. 1 ml of Bradford reagent was added to 20 µl of cell free extract. After incubation in the dark for 5 min, absorbance of reaction mixture was measured at 595 nm against a reagent blank. Protein concentration was determined from a calibration curve prepared using Bovine serum albumin (BSA) solution as standard.

3.9 Separation of proteins by non-denaturing gel electrophoresis for antioxidative enzyme assays: The cellular proteins (approximately 25 µg) obtained from *Anabaena* cultures treated and untreated with Cr(VI) in sulphate-sufficient and sulphate-limited $BG11_0$-medium were separated by non-denaturing polyacrylamide gel electrophoresis using a BioRad mini gel apparatus (BioRad Laboratories, California, USA). The non-denaturing gels (10 % resolving and 5 % stacking) were prepared according to Sambrook and Russel (2001). The gels contained 10 % glycerol, but no SDS. A 10 % resolving gel was prepared by adding 2 ml of Millipore water, 1.6 ml of 30 % acrylamide and N,N'-methylenebisacrylamide mix (1:29), 1.3 ml of 1.5 M Tris buffer (pH 8.8), 0.05 ml of 10 % ammonium persulphate, and 0.002 ml of TEMED. A 5 % stacking gel was prepared by adding 1.7 ml of Millipore water, 0.42 ml of 30 % acrylamide and N,N'-methylenebisacrylamide mix (1:29), 0.3 ml of 0.5 M Tris buffer (pH 6.8), 0.025 ml of 10 % ammonium persulphate, and 0.002 ml of TEMED. The sample loading dye contained 50 mM Tris buffer (pH 6.8), 0.1 % bromophenol blue and 10 % glycerol, and electrophoresis buffer contained 250 mM Glycine and 25 mM Tris buffer (pH 8.3). Electrophoresis of protein samples was carried out at 4 °C for 3 h under constant amperage.

3.10 In-gel staining for Superoxide dismutase (EC 1.15.1.1) activity assay:

Following separation of proteins by electrophoresis, gel was stained with 28 µM TEMED (N,N,N,N'- tetramethylethylenediamine) and 28 µM riboflavin in 36 mM potassium phosphate buffer (pH 7.8) for 30 min. Then, the gel was further stained for 10 min in 2.5 mM nitrobluetetrazolium and illuminated with a light intensity of 20 µmol m^{-2} s^{-1} for 15 min. The SOD activities appeared as colourless zones on purple-blue background of the gel (Beauchamp and Fridovich, 1971). To identify different SOD metalloforms, gel was soaked for 30 min with 10 mM H_2O_2, and then stained for SOD activity. The gel images were obtained using a Gel documentation system (Syngene,Cambridge,UK).

3.11 In-gel staining for Catalase (EC 1.11.1.6) activity assay: After electrophoresis (as described in section 2.9), gel was incubated with 10 mM H_2O_2 for 10 min, washed with Millipore water, and stained for catalase activity using 1 % ferric chloride and 1 % potassium ferricyanide solution (Weydert and Cullen, 2010) and photographed.

3.12 Determination of non-protein thiol concentration: The non-protein thiol content was estimated in the cell free extracts (obtained as mentioned in section 2.7) of *Anabaena* cultures treated and untreated with Cr(VI) in sulphate-sufficient and sulphate-limited $BG11_0$-medium. Following treatment with 50 % trichloroacetic acid, 100 µl cell free extracts was mixed with 850 µl of 20 mM EDTA and 50 µl of Ellman's reagent (5,5'-dithio-bis-(2-nitrobenzoic acid, DTNB), and the reagent mix incubated for 15 min followed by measurement of absorbance at 412 nm. The

non-protein thiol content was then calculated using a molar extinction coefficient of 14150 M^{-1} cm^{-1} (Sedlak and Lindsay, 1968).

3.13 Statistical analysis: All experiments were repeated at least thrice and the results are presented as mean±standard deviation. The significant differences between control and test samples were analyzed by Student's *t*-test (Microsoft word version 10), and the probability (P) values less than 0.05 were considered significantly different.

3.14 Chemicals: All the chemicals used in this study were obtained from Himedia, SRL, and Merck, India.

Chapter 4

Results and Discussion

4.1 Effect of sulphate limitation on Cr (VI) tolerance in *Anabaena*: Sulphur is an essential macronutrient used by all organisms to synthesize vital biomolecules such as amino acids cysteine and methionine, glutathione (a tripeptide antioxidant), vitamins, co-factors etc. Photoautotrophs like algae, plants and cyanobacteria acquire sulphur in the form of sulphate from the environment, and when sulphate availability becomes poor such organisms exhibit a number of adaptive responses, prominent among them is growth impairment accompanied by bleaching of photosynthetic pigments, also called chlorosis (Gutu et al., 2011). The standard concentration of sulphate used in $BG11_0$-medium is 300 µM (sulphate-sufficient medium) (Rippka et al., 1979). An initial experiment revealed that *Anabaena* requires a minimum of 15 µM of sulphate to sustain growth without becoming chlorotic, thus all experiments were conducted in BG 11_0-medium containing l5 µM sulphate (sulphate-limited medium).

To evaluate the effect of sulphate limitation on Cr(VI) tolerance, actively growing cultures of *Anabaena* were exposed to a range of concentrations of $K_2Cr_2O_7$ (0 to 150 µM) in sulphate-sufficient and sulphate-limited $BG11_0$ media, and changes in Chl *a* contents of such cultures were measured regularly. The concentration of $K_2Cr_2O_7$ used in this study was chosen based on previous reports on Cr (VI) tolerance thresholds in cyanobacteria (Gupta and Ballal, 2015; Tiwari et al., 2018). In sulphate-sufficient medium, Cr (VI) at 50, 100, 125 and 150 µM concentrations inhibited Chl *a* content in *Anabaena* by 20, 40, 60 and 80 %, respectively, as compared with control culture (100 %) without Cr(VI) after 4 days of incubation (Fig. 1A). Whereas, Chl *a* content in *Anabaena* after similar period of incubation with 50, 100, and 125 µM Cr(VI) in sulphate-limited medium, was inhibited by 25, 70, and 80 %, respectively, as compared with control culture (100 %) without Cr(VI) (Fig. 1B). These results suggested that Cr(VI) toxicity in *Anabaena* is dose-dependent, as has also been reported earlier (Gupta

and Ballal, 2015; Tiwari et al., 2018). Secondly, Cr(VI) toxicity is enhanced by sulphate limitation in this cyanobacterium. It is a known fact that Cr(VI)/chromate enter cells of cyanobacteria and various other organisms through sulphate uptake system (Aguilar-Barajas et al., 2011; Gupta et al., 2013). A competitive inhibition of Cr(VI) uptake by sulphate has earlier been shown in the unicellular cyanobacteria *Synechocystis* PCC 6803 and *Synechococcus* PCC 7942 (Gupta et al., 2013). Although not studied here, it is likely that enhanced Cr(VI) sensitivity in sulphate-limited conditions in *Anabaena* may have resulted from enhanced intracellular uptake and accumulation of Cr(VI). Such a mechanism of sulphate limitation induced enhancement of Cr(VI) sensitivity has also been reported in some plants (Schiavon et al., 2007, 2008).

4.2 Effect of Cr(VI) on heterocyst frequency in sulphate-sufficient and sulphate-limited growth conditions in *Anabaena*: To investigate if Cr(VI) affects heterocyst (N_2-fixing cells) frequency in *Anabaena*, light microscopic studies were conducted with 100 µM Cr(VI)-treated and untreated cultures grown in sulphate-sufficient and sulphate-limited $BG11_0$-medium for 4 days. The frequency of heterocyst in the filaments of *Anabaena* was found to be 5-6 %, irrespective of whether the cultures were grown in the presence or the absence of Cr(VI) (Fig. 2). These results suggest that individually or combined, sulphate limitation and Cr(VI) exposure did not lead to any significant drop in heterocyst levels. Thus, the cause of enhanced Cr(VI) sensitivity in sulphate-limited growth conditions may not be due to lack of heterocysts, but whether such heterocysts were functional in terms of N_2 fixation is not known.

4.3 Effect of Cr(VI) on accessory photosynthetic pigment concentrations in sulphate-sufficient and sulphate-limited growth conditions in *Anabaena*: To investigate if Cr(VI) affects accessory photosynthetic pigments such as phycocyanin (PC) and allophycocyanin (APC) in *Anabaena*, concentration of such pigments were evaluated in 100 µM Cr(VI)-treated and untreated cultures grown in sulphate-sufficient and sulphate-limited $BG11_0$-medium for 4 days. The results presented in Fig. 3A and B

shows that PC and APC levels were lower in sulphate-limited *Anabaena* cultures than in sulphate-sufficient cultures. Cr(VI) inhibited PC and APC levels in *Anabaena* under both sulphate-sufficient and sulphate-limited growth conditions compared to their respective Cr(VI)-untreated controls. The inhibitory effect of Cr(VI) was, however, more pronounced in sulphate-limited cultures. These results indicate that both sulphate limitation and Cr(VI) alone or in combination can cause deterioration in PC and APC levels in cells. Such effects may not only lead to reduced light absorption, but may also reduce photosynthetic activity in cyanobacteria. Similar effects of sulphur deficiency induced inhibition of photosynthetic pigment levels, and Cr(VI) inhibition of photosynthetic activity have been reported earlier in cyanobacteria (Gupta and Ballal, 2015; Gutu et al., 2011; Tiwari et al., 2018).

4.4 Effect of Cr(VI) on total hydroperoxide concentration in sulphate-sufficient and sulphate-limited growth conditions in *Anabaena*: Since Cr(VI) is known to trigger ROS formation in cyanobacteria (Bano et al., 2012;Gupta and Ballal, 2015), total intracellular hydroperoxide (an indicator of ROS level) was evaluated in 100 µM Cr(VI)-treated and untreated cultures of *Anabaena* grown in sulphate-sufficient and sulphate-limited $BG11_0$-medium for 4 days. The results presented in Fig. 4 shows that Cr(VI) enhanced hydroperoxide levels by 112 % compared with untreated control cultures (100 %) grown in sulphate-sufficient medium. In sulphate-limited cultures, Cr(VI) increased hydroperoxide level by 133 % compared to Cr(VI) untreated control. These results suggest that Cr(VI) in combination with sulphate limitation causes higher accumulation of hydroperoxides within cells. Hydroperoxides are potent oxidants that may be highly detrimental to cells (Latifi et al., 2009), and its increased production could be one of the main reasons underlying higher sensitivity of *Anabaena* to Cr(VI) under sulphate-limited growth conditions.

4.5 Effect of Cr(VI) on SOD activity in sulphate-sufficient and sulphate-limited growth conditions in *Anabaena*: SOD is a major antioxidative enzyme in

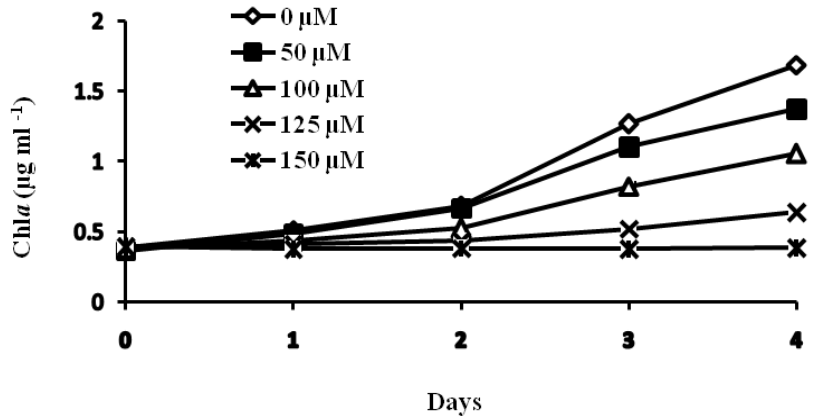
cyanobacteria, as it dismutates ROS $O_2^{\cdot -}$ into molecular oxygen and H_2O_2. The effect of Cr(VI) on SOD activity was evaluated in 100 µM Cr(VI)-treated and untreated *Anabaena* cultures grown in sulphate-sufficient and sulphate-limited $BG11_0$-medium for 4 days by in-gel analysis. The results presented in Fig. 5A shows that *Anabaena* produces four achromatic bands representing four SOD isozymes in all the cultures, regardless of whether they were exposed to Cr(VI) or not in sulphate-sufficient and sulphate-limited medium. The band with highest mobility was identified as FeSOD/SodB based on its H_2O_2 sensitive nature, whereas the bands designated as SOD I to III are MnSOD/SodA, as they are relatively more tolerant to H_2O_2 (Fig. 5B) These results suggest that sensitivity to Cr(VI) in *Anabaena* may not be due to deficiency of SOD activity, and are in agreement with the studies of Gupta and Ballal (2015).

4.6 Effect of Cr(VI) on catalase activity in sulphate-sufficient and sulpahte-limited growth conditions in *Anabaena*: Catalase is an important antioxidative enzyme in cyanobacteria, as it dismutates H_2O_2 to water and molecular oxygen. The effect of Cr(VI) on catalase activity was evaluated in 100 µM Cr(VI)-treated and untreated *Anabaena* cultures grown in sulphate-sufficient and sulphate-limited $BG11_0$-medium for 4 days by in-gel analysis. The results presented in Fig. 6 shows that *Anabaena* lacks catalase activity whether grown in the presence or in the absence of Cr(VI) in sulphate-sufficient or sulphate-limited conditions. Thus, catalase may not be involved in neutralizing Cr(VI)-induced oxidative stress in *Anabaena*. The results are in agreement with an earlier study reporting the absence of catalase activity in *Anabaena* (Banerjee et al., 2012).

4.7 Effect of Cr(VI) on non-protein thiol content in sulphate-sufficient and sulphate-limited growth conditions in *Anabaena*: The non-protein thiols such as cysteine and glutathione are important non-enzymatic components of antioxidative system in cyanobacteria (Latifi et al., 2009). The effect of Cr(VI) on non-protein thiol content was evaluated in 100 µM Cr(VI)-treated and untreated *Anabaena* cultures grown

in sulphate-sufficient and sulphate-limited $BG11_0$-medium for 4 days. The results presented in Table 1 shows that that Cr(VI) increased non-protein thiol content in *Anabaena* under sulphate-sufficient growth conditions compared to Cr(VI) untreated control, suggesting that this could be an important adaptive mechanism to combat Cr(VI) in *Anabaena*. The sulphate-limited cultures of *Anabaena* grown in the absence of Cr(VI) showed lower non-protein thiol content, which further decreased in the presence of Cr(VI). Sulphur requirement for biosynthesis coupled with Cr(VI)-mediated inhibition of sulphur assimilation pathway could well be the basis for reduced content of non-protein thiols (protective antioxidants with role in metal chelation) in cells (Viti et al., 2013). This, in turn, could have increased ROS formation and caused increased sensitivity to Cr(VI) in *Anabaena* during sulphate-limited growth. Non-protein thiols, particularly GSH, has been demonstrated to play a critical role in Cr(VI) tolerance also in *Synechococcus* sp. PCC 7942 (Gupta and Ballal, 2015).

A



B

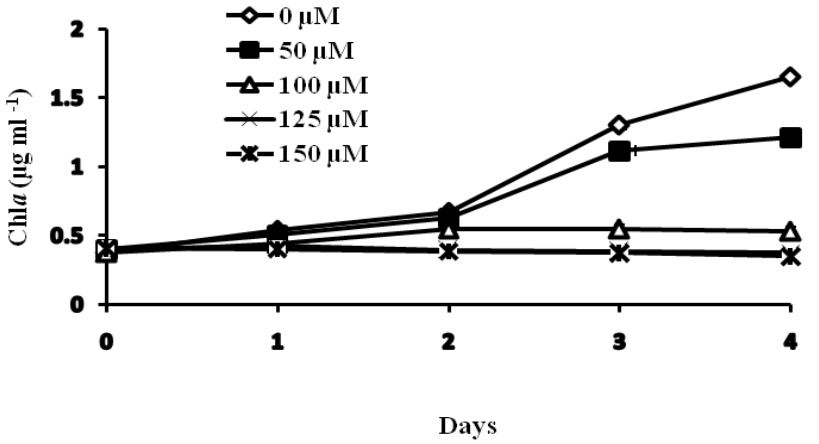


Fig.3.1 Effect of Cr(VI) on growth of *Anabaena* sp. PCC 7120 in sulphate-sufficient (300µM) (A) and sulphate-limited (15µM) medium (B). The actively growing cultures of *Anabaena* were washed twice with sterile $BG11_0$ sulphate-limited medium, harvested, and then transferred to fresh sulphate-sufficient and sulphate-limited media containing indicated concentrations of Cr(VI), followed by growth measurementin terms of Chl*a*. Error bars, sometimes smaller than the symbols, represent mean±standarddeviation of three independent experiments.

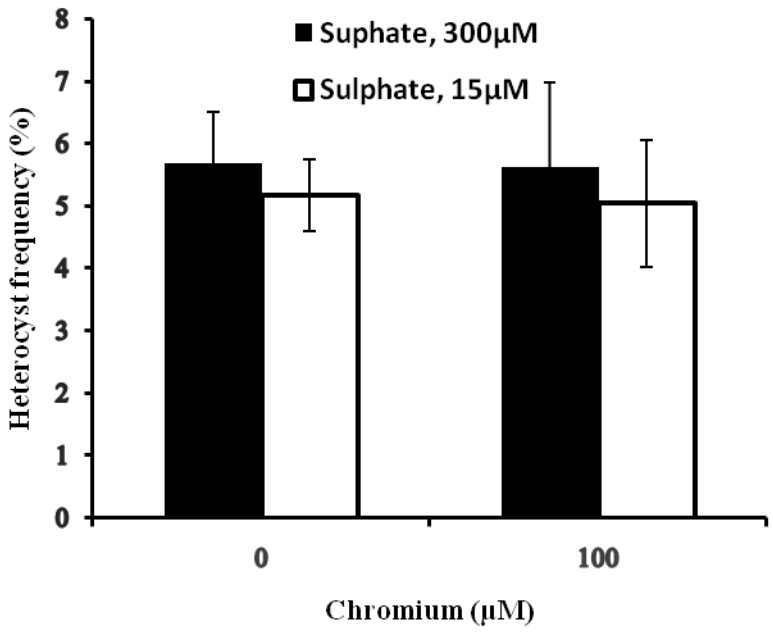
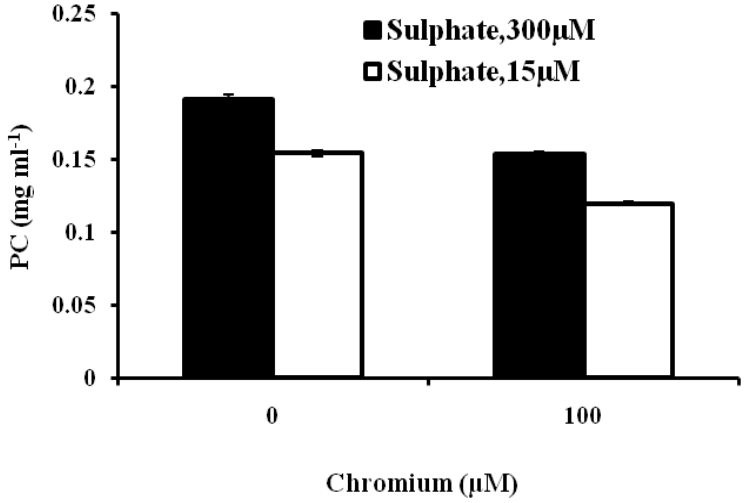


Fig.3.2 Effect of Cr(VI) on heterocyst frequency of *Anabaena* sp. PCC 7120 cultures grown in sulphate-sufficient and sulphate-limited medium. The frequency of heterocysts was counted in filaments of *Anabaena* after 4 days of incubation by light microscopy, and presented as percentage of total cells. Error bars represent mean±standard deviation of three independent experiments.

A



B

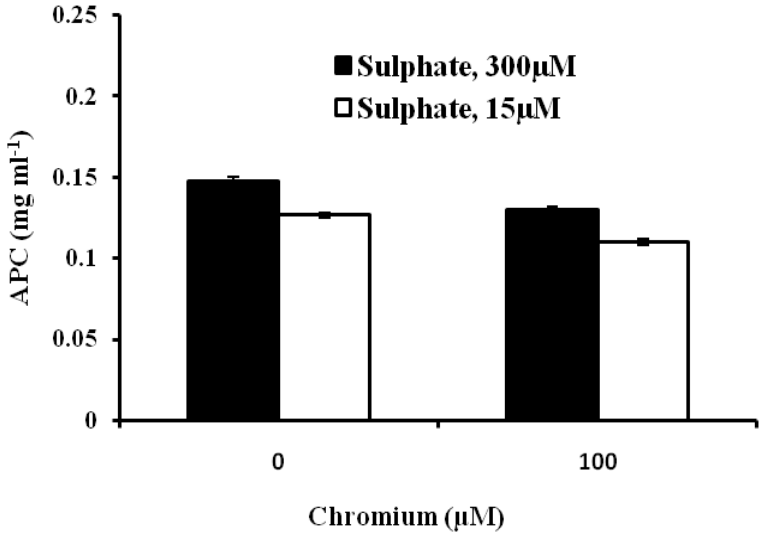


Fig. 3.3 Effect of Cr(VI) on phycocyanin (PC) (A) and allophycocyanin (APC) (B) levels in *Anabaena* sp. PCC 7120 grown in sulphate-sufficient and sulphate-limited medium (B). The PC and APC levels were determined in cell free extracts of *Anabaena* after 4 days of incubation. Error bars, sometimes smaller than the symbols, represent mean±standard deviation of three independent experiments.

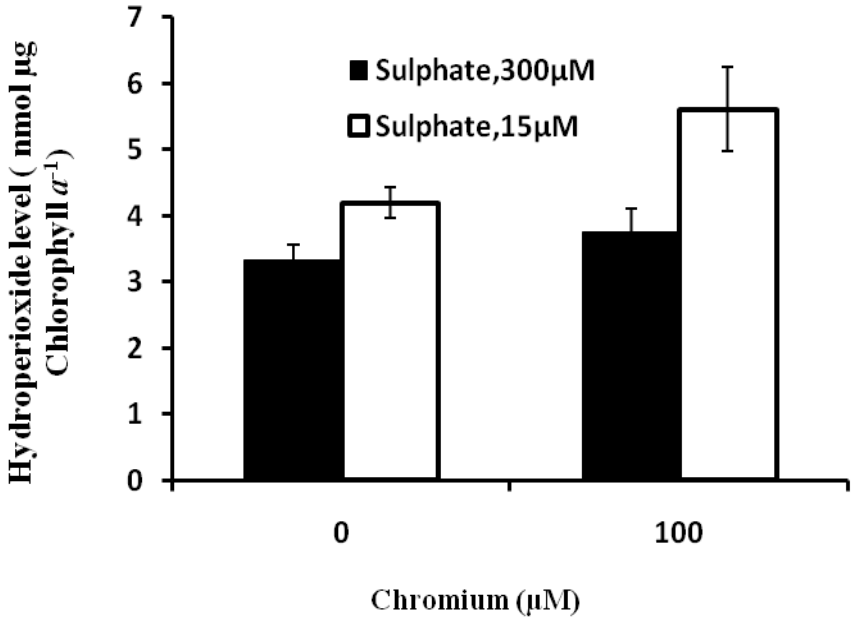


Fig. 3.4 Effect of Cr(VI) on total hydroperoxide levels in *Anabaena* sp. PCC 7120 grown in sulphate-sufficient and sulphate-limited medium. The hydroperoxide levels were determined in whole cells of *Anabaena* after 4 days of incubation. Error bars represent mean ± standard deviation of three independent experiments.

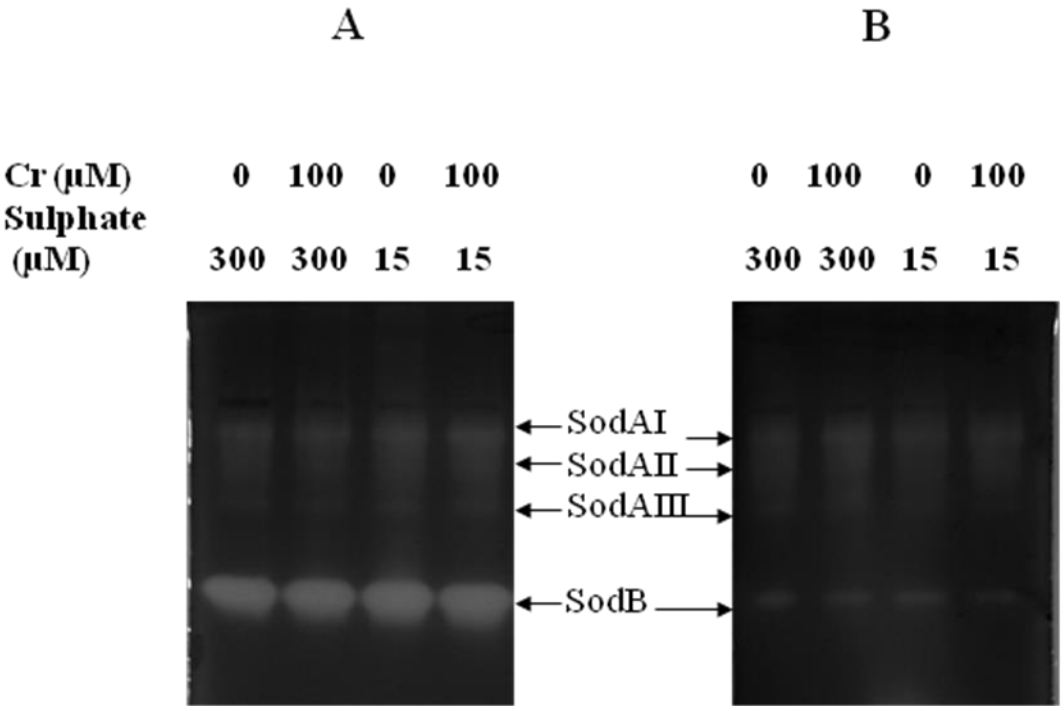


Fig. 3.5 In-gel assay of Superoxide dismutase activity in *Anabeana* sp. PCC 7120 grown in sulphate-sufficient and sulphate-limited conditions in the presence and absence of Cr(VI) (A). Total protein extract from the cultures (25 µg) were loaded on 10 % non-denaturing gel, separated by electrophoresis and stained for SOD activity. SOD isozymes were identified by soaking the gel in 10 mM H_2O_2 for 30 min prior to staining (B). The experiment was repeated at least thrice with similar results, and a representative gel is shown.

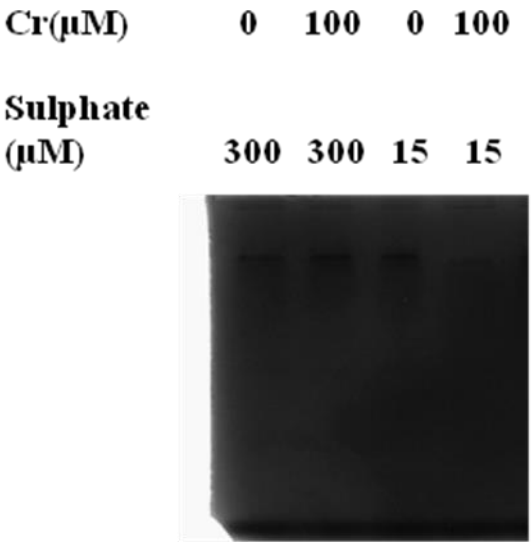


Fig. 3.6 In-gel assay of Catalse activity in *Anabeana* sp. PCC 7120 grown in sulphate-sufficient and sulphate-limited conditions in the presence and absence of Cr(VI). Total protein extract from the cultures (20 µg) were loaded on 10 % non-denaturing gel, separated by electrophoresis and stained for catalase activity. The experiment was repeated at least thrice with similar results, and a representative gel is shown.

Growth conditions	Non protein thiol content (µM)
Sulphate-sufficient (300µM) without Cr(VI)	43±0.011
Sulphate-sufficient (300µM) with Cr(VI)	57±0.007
Sulphate-limited(15 µM) without Cr(VI)	25.5±0.011
Sulphate-limited(15 µM) with Cr(VI)	10.55±0.006

Table 1. Non-protein thiol content in *Anabaena* sp. PCC 7120 grown in sulphate-sufficient and sulphate-limited medium with and without 100 µM Cr(VI)
Data represent the mean±standard deviation of three independent experiments.

CHAPTER 5

Summary

Heavy metal such as Chromium (Cr) is one of the most common pollutants present in many aquatic and terrestrial ecosystems. Cr pollution is mainly caused by industries (electroplating, steel and automobile manufacturing, wood treatment, leather tanning, textile dyeing etc.), which use Cr in large amounts and discharge Cr-containing wastes in aquatic bodies. Most of the Cr in such aquatic bodies exists in the form of chromite [Cr(III)] and chromate [Cr(VI)]. Due to water soluble nature, Cr(VI) spreads quickly within the environment and may severely affect growth and survival of resident biota with serious implications for ecosystem structure and function. Cr(VI) toxicity in organisms may, however, be influenced by availability of nutrients such as sulphate, an essential macronutrient structurally analogous to Cr(VI).

The oxygen-evolving photosynthetic cyanobacteria are abundant and functionally important (primary producers) constituents of microbial population in various ecosystems. Thus, their response to Cr(VI) merits investigation. In the present study, the response of an eco-physiologically important photoautodiazotrophic filamentous heterocystous cyanobacterium *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) to Cr(VI) was characterized in relation to sulphur/sulphate availability. *Anabaena* was exposed to Cr(VI) by addition of a range of concentrations of potassium dichromate ($K_2Cr_2O_7$) from 0 to 150 µM in $BG11_0$-growth medium with varying sulphate levels, named as sulphate-sufficient (300 µM) and sulphate-limited (15 µM) growth media. Growth of *Anabaena*, as measured in terms of Chlorophyll *a* (Chl *a*) content, was similar in sulphate-sufficient and sulphate-limited growth conditions in the absence of Cr(VI). Following exposure to Cr(VI), growth of *Anabaena* was inhibited in

a concentration and time-dependent manner. However, growth inhibition was markedly higher under sulphate-limited condition, as compared with sulphate-sufficient condition, suggesting that sulphate limitation enhances Cr(VI) sensitivity in *Anabaena*. The levels of accessory photosynthetic pigments such as phycocyanin and allophycocyanin were also inhibited by Cr(VI) under sulphate-sufficient and sulphate-limited growth conditions, but heterocyst frequency (N_2-fixation sites) remained unaffected. Evaluation of total intracellular hydroperoxides [indicator of reactive oxygen species (ROS) level] indicated that Cr(VI) enhanced hydroperoxide level, but did so more in sulphate-limited grown cells than in sulphate-sufficient cells, as compared to respective untreated controls, suggesting that Cr(VI) generates oxidative stress, which is further aggravated by sulphate limitation. The activity of superoxide dismutase (SOD, an important antioxidative enzyme involved in detoxification of ROS superoxide radical) by native-PAGE (polyacrylamide gel electrophoresis) assay revealed no change in SOD isozyme composition (one iron-containing and three manganese-containing SODs) and/or levels (visually judged from intensity of bands) in Cr(VI) treated and untreated *Anabaena* cells grown in sulphate-sufficient and sulphate-limited conditions. This suggests that sensitivity to Cr(VI) is not due to lack of SOD activity. Further, no catalase [hydrogen peroxide (ROS) detoxifying enzyme] activity was detected in *Anabaena*, irrespective of its growth in the presence or absence of Cr(VI) and sulphate sufficiency or limitation. However, determination of intracellular non-protein thiol content (cysteine and/or non-enzymatic antioxidant glutathione) revealed that Cr(VI) treatment increased non-protein thiol content in sulphate-sufficient cells of *Anabaena*, as compared to untreated control, suggesting an important role for this class of molecules in management of Cr(VI) stress. In contrast, sulphate limitation individually and in combination with Cr(VI) treatment inhibited non-protein thiol content considerably, the effect being more pronounced under latter condition. This could be due to the fact that non-protein thiols are sulphur containing compounds, and their synthesis would thus be affected by sulphate limitation. Overall, the results suggest that Cr(VI) sensitivity is exacerbated by sulphate limitation in *Anabaena* due to enhanced production of ROS and/or reduced synthesis of

antioxidative protective non-protein thiols. These findings indicate that sulphate availability may play a critical role in growth and survival of *Anabaena* in Cr(VI) polluted ecosystem.

Conclusions

Response to Cr(VI) was evaluated in the present study in *Anabaena* in relation to sulphate availability. The results suggest that sensitivity to Cr(VI) increases in this cyanobacterium under sulphate-limited growth condition accompanied by inhibition of Chl *a*, phycocyanin and allophycocyanin, and increase in ROS formation and reduced synthesis of protective antioxidative non-protein thiols. These findings indicate that sulphate availability may play a critical role in fitness of *Anabaena* polluted ecosystems.

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ABBREVIATIONS

ºC	Degree Celsius
M	Molar
Min	Minute (s)
ml	millilitre
mM	Millimolar
N_2	Dinitrogen
nm	Nanometre
U	Unit
%	Percent
gm	gram
µg	Microgram
µl	Microlitre
µm	Micromole
µM	Micromolar

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DEGREE	: Master of Philosophy
DPARTMENT	: Biotechnology
TITLE OF DISSERTATION	: Role of sulphur in modulating Chromium (CrVI) toxicity in the *Anabaena* sp. PCC 7120
DATE OF ADMISSION	: 07/08/2018
COMMENCEMENT OF SECOND SEM/DISSERTATION	: Feb 2019
APPROVAL OF RESEARCH PROPOSAL	
1. *DRC*	: 07/05/2019
2. *BOS*	: 07/05/2019
3. *SCHOOL BOARD*	: 07/05/2019
MZU REGISTRATION NO.	: 1505352
M.Phil. REGISTRATION NO. & DATE	: MZU/M.Phil./534 of 17.05.19
DATE OF SUBMISSION	: 28 th January2021
EXTENSION	: Feb 20-Jan 21

Head

Department of Biotechnology

ABSTRACT

ROLE OF SULPHUR IN MODULATING CHROMIUM (CrVI) TOXICITY IN THE CYANOBACTERIUM *ANABAENA* SP.PCC 7120

Dissertation submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

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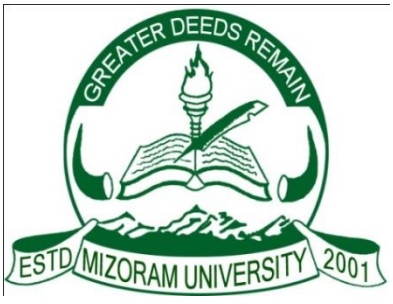

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January, 2021

Abstract

Heavy metal such as Chromium (Cr) is one of the most common pollutants present in many aquatic and terrestrial ecosystems. Cr pollution is mainly caused by industries (electroplating, steel and automobile manufacturing, wood treatment, leather tanning, textile dyeing etc.), which use Cr in large amounts and discharge Cr-containing wastes in aquatic bodies. Most of the Cr in such aquatic bodies exists in the form of chromite [Cr(III)] and chromate [Cr(VI)]. Due to water soluble nature, Cr(VI) spreads quickly within the environment and may severely affect growth and survival of resident biota with serious implications for ecosystem structure and function. Cr(VI) toxicity in organisms may, however, be influenced by availability of nutrients such as sulphate, an essential macronutrient structurally analogous to Cr(VI).

The oxygen-evolving photosynthetic cyanobacteria are abundant and functionally important (primary producers) constituents of microbial population in various ecosystems. Thus, their response to Cr(VI) merits investigation. In the present study, the response of an eco-physiologically important photoautodiazotrophic filamentous heterocystous cyanobacterium *Anabaena* sp. PCC 7120 (hereafter *Anabaena*) to Cr(VI) was characterized in relation to sulphur/sulphate availability. *Anabaena* was exposed to Cr(VI) by addition of a range of concentrations of potassium dichromate ($K_2Cr_2O_7$) from 0 to 150 µM in $BG11_0$-growth medium with varying sulphate levels, named as sulphate-sufficient (300 µM) and sulphate-limited (15 µM) growth media. Growth of *Anabaena*, as measured in terms of Chlorophyll *a* (Chl *a*) content, was similar in sulphate-sufficient and sulphate-limited growth conditions in the absence of Cr(VI). Following exposure to Cr(VI), growth of *Anabaena* was inhibited in a concentration and time-dependent manner. However, growth inhibition was markedly higher under sulphate-limited condition, as compared with sulphate-sufficient condition, suggesting that sulphate limitation enhances Cr(VI) sensitivity in *Anabaena*.

The levels of accessory photosynthetic pigments such as phycocyanin and allophycocyanin were also inhibited by Cr(VI) under sulphate-sufficient and

sulphate-limited growth conditions, but heterocyst frequency (N_2-fixation sites) remained unaffected. Evaluation of total intracellular hydroperoxides [indicator of reactive oxygen species (ROS) level] indicated that Cr(VI) enhanced hydroperoxide level, but did so more in sulphate-limited grown cells than in sulphate-sufficient cells, as compared to respective untreated controls, suggesting that Cr(VI) generates oxidative stress, which is further aggravated by sulphate limitation.

The activity of superoxide dismutase (SOD, an important antioxidative enzyme involved in detoxification of ROS superoxide radical) by native-PAGE (polyacrylamide gel electrophoresis) assay revealed no change in SOD isozyme composition (one iron-containing and three manganese-containing SODs) and/or levels (visually judged from intensity of bands) in Cr(VI) treated and untreated *Anabaena* cells grown in sulphate-sufficient and sulphate-limited conditions. This suggests that sensitivity to Cr(VI) is not due to lack of SOD activity. Further, no catalase [hydrogen peroxide (ROS) detoxifying enzyme] activity was detected in *Anabaena*, irrespective of its growth in the presence or absence of Cr(VI) and sulphate sufficiency or limitation. However, determination of intracellular non-protein thiol content (cysteine and/or non-enzymatic antioxidant glutathione) revealed that Cr(VI) treatment increased non-protein thiol content in sulphate-sufficient cells of *Anabaena*, as compared to untreated control, suggesting an important role for this class of molecules in management of Cr(VI) stress. In contrast, sulphate limitation individually and in combination with Cr(VI) treatment inhibited non-protein thiol content considerably, the effect being more pronounced under latter condition. This could be due to the fact that non-protein thiols are sulphur containing compounds, and their synthesis would thus be affected by sulphate limitation. Overall, the results suggest that Cr(VI) sensitivity is exacerbated by sulphate limitation in *Anabaena* due to enhanced production of ROS and/or reduced synthesis of antioxidative protective non-protein thiols. These findings indicate that sulphate availability may play a critical role in growth and survival of *Anabaena* in Cr(VI) polluted ecosystems.