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Research Article

REMEDIATION OF HEAVY METALS FROM TANNERY EFFLUENTS AND ITS EFFECTS ON ENZYMATIC AND NON-ENZYMATIC ANTIOXIDANTS OF CYANOBACTERIA

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ABSTRACT

Cyanobacteria account for most of the biologically sequestered heavy metals in terrestrial as well as aquatic environments. Based on previous research of our lab and prevailing literature, Cyanobacteria's ability to absorb and metabolize heavy metals can be associated with the presence of high-affinity metal binding groups on the cell surface and efficient metal uptake and storage systems. Our study dealt with the uptake of heavy metals dissolved in Tannery effluent by freshwater Cyanobacteria. Various microscopic and viability studies were performed along with studies on the effects of respective heavy metals on the non-enzymatic anti-oxidative pigments and anti-oxidative enzymes of Cyanobacteria. Maximum metal uptake was observed in case of Fe and Cr compared to Cd or Pb at different concentrations and conditions. The given study shows how freshwater Cyanobacteria are an economical, productive alternative of the prevailing remediation techniques for the remediation of heavy metals.

Keywords: Anti-oxidative enzymes, Atomic Absorption Spectrometry, Bioremediation, Camera Lucida, Cyanophycean media, Non-enzymatic pigments.

1. INTRODUCTION

The tanning process undertaken by Tanneries is almost entirely a wet process that consumes large amounts of water, and generates almost 90% of the used water as effluent. Tannery effluents carry heavy pollution loads due to a vast presence of highly colored compounds, Sodium Chloride and Sulphate, different organic and inorganic substances, toxic metallic compounds etc. These effluents disrupt the standard lives of the water bodies and land surfaces into which they are drained.

Two of the major tanning techniques that contribute to high heavy metal concentrations are:

- Chrome tanning-Chromium (III) sulfate ($[Cr(H_2O)_6]_2(SO_4)_3$) has long been considered as the most efficient and effective tanning agent
- Alternative chemicals-Wet white (an off-white semi finished stage of leather) can be produced using aldehydes, aluminum, zirconium, titanium, or iron salts, or their combination. Researches regarding more efficient wet white methods to reduce any toxic chromium (VI), that may form during the tanning process have been actively pursued [1].

The chrome tanning effluents contain sulphuric acid, chromium, chlorides, sodium bicarbonate and sulphates. Of the large number of chemicals used, only about 20% is absorbed by leather in the tanning process and the rest is released as wastes.

Heavy metal ions, like Zn, Cu, Mg, Cr, Cd, Pb, Fe and As, are commonly detected in tannery sludge samples [2]. The average concentrations of Na and Cr in tannery sludge can sometimes reach around 6070 and 10,000 mg/kg and they are far above the standard limits. Due to the addition of large amounts of chromium salts in the chrome tanning process, presence of excessively high concentrations of Cr in the sludge is observed [3-6].

Among Biological treatments, the chromium remediation ability of bacteria *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae* in consortia and in their immobilized forms have been studied [7-9]. Studies on potential degradation of industrial effluents by environmental species of Cyanobacteria showed biodegradation and biosorption capacity of some potential cyanobacterial species: *Oscillatoria* sp., *Synechococcus* sp., *Nodularia* sp., *Nostoc* sp. and *Cyanothece* sp. with varying sensitivity [10-11]. Uses of cyanobacterial mat have also been mentioned in a number of literatures to remove hexavalent chromium [12-13]. A mat of consortium of cyanobacteria/blue-green algae such as *Chlorella* sp.,

Phormidium sp. and *Oscillatoria* sp. has been proved to be very effective [14-16].

Defense Mechanism of Cyanobacteria:

- Uptake and adsorption of metal through Gelatinous sheath.
- Efflux of heavy metals from cytoplasm.
- Complexation of heavy metal ions inside the cell by various substances, *e.g.,* Organic acids, Amino acids, Ferritins, Phytochelatins, heat shock proteins and Metallothionines.
- Biochemical stress defense responses such as induction of oxidative enzymes.

In this paper research work on remediation of heavy metals from tannery effluents and its effects on enzymatic and non-enzymatic antioxidants of Cyanobacteria have been discussed.

2. MATERIAL AND METHODS

2.1. Collection of Water Sample containing Cyanobacteria

To culture Cyanobacteria, water samples were collected from Kolkata and its outskirts. Water samples were taken from stagnant greenish coloured ponds during 7-9 am.

2.2. Culture and Isolation of Cyanobacteria:

The BG11 and Cyanophycean media was prepared, autoclaved, cooled and pH maintained at 7.5. Antibiotic Cycloheximide 50µg/ml was initially added [17-18] to prevent undesirable fungal growth. Water samples collected were taken in tarson centrifuge tubes (15ml) and centrifuged at 2500 rpm for 10mins. The process was repeated a number of times in order to get considerable amount of green pellet. The green pellet were then used to inoculate the media, in 1L conical flasks, maintaining a sterile condition and were incubated under white fluorescent light (40W) for 16 hrs of light and 8hrs of darkness at room temperature in undisturbed condition for 14-21days.

2.3. Morphological identification of Cyano-bacteria

Camera Lucida [19] drawings and staining with Cotton blue and Lactophenol was performed with the crude sample as well as the cultured sample.

2.4. Collection of water sample containing Heavy Metals

Tannery effluents containing heavy metals were collected from outskirts of Kolkata.

2.5. Analysis of heavy metals present in Tannery Effluent

AAS was performed to measure the concentrations of dissolved heavy metal residues. Stock solutions of 1% and 10% concentration were prepared from the raw effluent.

2.6. Determination of the amount of heavy metals taken up by Cyanobacteria

The experimental set up consisted of sterile BG11 medium (pH7.4), sodium acetate buffer (pH 5.6), effluent concentrations prepared (1%, 10% and raw) and inoculum (filamentous cyanobacteria). The control set up consisted of all the constituents except the inoculum. The control and the experimental set up were incubated for 21 days at 16 hrs of light and 8 hrs of darkness at room temperature in an undisturbed condition. AAS was performed to measure the concentrations of heavy metals and calculate the metal uptake by Cyanobacteria (Blank=Medium without heavy metals and inoculum; Control=Medium with heavy metals but without inoculum) [20-21].

2.7. Determination of effect of heavy metals on Cyanobacterial Chlorophyll content

The chlorophyll was measured at 665nm [22]. For carotenoid estimation, absorbance was measured at 470nm (Lichtenthaler method). Staining of the metal treated Cyanobacteria was performed by Cotton blue-Lactophenol.

2.8. Determination of the effects of Heavy metals on Cyanobacterial Antioxidants

To analyze the effects of heavy metal treatment on the Cyanobacteria, Enzymatic and Non-enzymatic Anti-oxidants were estimated. The major non-enzymatic antioxidative pigment Phycobillin (Phycoerythrin, Phycourobilin, Phycoviolobilin and Phycocyanobilin) was estimated by the method described by Bennett and Bogorad [23-24]. Assays of Antioxidative enzymes like Superoxide Dismutase, Catalase, Ascorbate Peroxidase [25-31] were performed.

3. RESULTS AND DISCUSSION

3.1. Culture and isolation of Cyanobacteria

BG11 showed profuse growth compared to other media. Growth in solid Cyanophycean media occurred after an incubation of more than 45 days. The cultures on solid media was maintained by repeated streak plate and spread plate methods. To inhibit the undesirable growth of eukaryotic organisms such as diatoms, green algae and

fungi antibiotic Cycloheximide (Himedia) 50µg/ml was added to both liquid and solid media initially [17-18, 32]. Later on, the use of antibiotic was discontinued once cyanobacterial genera were obtained. Upon staining with Lactophenol Cotton blue under low and high power objectives, irregular compact or loose aggregated colonies were observed under the microscope (depending on the media type). Cells in the colonies were embedded in mucilage and gelatinous sheath. Filamentous and colonial forms of Cyanobacteria were observed after 14-21 days of culture. A number of genera such as *Microcystis, Nostoc, Anabaena, Oscillatoria, Lyngbya, Gloeotrichia, and Merismopedia* along with some diatoms, green algae were observed in the crude water sample. Further experiments were performed with filamentous sample.

3.2. Analysis of Heavy Metals in Tannery Effluent

The water sample collected from the Leather Complex in Kolkata was subjected to AAS to measure the concentrations of different types of heavy metals present [33].

The AAS results determining the concentrations of the various dissolved heavy metals are shown in Table 1. The results showed that the water sample collected from the tannery (tannery effluent) initially contained high amounts of Cr (35.7mg/l), followed by Fe (4.34mg/l), whereas the Pb and Cd concentrations were considerably less. After incubation with Cyanobacteria for 21 days another AAS interestingly revealed that there was a drastic reduction in the concentration of total Cr (0.06 mg/l) and Fe (<0.1 mg/l), although there was almost no change in the concentrations of Pb and Cd.

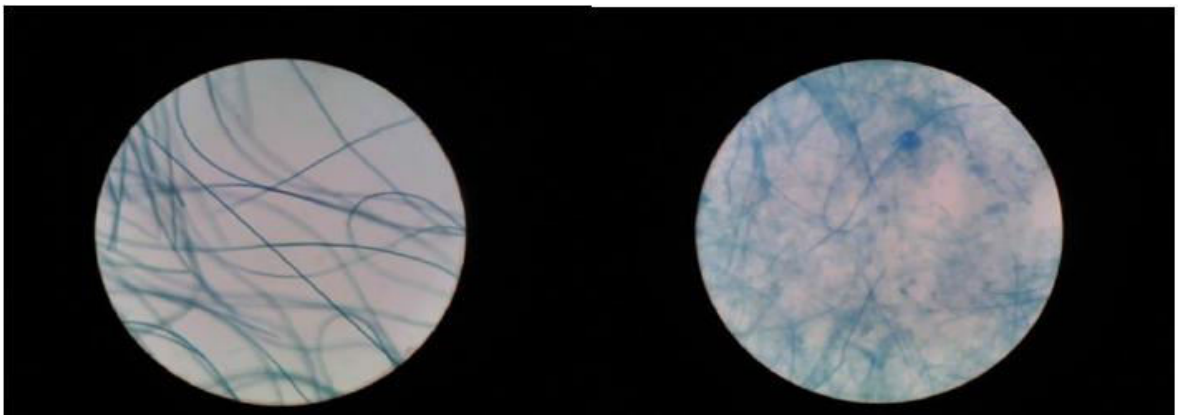
3.3. Morphological analysis of treated Cyanobacteria under Light microscope and Scanning Electron Microscope

The Cotton blue and Lactophenol staining (fig. 1) showed that the filaments of Cyanobacteria had ruptured and the mucilaginous sheath was less dense when treated with the 1% dilution of the effluent but with the increase in concentration of the effluent (*i.e.* at 10% and 100%) the filaments were fully fragmented, decreased in number, along with the disappearance of the sheath after 21 days.

Scanning electron micrograph, shown in Fig. 2, explains the morphology of the Cyanobacteria, before and after biosorption. In treated Cyanobacteria, intact filaments were broken, their numbers were reduced, and the thick, dense mucilaginous sheath of Cyanobacteria was highly reduced after 21 days upon incubation in raw tannery effluent [34].

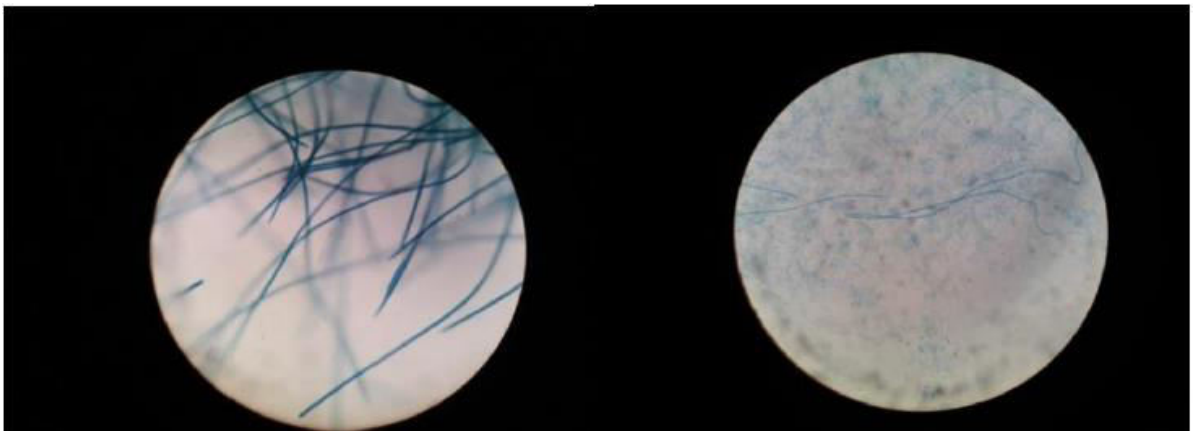
Table 1: AAS results determining concentrations of heavy metals in effluent sample before and after Cyanobacterial treatment

Sl. No.	Parameters	Unit	Minimum detection limit	Result (before incubation with Cyanobacteria)	Result (after incubation with Cyanobacteria)	Test Method Specification
1	Total Fe	mg/L	0.1	4.34	<0.1	APHA 23rd Edition, 3500-Fe B
2	Total Cd	mg/L	0.02	0.283	0. 283	APHA 23rd Edition, 3111B
3	Total Pb	mg/L	0.1	0.088	0.088	APHA 23rd Edition, 3111B
4	Total Cr	mg/L	0.05	35.7	0.06	APHA 23rd Edition, 3111B



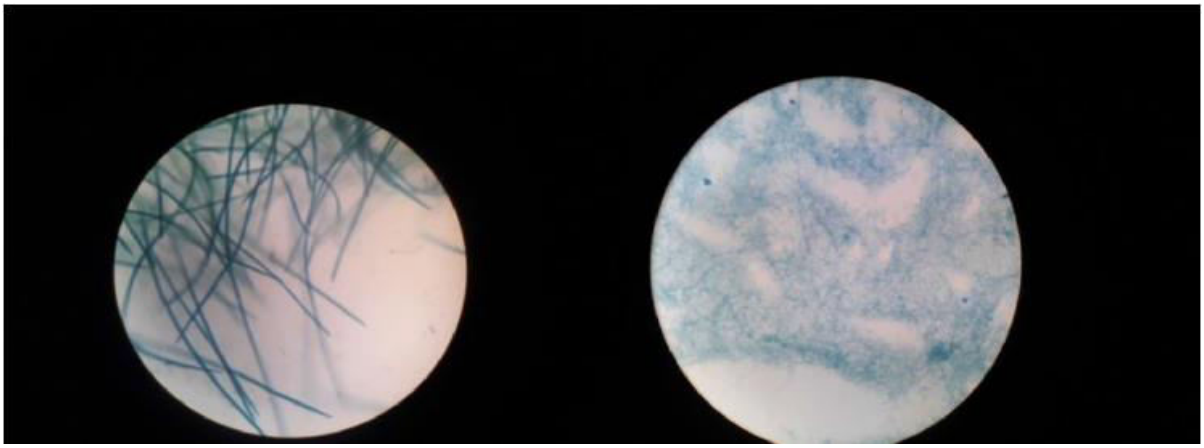
i. Before treatment **ii. After Treatment**

a) Tissue subjected to 1% effluent concentration viewed under light microscope (40X)



i. Before Treatment **ii. After Treatment**

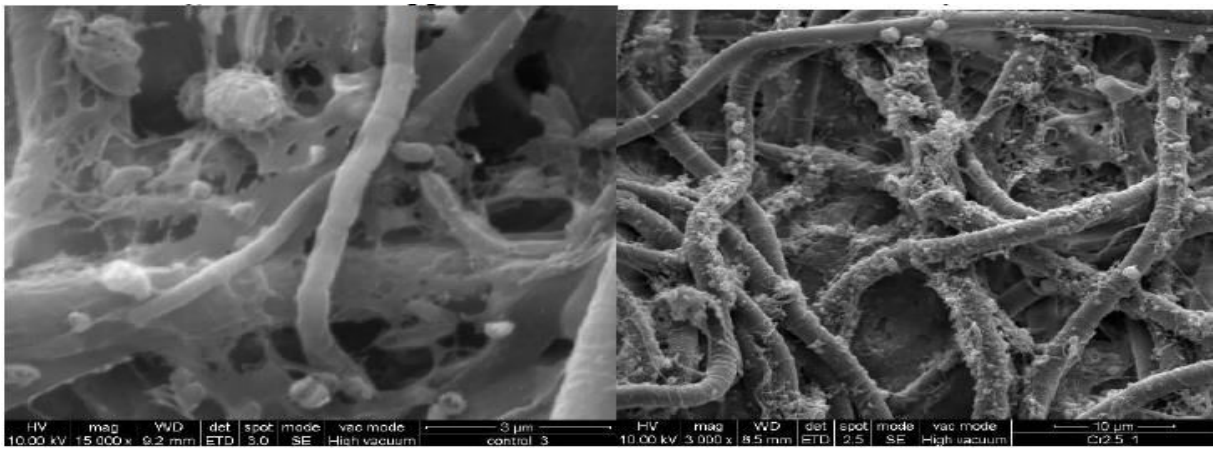
b) Tissue subjected to 10% Effluent concentration viewed under light microscope (40X)



i. Before Treatment **ii. After Treatment**

c) Tissue subjected to Raw Effluent viewed under light microscope (40X)

Fig. 1: Light Microscope fields of Cyanobacterial tissue before being subjected to heavy metals and after 21 days of exposure to heavy metals



a) Untreated Cyanobacteria **b) Treated Cyanobacteria**

Fig. 2: Scanning Electron Microscope fields of Cyanobacterial tissue before being subject to heavy metals and after 21 days of exposure to heavy metals

3.4. Effect of heavy metals on Chlorophyll content of Cyanobacteria after 21 days

The bar diagram (fig. 3) shows that there is a decrease in the chlorophyll content with the increasing concentration of heavy metals. A drastic decrease of chlorophyll content is observed in case of Cyanobacteria treated with 10% and raw effluent. A decrease in Chlorophyll levels is a direct indicator of Cyanobacteria responding to the heavy metal stress.

3.5. Effect of heavy metals on Phycobiliproteins (non-enzymatic anti-oxidative pigments) of Cyanobacteria after 21 days

It could be inferred from the bar diagram (fig. 4) that in case of raw sample, allophycocyanin was the least affected compared to phycocyanin and phycoerythrin. In case of 10% and 1% tannery effluent, phycocyanin was most affected, compared to allophycocyanin and phycoerythrin was least affected.

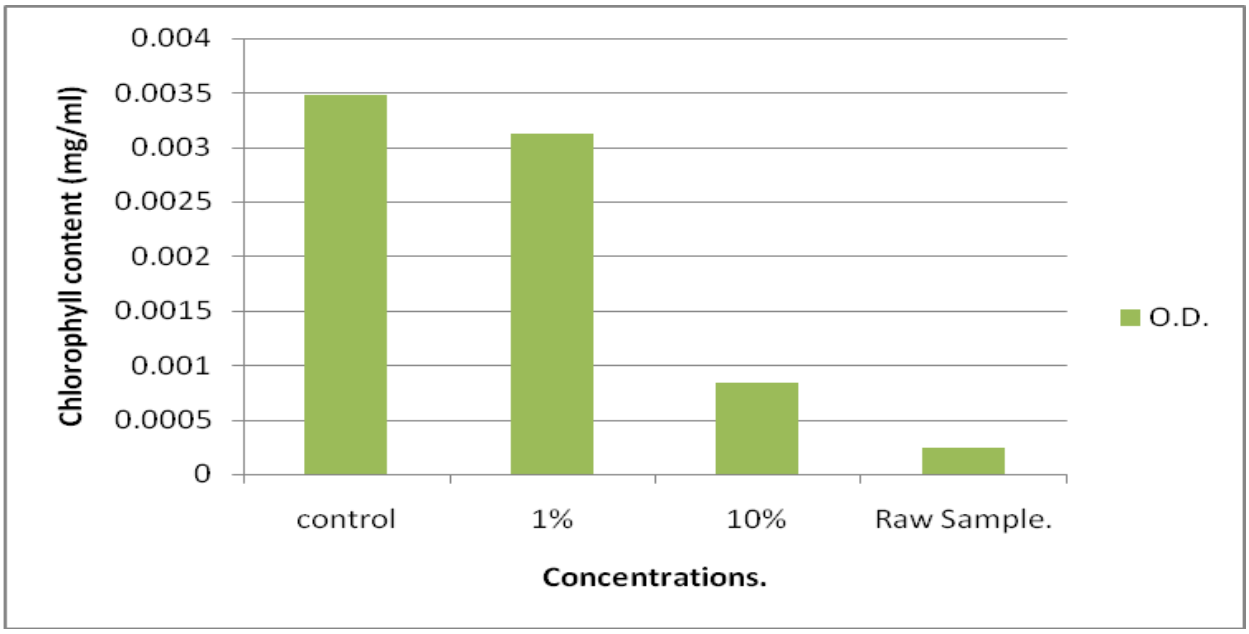


Fig. 3: Cyanobacterial content of Cyanobacterial cells

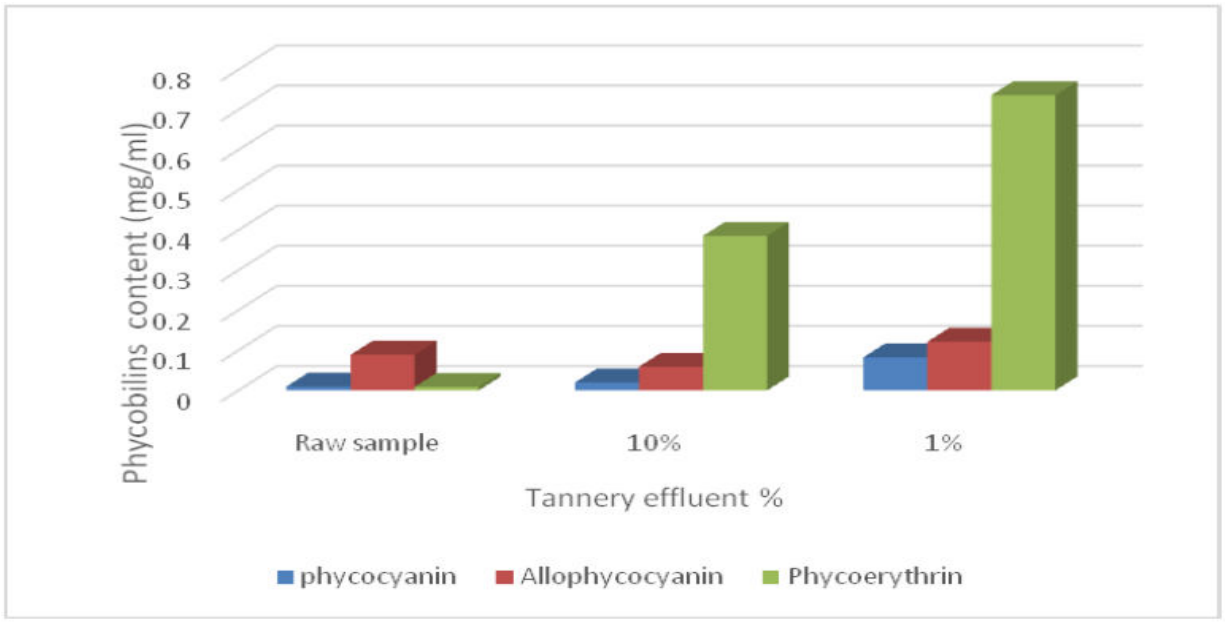


Fig. 4: Effect of Tannery effluent on Phycobilins of Cyanobacteria

3.6. Effect of heavy metals on Antioxidative Enzymes of Cyanobacteria after 21 days

From the bar diagram derived by performing the assay of Super Oxide Dismutase (Fig. 5), it can be seen that the concentration of SOD has steadily increased with gradual increase in the concentration of heavy metals. Thus, as the stress of heavy metals increases, SOD secreted by the cells increase likewise. These results can be described by the fact SOD is the 1st line of defense against oxidative stress, as it converts Superoxide anion to Peroxide, which can be converted to water by Catalase or Peroxidase.

From the bar diagram derived by performing the assay of Ascorbate peroxidase (Fig. 6), it can be seen that there is a considerable increase in APX in case of raw sample and least in case of 1% tannery effluent compared to that of control. Thus, as the stress of heavy metals increases, APX secreted by the cells increases likewise [35].

From the bar diagram derived by performing the assay of Catalase (Fig. 7), it can be seen that a steady decrease in the concentration of the Catalase enzyme with increase in metal concentration can be observed. Thus, as the stress of heavy metals increases, Catalase secreted by the cells keeps decreasing. We can infer that increase in metal concentration is detrimental to the production of Catalase by Cyanobacteria.

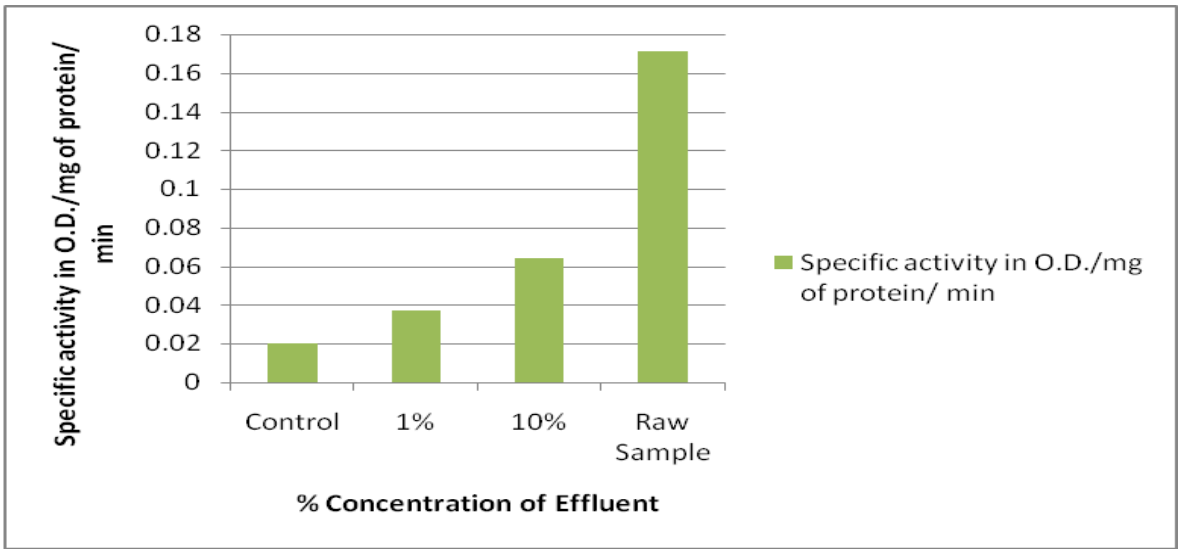


Fig. 5: Assay of Superoxide Dismutase

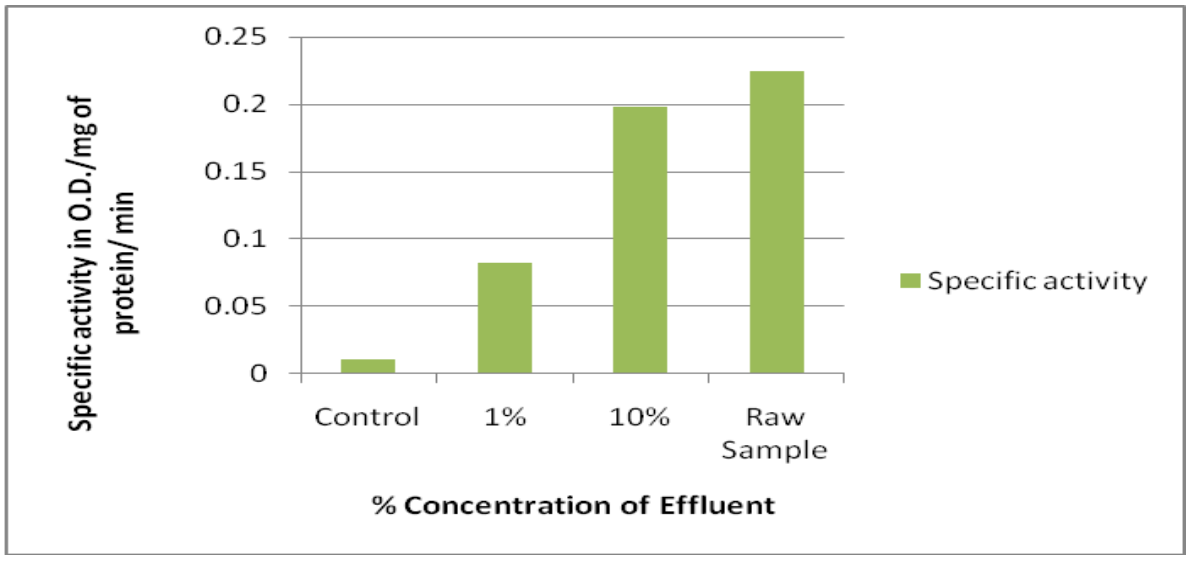


Fig. 6: Assay of Ascorbate Peroxidase

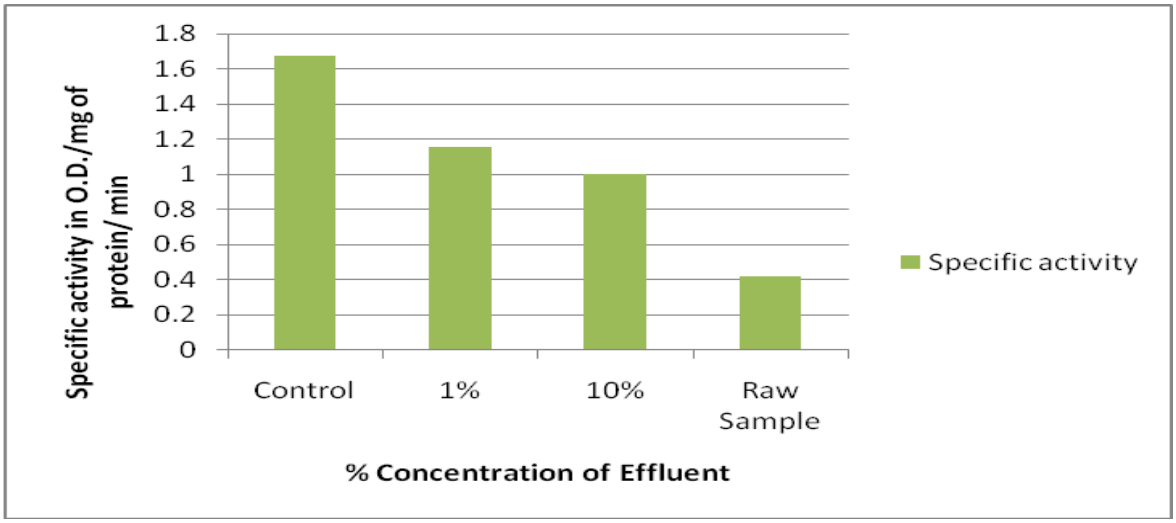


Fig. 7: Assay of Catalase

4. CONCLUSION

From the data obtained from the experiments performed, we can conclude that Cyanobacteria is capable of uptaking heavy metals like chromium and iron from the tannery effluent, thus aiding significantly in Bioremediation. The cyanobacterial samples used were able to uptake the total Chromium and Iron from the Tannery effluent efficiently as compared to Lead and Cadmium. After staining with Lactophenol and Cotton blue it was found that the incubated Cyanobacterial filaments were ruptured, along with reduction of mucilaginous sheath in case of raw effluent compared to that of 1% and 10% effluents. Analysis by Scanning Electron Microscopy showed intact filaments were broken, their numbers reduced, mucilaginous sheath was highly reduced upon incubation in raw tannery effluent for 21 days, compared to that of untreated Cyanobacteria. Chlorophyll content was most affected by raw effluent sample followed by 10% and 1% tannery effluent. In case of raw sample, Allophy-cocyanin was least affected compared to Phycocyanin and Phycoerythrin. In case of 10% and 1% tannery effluents, Phycocyanin was most affected compared to Allophycocyanin and Phycoerythrin as non-enzymatic antioxidants. The antioxidative enzymes Superoxide Dismutase (SOD) and Ascorbate Peroxidase (APX) provided sufficient protection to Cyanobacteria while Catalase was highly affected due to the various concentrations of tannery effluent. Thus the study showed that the isolated freshwater filamentous Cyanobacteria is an effective, economical alternative for the degradation of dissolved heavy metals in tannery effluents.

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Conflict of interest

None declared

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