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Lead Toxicity on Growth and Nitrate Reductase Activity of the Rice Field Cyanobacterium *Scytonema*: Ameliorating Effect of pH, Algal Filtrate, Phosphate and Ascorbic Acid.

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ABSTRACT

Heavy metals are ubiquitous in the biosphere where they occur as part of the natural constituents of chemicals to which biota and human beings are frequently exposed. This results in introduction of substantial amounts of potentially toxic metals into the food chain. Microorganisms form a group of inseparable interacting communities, which are subjected to such unfavorable alteration of the aquatic and other ecosystems. Cyanobacteria, one of the micro living organisms, with their nitrogen fixing capacity and photoautotrophic nature have a very important place in every ecological niche and in many other biotechnological processes, with equally good potentials for eliminating the heavy metals from the ecosystems. In the present investigation a comparative study of growth and nitrate reductase activity has been made with free and immobilized *Scytonema* cells subjected to lead toxicity. Effect of different pH, algal filtrate, high phosphate and oxalic acid concentrations have also been studied. Results show that immobilization could reduce Lead toxicity in *Scytonema*. pH, algal filtrate phosphate and ascorbic acid not only ameliorated toxicity but seemed to have a stimulatory effect on growth and nitrate reductase activity of *Scytonema.*

INTRODUCTION

Present day pollution is the contamination of Earth's environment with materials that interferes with human health, the quality of life, or the natural functioning of ecosystem (living organism and their physical surroundings). Industrializations and urbanization has substantially enhanced the degradation of our environment by polluting two basic amenities of life – air and water. As a result, a huge amount of pollutants is ejected into the environment and creates a danger for all living organisms. Approximately 40 heavy metals (HMs) of the periodic system are potentially toxic to humans, animals, plants, and microorganisms; their high concentrations accumulate in the ecosystems nutrient chain (Nalimova *et al.,* 2005; De Schamphelaere *et al* 2014; Sulaymon *et al* 2013). Among the heavy metals (HMs), Lead pollution is a worldwide phenomenon and is associated with a broad spectrum of human activities, ranging from the most basic agricultural practices to the most high tech industrial processes. Lead is one of the oldest known major heavy metal of antiquity and has gained considerable importance as a potent environmental pollutant which is non-degradable and thus persistent. It is non-essential element, highly toxic to microorganisms due to its ability to replace functional metals leading to denaturation of proteins, nucleic acids, and enzymes.

Many chemical methods are currently known for removing HMs from aqueous solutions, but the disadvantages of these methods are expensive, low economic efficiency, especially during removing small amounts of HMs. With the advance in biotechnology, bioremediation has become one of the major developing field of environmental restoration, utilizing microorganisms to reduce, eliminate, contain and transform to benign products, contaminants present in soil, water, air or sediments (Henry et al 2005, Prasanna *et al* 2008). In this connection many algae have immense capability to sorbs metals, and there is considerable potential for using them to treat wastewaters.

Cyanobacteria represent an ancient group of oxygenic, photosynthetic prokaryotes having bio remedial capacity. In the aquatic environment and flooded paddy fields cyanobacteria can act as biological sorbents because of their affinity to bind dissolved metals, thus playing an important role in metal sequestration and affecting metal speciation (Narula P *et al*., 2015; Li *et al.,* 2004; Zhou *et al*., 2004; Banerjee *et al.,* 2004). Cyanobacteria are effective biological metal sorbents, representing an important sink for metals in aquatic environment (Baptista Mafalda, 2006). Characterization of metal-cyanobacteria sorption reactions has demonstrated that the cyanobacterial surfaces are complex structures, which contain distinct surface layers, each with unique molecular functional groups and metal binding properties (Dittrich and Sibler, 2005; Kretschmer *et al*., 2004). Technological options should not just be confined to remediation strategies, but concentration on mitigation strategies through reduction either by total replacement of heavy metals by alterations or refining the existing technologies for reducing the requirement. Among the remediation technologies available for contaminated sites, *in situ* immobilization techniques are of particular interest because immobilized algal cultures were found to survive the heavy metal stress better than the free cells and these cells were found to remarkably improve functional longevity, easy regeneration and reuse, high biomass loading, provide metal binding sites for metal ions, higher nitrogen fixation, H_2 evolution and NH_3 production rates, in cyanobacteria.

Lead was chosen for the present study because it is found abundantly in water and soil ecosystem from leaching of factories related to paint industries, batteries, electroplating and also from immersion of idols and proof to be quiet hazardous, if present above the normal level for both humans and other flora and fauna. Since paddy fields are watered from these water bodies, it poses to be a threat for the naturally present flora especially the cyanobacteria which are natural contributors of N and P in these ecosystems. So, any deleterious effect on these organisms can greatly reduce the crop yield.

In the present work the cyanobacterium *Scytonema* which has been isolated from a paddy field in West Bengal that receives water from a pond in which the idols after festivals are immersed has been used as the test organism. Effect of lead on growth, nitrate reductase activity has been studied on free and immobilized cells. Also lead accumulation and effect of different pH and concentrations of algal filtrate, high phosphate concentration have been studied and used to extrapolate the data to rice field conditions. The whole protein profile was also seen with and without Lead treatment on free and immobilized cells using SDS-PAGE to document changes in protein profile of the organism with lead treatment.

MATERIALS AND METHODS

Incubation and Maintenance of Culture: This work was performed with a cyanobacterium *Scytonema* that was obtained from Indian Agriculture Research Institute, New Delhi, India, that was isolated from a paddy field receiving lead contaminated water and grown in BG11 and maintained at 27±ºC, 2500± 200 lux light intensity and 14:10 hour light and dark rhythm.

Determination of lethal and sub lethal concentration of the metal The lethal and sub lethal values of lead treated cyanobacterium were scored by Standard plate / Colony count method with an exposure time to the metal equivalent to the generation (doubling) time of the organism. 20 ppm was the sub lethal dose and 40 ppm was lethal for *Scytonema*.

Metal uptake studies

The uptake of lead was under-taken in an Atomic Absorption Spectrophotometer Model 2380 Perkin Elmer and expressed in mgl^{-1} by the method described by Singh et al 1989. The result has been calculated by the difference

observed in the concentration of both metals comparing the initial value (mgl^{-1}) added in culture medium containing the cyanobacterium/immobilized beads and the value (mgl^{-1}) remaining in the medium after 8 days.

Immobilization procedure: Immobilization was done by the cell entrapment method using calcium alginate as described by Singh et al. 1989.

Metal solution**:** Lead solutions with different concentrations were prepared by dissolving $PbCl_2$ in conc. HCl diluted in double distilled water (1:99) and were filtered with 0.45 µ Millipore filter before adding to the medium.

Growth measurement**:** Growth of *Scytonema* was studied in free cells and immobilized cells and compared. Growth measurement was conducted by chlorophyll *a* extraction and the optical density read in a Systronic 169 Spectrophotometer at 663 nm. The amount of chlorophyll *a* was calculated by the growth equation of McKinney 1941.

Protein Profiling: Total protein isolation performed as described earlier (Sinha *et al* 1995). SDS-PAGE was done by the method of Laemmli, 1970.

Nitrate reductase measurement: The estimation of *in vivo* nitrate reductase activity was measured by the method of Camm and Stein, 1974 and as slightly modified by Kumar and Kumar, 1980.

Toxicity mitigating studies: An experiment was conducted with different ratio of algal filtrate: medium with lead. The algal filtrate was prepared by centrifuging two month old culture of *Aulosira fertilissima* and using the supernatant. This filtrate was added in different ratio to the medium (e.g. 25:75, 50:50, 75:25 of filtrate and medium respectively).

The different pH of the medium was adjusted by adding 2N, NaOH or HCl and monitoring in Systronic 335 digital pH meter.

The effect of phosphate was analyzed by dissolving different concentration of phosphate (1%, 5% and 10%) in the form of potassium dihydrophosphate in BG-11 medium.

RESULT

Metal Uptake Studies

The metal uptake studies of Lead in *Scytonema* revealed that Lead was taken up in significant quantity. The percentage of uptake by free cells was 75%, in immobilized cells uptake was 87% (Fig 1).

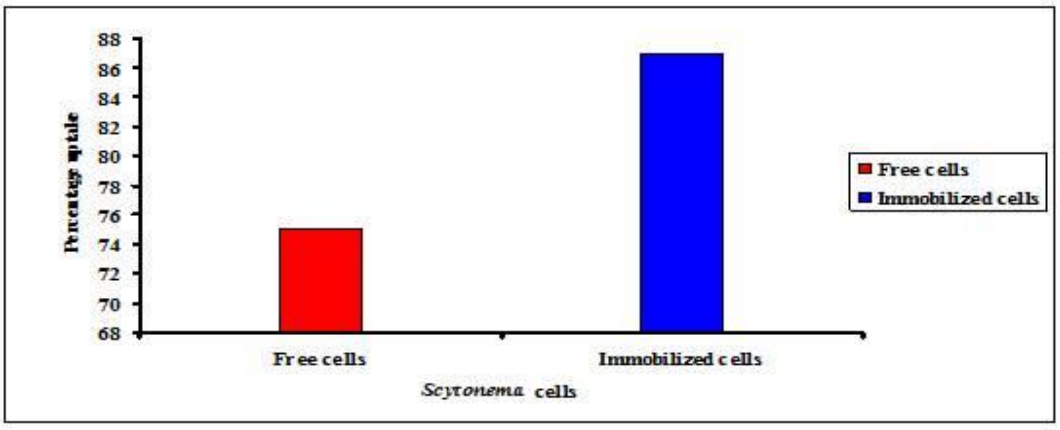

Fig 1:- Uptake of Lead added to the medium as lethal doses after 8 days of growth of free and immobilized cells of *Scytonema*

Growth

The growth of *Scytonema* was significantly inhibited with the increasing concentrations of the heavy metals. LC_{50} value of the organism for lead was found to be 20 ppm after exposure to the metals for a period equivalent to the generation time of the cyanobacterium. The generation time of *Scytonema* in free cell condition was found to be 58 h whereas in immobilized condition is was 52 h. The effect of different factors on growth pattern was studied. Different pH (5, 7, 9, 10 and 11) in *Scytonema* with and without added Lead in free and immobilized conditions was studied. The alkaline pH (pH 10) produced best results in both Lead treated free and immobilized cells with 20 ppm lead as compared to control (pH 8.2). The percentage increase in growth of Lead free cells compared to control pH 8.2 was 61% and 50% in immobilized and free cells respectively at 96 h. In 20 ppm, Lead treated cells at pH 10; there was 66% and 56% increase in immobilized and free cells respectively over control (pH 8.2) at 96 h (Fig 2). The growth rate of 40 ppm Lead treated cells was 13% and 7.43 % in immobilized and free cells respectively as compared to control (pH 8.2) cells at 96 h (Fig 2).

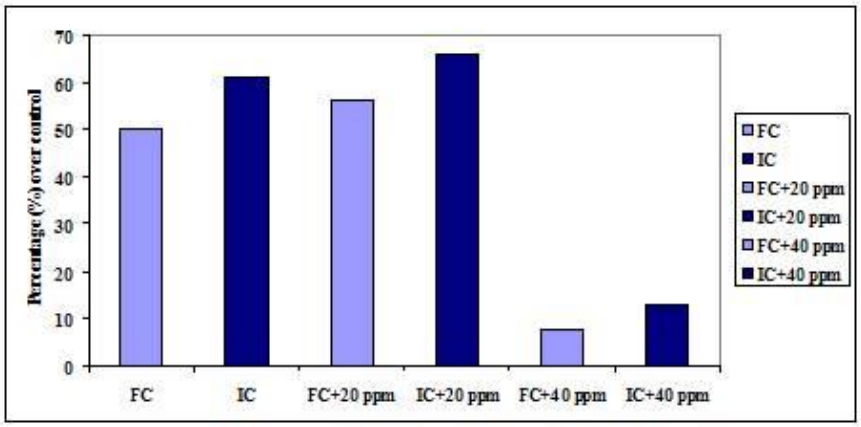

Fig 2: Percentage increase in the growth of free and immobilized of *Scytonema* in pH 10 at 96 h.

The effect of different ratio of algal filtrate (25F:75M, 50F:50M, 75F:25M) was studied on growth of *Scytonema* with and without Lead in free and immobilized conditions. It was found that there was an increase in growth with ratio of 75F:25M, in both Lead free and treated free and immobilized *Scytonema* cells and produced significant amelioration of the Lead toxicity. In Lead free cells, the growth rate increased to 72% and 84% in free and immobilized cells respectively as compared to control cells at 96 h. With 20 ppm Lead treated cells the growth was significantly increased, it was 79.5% and 90.5% increase in free and immobilized cells respectively over control cells at 96 h (Fig 3). Even in 40 ppm Lead treated cells there was an increase of 52% and 54.5% in free and immobilized cells respectively at 96 h with 75F:25M (Fig 3).

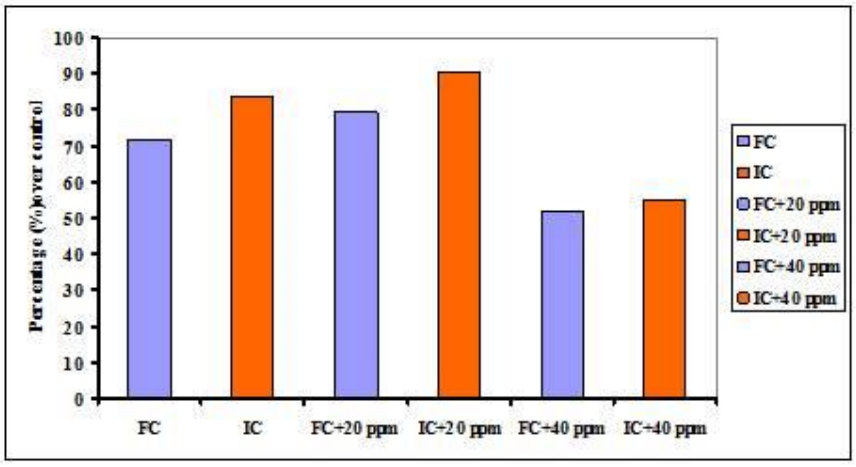

Fig 3: Percentage increase in the growth of free and immobilized of *Scytonema* in algal filtrate (75F:25M) at 96 h.

The effect of different concentration of phosphate (1%, 5% and 10%) was studied on growth of *Scytonema*. Addition of 5% phosphate to Lead treated free and immobilized cells produced maximum amelioration of metal toxicity. Without Lead immobilized and free cells, there was no significant increase in growth of *Scytonema*. Addition of 5% KH_2PO_4 increased the growth of 20 ppm Lead treated cells, to 5.2% and 10.11% in free and immobilized cells respectively as compared to control cells at 96 h (Fig 4). However, in the case of 40 ppm Lead treated cells addition of 5% KH_2PO_4 brought about a marked increase in the growth to 40 % and to 35 % in free and immobilized cells respectively as compared to control cells at 96 h (Fig 4).

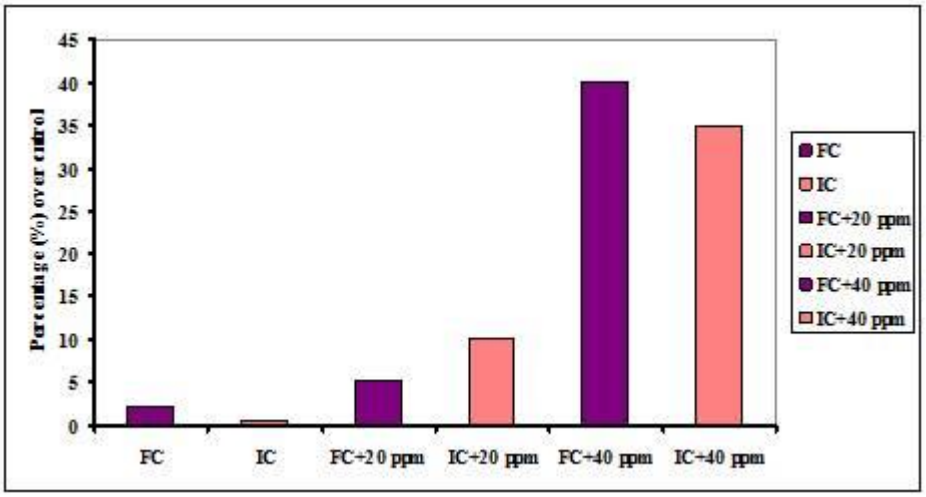

Fig 4: Percentage increase in the growth of free and immobilized of *Scytonema* in KH_2PO_4 (5%) at 96 h.

The effect of different concentration of oxalic acid (1% and 5%) was studied on growth of *Scytonema*. Addition of 1% of Oxalic acid to lead treated free and immobilized cells brought about maximum mitigation. Without lead immobilized and free cells, there was no significant increase in growth of *Scytonema*. But, in 20 ppm lead treated cells, addition of 1% Oxalic acid showed 31% and 38% increase in free and immobilized cells respectively as compared to control cells at 96 h (Fig 5). In 40 ppm, Lead treated cells, addition of 1% Oxalic acid showed 55% and 47% increase in free and immobilized cells respectively as compared to control cells at 96 h (Fig 5).

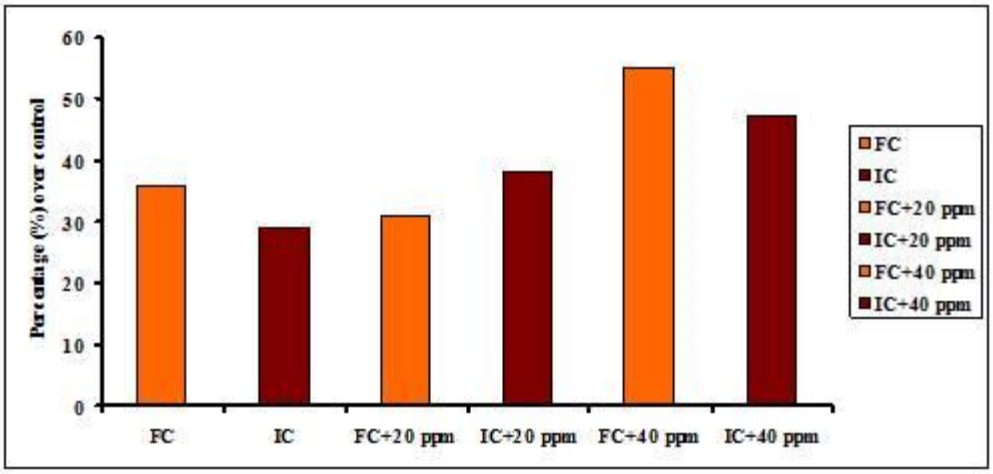

Fig 5: Percentage increase in the growth of free and immobilized of *Scytonema* in oxalic acid (1%) at 96 h.

Protein Profiling:- SDS-PAGE

SDS – PAGE analysis of whole cell protein after cell exposure to lethal concentration of Lead (40 ppm) for 144 h is shown in (Plate I). Almost every band in the control non-treated cells in lane 2, 3 and non- treated immobilized cells (lane 4) showed all polypeptide between 14 to above 66 kDa. Treatment with 40 ppm Pb in immobilized cells (lane 5) and free cells 6 &7 showed decrease in protein bands between 16 to 22 kDa and between 24 to 45 kDa. The loss however was more drastic in free cells with regard to intensity of the bands. The low molecular mass proteins between 16 to 22 kDa represent the phycocyanin α β monomers and evolved in photosystem (PS) II were missing after exposure to 40 ppm Lead (Plate 1).

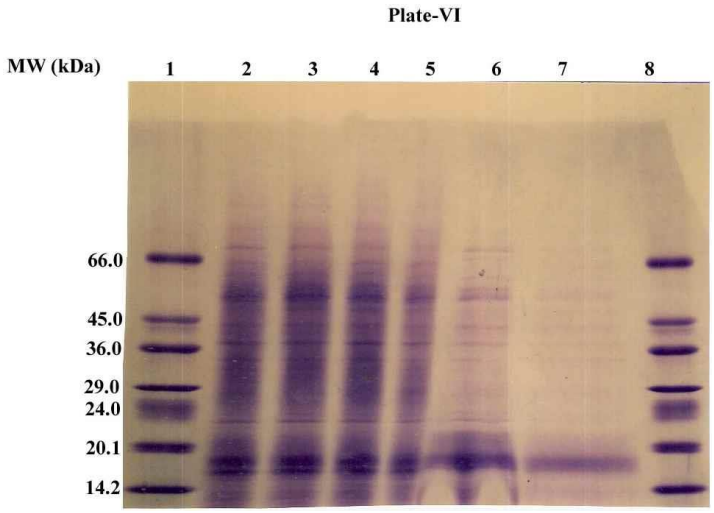

Plate-1. Vertical SDS-PAGE. (Gradient SDS-PAGE, 5-15% of *Scytonema* following exposure to heavy metal Lead (40 ppm lethal dose). Lane 1 and lane 8 marker proteins, lane 2-3 control non-treated free cells, lane 4 control non-treated immobilized cells, lane 5 40 ppm Lead treated immobilized cells, lane 6&7 40 ppm Lead treated free cells.

Nitrate reductase activity

When nitrate reductase activity was studied in free and immobilized Lead treated and Lead free cells of *Scytonema,* the highest activity was observed at 96 h.

The nitrate reductase activity was studied with different pH (5, 7, 9, 10 and 11) in Lead free and Lead treated free and immobilized cells of *Scytonema*. The maximum nitrate reductase activity was observed at pH 10, in Lead treated immobilized and free cells as compared to control (pH 8.2). In Lead free cells, there was no significant nitrate reductase activity. With 20 ppm of Lead, at pH 10; there was 20% and 27% increase in free and immobilized cells respectively over control cells at 96 h (Fig 6). The nitrate reductase activity of 40 ppm Lead treated cells was 3.4% and 12.36 % in free and immobilized cells respectively as compared to control cells at 96 h (Fig 6).

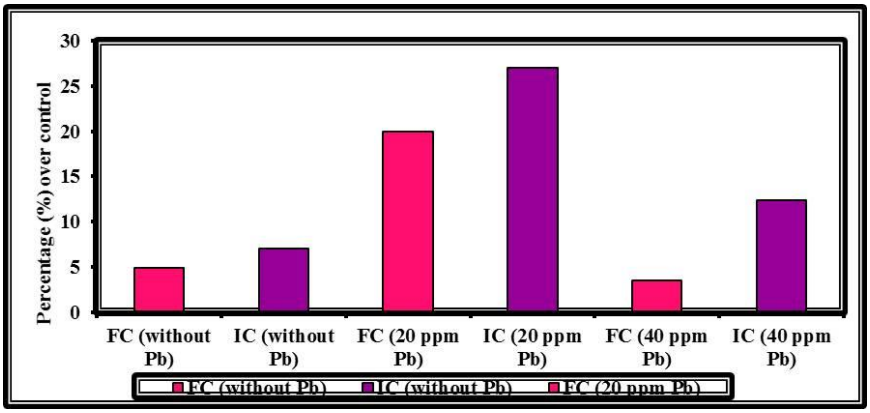

Fig 6: Percentage increase in the nitrate reductase activity of free and immobilized *Scytonema* with pH 10 at 96 h.

Different ratios of algal filtrate (25F:75M, 50F:50M, 75F:25M) was studied on nitrate reductase activity of *Scytonema* with and without Lead in free and immobilized conditions. It was found that the maximum nitrate reductase activity was found with ratio of 75F:25M, in both Lead free and treated free and immobilized conditions. In Lead free cells, the nitrate reductase activity increased to 42% and 50% in free and immobilized cells respectively as compared to control cells (without added filtrate) at 96 h. In the case of 20 ppm Lead treated cells the nitrate reductase activity was significantly increased, it was 65% and 70.5% increase in free and immobilized cells respectively over control cell at 96 h (Fig 7). In 40 ppm Lead treated cells with the addition of algal filtrate the increase was 42% and 45% in free and immobilized cells respectively at 96 h (Fig 7).

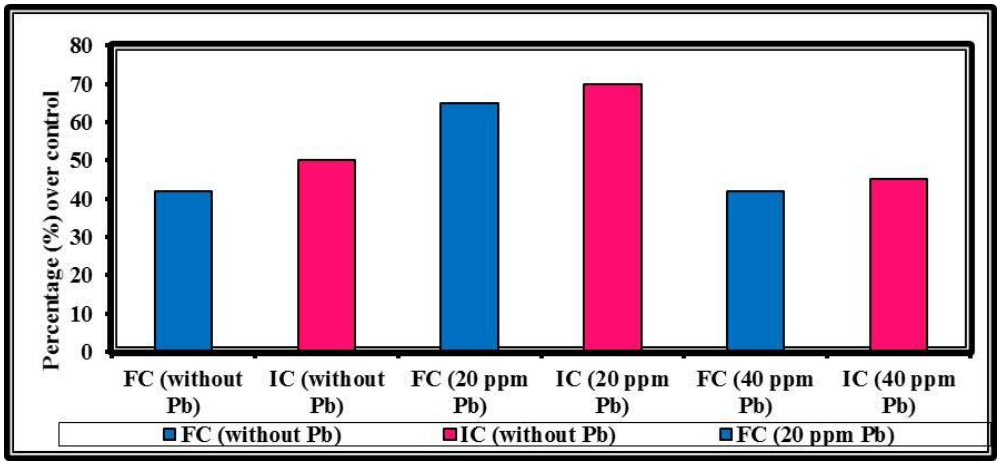

Fig 7: Percentage increase in the nitrate reductase activity of free and immobilized of *Scytonema* with algal filtrate (75F:25M) at 96h.

The effect of different concentration of phosphate (1%, 5% and 10%) was studied on nitrate reductase activity of *Scytonema*. Maximum enzyme activity was noted at 5% in Lead treated free and immobilized cells. Addition of 5% PO_4 increased the nitrate reductase activity of 20 ppm Lead treated cells to 21% and 36% in free and immobilized cells respectively as compared to control cells (containing the concentration of KH_2PO_4 as found in the medium) at 96h (fig 8). However in the case of 40 ppm Lead treated cells addition of 5% PO_4 increased the nitrate reductase activity to 34% and to 47% in free and immobilized cells respectively as compared to control cells at 96h (Fig 8).

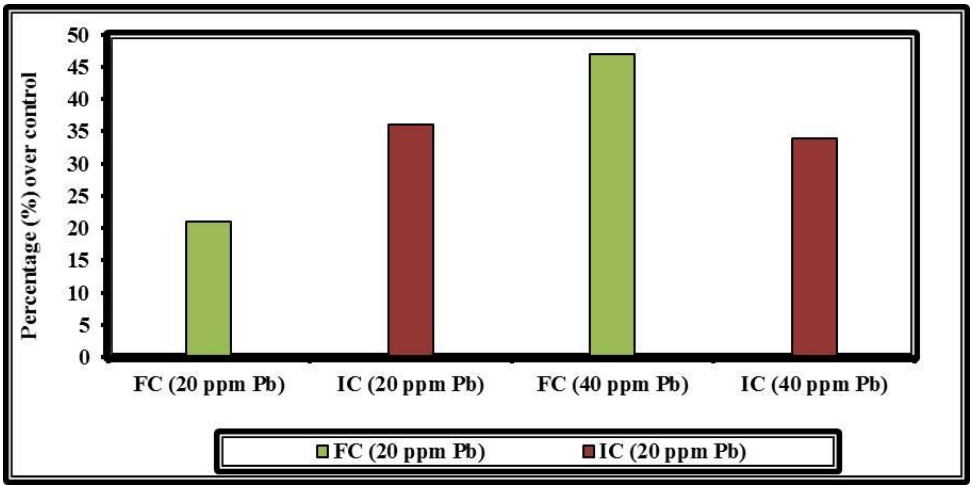

Fig 8: Percentage increase in the nitrate reductase activity of free and immobilized *Scytonema* with KH_2PO_4 (5%) at 96 h.

The effect of different concentration of oxalic acid (1% and 5%) was studied on nitrate reductase activity of *Scytonema*. Maximum enzyme activity was noted at 1% in Lead treated free and immobilized cells. In Lead free, immobilized and free cells, there was no significant increase in nitrate reductase activity of *Scytonema*. In 20 ppm, Lead treated cells, addition of 1% oxalic acid showed 28% and 31% increase in free and immobilized cells respectively as compared to control cells (without added oxalic acid) at 96 h (fig 9). In 40 ppm, Lead treated cells, addition of 1% Oxalic acid showed 36% and 40% increase in free & immobilized cells respectively as compared to control cells (without added oxalic acid) at 96 h (Fig 9).

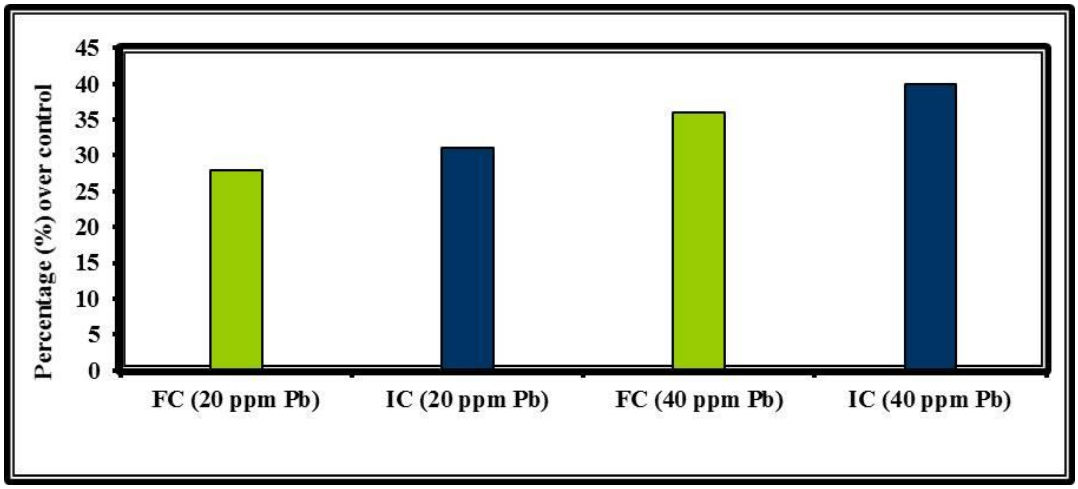

Fig 9: Percentage increase in the nitrate reductase activity of free and immobilized of *Scytonema* with oxalic acid (1%) at 96 h.

DISCUSSION

The result of the present study clearly indicates that immobilization has a great protective effect on *Scytonema* against lead toxicity. Also an interesting observation is that different environmental factors like pH, algal filtrates and phosphate and ascorbic acid not only overcome the toxic effect but also stimulate the growth and nitrate reductase activity. The mechanism of toxicity of metals to cyanobacteria are not fully known but several heavy metals retard the flow of electrons in electron transfer reaction in mitochondria and chloroplast and thus can be expected to have a detrimental effect on respiration, photosynthesis and other processes related to it.

Alginate, which is a mixture of polyguluronic acid and polymannuronic acid, has abundant hydroxyl groups which bind the metal ions and prevent them from entering the cells in full concentration thereby protecting against the decrease of chlorophyll *a* content and nitrate reductase activity as observed in free cells. So it is likely that the observed protection be due to calcium alginate immobilization on growth and nitrate reductase activity at 20 ppm and to a certain extent in 40 ppm of lead.

The increase in nitrate reductase activity may also be due to change in the cellular behavior by directly modifying the intrinsic characteristics of the culture as beads by forming a monolayer on the surface of the culture medium thus increasing not only surface area but also increasing availability of other important environmental parameters like light and oxygen concentration.

The observations that the addition of the algal filtrate had marked stimulatory effect comparable to immobilized control cells with Lead suggest the complexing of the metals with organic extracellular material in the filtrate. Cyanobacteria are known to release organic substances that can chelate free metal ions. The analysis of algal filtrate of *Aulosira fertilissima* revealed the presence of some major amino acids along with polysaccharides. Banerjee and Sharma, 1994 also reported the mitigating effect of algal filtrate on Lead toxicity in *Anabaena flos aquae* and Banerjee and Mishra, 2004 in *Aulosira fertilissma*. The presence of methionine as one of the amino acids is very significant, which may result in binding of the metals with sulphydryl groups. Further, the polysaccharides may act as chelators.

Addition of phosphate resulted in increased growth and nitrate reductase activity probably because as an essential nutrient it promotes growth and also addition of extracellular phosphate could account for the increase in ATP formation. Also high concentration of phosphate could chelate the heavy metal.

The study on *Scytonema* is of great significance because many species of cyanobacteria occurs naturally in an almost immobilized state i.e. mats or aggregates. *Scytonema* is one such cyanobacerium found as a sheath embedded in mucilage forming an immobilized layer totally covering paddy fields and is a classic example of naturally immobilized system. Under these natural conditions also this organism can probably accumulate HMs, if present. No such study has been done before. The study shows that, if this observation extrapolated to the paddy field is extremely significant with respect to the nitrogen economy of the rice field. Presence of HMs in that case would not have any detrimental effect on the enzymes of the nitrogen assimilating pathway because we cannot forget that these cyanobacteria play an important role in maintaining the fertility of paddy fields. Also it is pertinent to note that Lead addition has no effect on nitrate reductase activity under immobilized conditions; rather there is an increase in the enzyme activity.

Therefore this cyanobacterium could be playing a very important role in protecting the rice paddies from the toxic effects of heavy metals and maintaining the nitrogen economy of the fields in spite of the presence of heavy metals. Role of cyanobacteria as biofertilizer gain tremendous important in the present scenario.

CONCLUSION

In view of the present findings, it is concluded that *Scytonema* can tolerate higher levels of Lead and shows pronounced nitrate reductase activity in the presence of heavy metal Lead and therefore may serve as one of the most promising and effective cyanobacterial strain for application in the rice fields as bio fertilizers by providing nitrogen by its nitrogen fixing capacity and as a potent organism in bioremediation processes in rice fields.

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