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MITIGATION OF SELENIUM TOXICITY BY THE IMMOBILIZED CYANOBACTERIUM *HAPALOSIPHON SP*. FROM THE RICE FIELD ECOSYSTEMS

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ABSTRACT
Today industrialization and advance technology increases the environmental issues like metal pollution, which is one of the most serious problem facing humanity and other forms of life on the earth. Selenium (Se) is one of the most sever pollutant released into the environment through different anthropogenic sources and has strong potential to bioaccumulate severe toxic levels in the living environment. It is a regulatory problem and assuming increasing importance due to increased evidence of selenium contamination of the lentic systems which are more at risk of selenium poisoning such as rice fields than the lotic systems. There are number of methods available for the removal of Se from the environment. In this connection unique ability of cyanobacteria to tolerate and accumulate metal toxicity from the environment have made them an active research subject in the past few years. With this aim the current research is an attempt to examine the role of immobilized rice field cyanobacterium *Hapalosiphon sp*. in mitigating selenium toxicity in the laboratory by simulating a rice field condition. Rice fields are Se contaminated from agricultural soils and drainage waters released into nearby ponds that forms the main sources of irrigation for the fields. The observations of the present study clearly demonstrate that the cell immobilization could protect the growth and enzyme activity of cyanobacterium *Hapalosiphon sp*. as compared to free cells against the toxicity of Se at 200µg/ml (LC-50). This paper also concludes that the presence of selenium in any ecological niche with naturally immobilized cyanobacteria, a common occurrence, will not affect the nitrogen economy of that ecosystem. Hence, it is recommended that, this bioremediation technology is a cleaner and useful method for the removal of Se from the environment and maintaining a healthy ecosystem.

KEY WORDS- *Hapalosiphon sp.*, immobilization, selenium toxicity.

INTRODUCTION

During the last century, a fast pace of industrialization, modern civilization and reckless exploitation of natural resources, various wastes containing different toxic metal ions are directly or indirectly discharged into the environment creating severe threat to the environment and all forms of life (Poonam Narula *et al* 2015; Kaoutar Ben Chekroun and Mourad Baghour 2013; Arunakumara and Xuecheng, 2008). Environmental pollution with toxic metals is one of the serious problems facing humanity today and is responsible for several environmental problems, including human health, decrease of microbial activity, soil fertility and crop yield. Toxic metals are also reported to exert an adverse effect on the qualitative and quantitative structures of microbial communities, including the diversity, biomass and activity of soil microorganisms (Ming-He Mo *et al.,* 2007; Banerjee and Gole, 2009). Hence, metal pollution become a major serious global problem, which needs to be addressed in the present scenario and has gained the attention of natural resource managers around the world, due to their incremental accumulation in the food chain and continued persistence in the ecosystem (Srivastava and Dumka, 2001; Banerjee *et al.*, 2004; Chen and Pan, 2005; Banerjee *et al.*, 2008; Banerjee and Gole, 2009; Chouhan and Banerjee 2010). Selenium is one of the major environmental contaminant and one of the widespread toxic metals, generally known as "essential toxic" because it is required in trace amounts for growth and metabolism of all living organisms, but it is toxic at higher concentration (Hockin and Gadd, 2003; Germ and Stibilj, 2007). It is one of the most severe pollutant released into the environment through different pollution sources and has previously led to several environmental damages (USEPA, 1998) and could cause severe problems on a global scale in the future (Hamilton, 2002; Lemly, 2004). Its toxicity mainly linked to its propensity to bioaccumulate within the base of food webs (Hamilton, 2004; Morlon *et al.,* 2005). For many years, selenium has been largely an unrecognized pollutant, particularly in developing nations, and has been overshadowed by other pollutant issues. But in recent years, there has been an escalation in selenium pollution episodes on a global scale. As a result, selenium has globally emerged as an important environmental contaminant that can be addressed through several approaches. There are number of conventional methods for the remediation of contaminated sites, but these are so expensive, ineffective and not eco-friendly (Gupta *et al.,* 2000; Kretschmer *et al.,* 2003). In this context, unique ability of microorganism especially cyanobacteria are effective biological metal sorbents, representing an important sink for the removal of variety

of effluents or certain toxic metals because they tolerate, accumulate, sequester, detoxify and interact with pollutants and metabolize or use them as nutrient from the environment (Li *et al*, 2004; Banerjee *et al.*, 2004; Wang *et al.*, 2005;).

In recent years the method of immobilization of living cells has gained a wide range of applications because immobilized culture was found to survive the heavy metal stress better than free cells. But what has not been attempted is the study of naturally immobilized cyanobacteria such as *Hapalosiphon* and others with form a permanent layer on the rice field soils naturally, in ameliorating metal toxicity using similar and simulated immobilized conditions in the laboratory. With this aim the present study was undertaken to investigate the effect of selenium on growth, nitrate reductase activity and nitrogenase activity of free and immobilized cells of *Hapalosiphon sp.* and its possible role in ameliorating selenium toxicity in the rice fields.

MATERIALS & METHODS

Incubation and maintenance of culture

Filamentous cyanobacterium *Hapalosiphon sp.* was collected and isolated from rice fields of Sehore M.P. The isolation and identification of culture were made with the aid of available taxonomic keys (viz Geitler, 1932; Desikachary, 1959; Rippka *et al*., 1979). The cloning and purification was done with the aid of standard microbiological techniques and grown axenically in BG-11 medium and maintained at 25±2ºC, 2500-3000 lux light intensity and 14:10 hour light and dark rhythm.

Metal solution

Selenium solutions with different concentrations were prepared by dissolving selenium dioxide (SeO_2) in different required quantities in BG-11 medium and were sterilized by filtration through Millipore membrane filter (0.45µm). LC_{50} (200 µg/ml) and LC (250µg/ml) values of selenium treated algae were scored by standard plate/colony count method with an exposure time to the metal equivalent to the generation time of the organisms. Sublethal (LC_{50}) concentration of selenium was used in all further experiments.

Immobilization procedure

The exponentially growing algal cultures were agitated vigorously with sterilized glass beads in test tubes, to disrupt the clumps formed. The separated algal filaments were harvested by centrifugation and washed thoroughly with double distilled water. Immobilization was done by cell entrapment method using calcium alginate beads as described by Singh *et al.*, 1989. Growth, nitrate reductase activity and nitrogenase activity of cyanobacteria in free and immobilized cells were compared.

Growth measurement

Growth experiment was carried out in culture tubes each containing 10 ml basal medium and growth was measured by Chlorophyll-*a* extraction in 100% methanol saturated with $MgCO_3$. The optical density of the extract (Chl-*a*) was measured with a digital Systronic 169 spectrophotometer at 665nm. The amount of Chl-*a* extracted was calculated according to the equation of Mackinney, 1941.

Chlorophyll-*a* µg/ml = optical density × 12.63 × dilution factor. The generation time was calculated by growth equation of Kratz and Myers, 1955.

$K = 2.303 (\log N_2 - \log N_1) / T_2\text{-}T_1$

Where, N_1 = Initial OD / Protein concentration at time T_1, and N_2 = Final OD / Protein concentration at time T_2.

Nitrate reductase measurement

In vivo nitrate reductase activity was estimated by the method of Camm and Stein, 1974 and as slightly modified by Kumar and Kumar, 1980. The activity is based on the total nitrite formed and it was measured by the diazocoupling method of Lows and Evans, 1964. For the estimation of enzyme activity known amount of algal suspension/ immobilized beads was centrifuged and washed 3-4 times with sterile double distilled water and suspended in culture medium containing 5mM $NaNO_3$ (pH 8.2). For different time intervals, different sets of culture tubes containing 1 ml of culture (final) were incubated. Standard curve was plotted with varying concentrations of $NaNO_2$ dissolved in test medium. The nitrite present in the sample was expressed in µg $NaNO_2$ / µg Chl-*a* using a standard curve.

Nitrogenase measurement

Estimation of nitrogenase activity was done by Acetylene Reduction Assay given by Stewart *et al*., 1968. The enzyme activity is totally based on the reduction of acetylene to ethylene. Aliquots (2 ml) of exponentially growing cultures were placed in 8 ml vacutainer tubes and sealed with washed serum stoppers. Then, 10% acetylene gas was injected into the tubes and reaction was run for 60 min at 28ºC and 2500 lux light intensity. The reaction was terminated by injecting 0.2 ml of 2N NaOH. The ethylene produced was analyzed in a Gas Liquid Chromatograph fitted with a Porapak R. column and a hydrogen flame ionization detector. The nitrogenase activity was expressed in terms of n moles C_2H_4 μg^{-1} Chl-$a^{-1}h^{-1}$.

Metal uptake studies

The uptake of selenium metal 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.

All chemicals were of analar grade available at highest purity level from British Dry House (Glaxo) Mumbai. The culture medium and vessels were sterilized at 15 $Ib/inch^2$ pressure at 121ºC for 15 minutes and chemicals were sterilized through Millipore filter with pore size of 0.45 µm.

Statistical analysis

Mean values and standard deviations, given in figures and tables, were compared and analyzed statistically using student-t test at α=0.01 and 0.05.

RESULT

The growth of *Hapalosiphon* was studied in BG-11 medium supplemented with graded concentration of the toxic metalloid Selenium (Se). The study revealed that LC-50 value of the organism for Selenium was 200 ppm and LC value of Selenium was 250 ppm after exposure to the metal for a period equivalent to the generation time of the cyanobacterium. The growth of free and immobilized cells of *Hapalosiphon* with and without Selenium treatment showed increasing trend till 72 hours after which

the growth was declined. Table-1 shows the comparative growth study in both selenium treated and non-treated free as well as immobilized cells of *Hapalosiphon* at 72 hours. Growth in Selenium non-treated free and immobilized cells of *Hapalosiphon* was found to be 1.516 and 1.617 µg Chl-*a* ml^{-1} respectively at 72 hours. When free and immobilized cells were compared, there was an increase of 6.6% in the growth of immobilized cells over the free cells. In case of 200 ppm Selenium treated free and immobilized cells, there was a significant increase in growth in comparison with the non-treated free and immobilized cells and it was found to be 1.730 and 1.932 µg Chl-*a* ml^{-1} respectively at 72 hours. When 200 ppm Selenium treated free and immobilized cells were compared, the growth was found to be increased by 11.6% in immobilized cells over the Selenium treated free cells. But, in case of 250 ppm Selenium treated free and immobilized cells, the growth decreased to 1.162 and 1.288 µg Chl-*a* ml^{-1} respectively at 72 hours as compared to non-treated free and immobilized cells. A comparative growth curve of free and immobilized cells of *Hapalosiphon* with and without Selenium treatment at 72 hours is given in Fig.1.

TABLE 1. Effect of selenium on the growth of *Hapalosiphon* sp. in free and immobilized cells condition at 72 hour measured as chlorophyll content.

Condition	Free Cell (FC ± S.D) µg Chl-*a*/ ml	Immobilized Cell (IC ± S.D) µg Chl-*a*/ ml
Control (without Se)	1.516 ± 0.06	1.617 ± 0.06
Sublethal (with Se 200ppm)	1.730 ± 0.07	1.932 ± 0.09
Lethal (with Se 250ppm)	1.162 ± 0.05	1.288 ± 0.04

(Results mean ±SD, n=3)

TABLE 2: Effect of selenium on nitrate reductase activity of *Hapalosiphon* sp. in free and immobilized cells condition at 48 hour.

Condition	Free Cell (FC ± S.D) µgNO_2/ µgChl-*a*	Immobilized Cell (IC ± S.D) µgNO_2/ µgChl-*a*
Control (without Se)	2.385 ± 0.13	2.692 ± 0.16
Sublethal (with Se 200ppm)	2.846 ± 0.17	3.077 ± 0.20
Lethal (with Se 250ppm)	1.529 ± 0.05	1.720 ± 0.06

(Results mean ±SD, n=3)

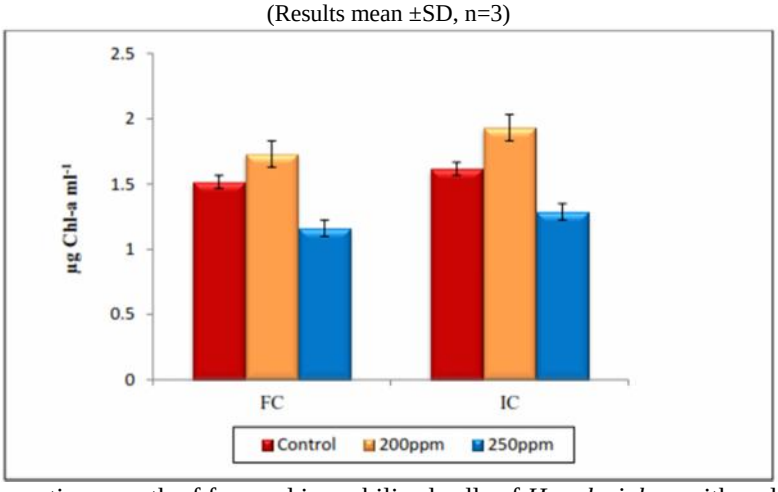

FIGURE 1.Comparative growth of free and immobilized cells of *Hapalosiphon* with and without Selenium treatment at 72 h.

A time course study of nitrate reductase activity and nitrogenase activity of free and immobilized cells of *Hapalosiphon* without Selenium treatment showed the increasing trend till 48 hours after which the activity declined. Table-2 shows the nitrate reductase activity in both selenium treated and non-treated free as well as immobilized cells of *Hapalosiphon*. The nitrate reductase activity in Selenium non-treated free and immobilized cells of *Hapalosiphon* was found to be 2.385 and 2.692 µg NO_2 µg Chl-a^{-1} h^{-1} respectively at 48 hours. When free and immobilized cells were compared, there was an increase of 12.8% in the activity of immobilized cells over the free cells. In case of 200 ppm Selenium treated free and immobilized cells, there was a significant increase in nitrate reductase activity in comparison with the non-treated free and immobilized cells and it was found to be 2.846 and 3.077 µg NO_2 µg Chl-a^{-1} h^{-1} respectively at 48 hours. When 200 ppm Selenium treated free and immobilized cells were compared, the activity was found to be increased by 8.1% in immobilized cells over the Selenium treated free cells. But, in case of 250 ppm Selenium treated free and immobilized cells, the activity decreased to 1.529 and 1.720 µg NO_2 µg Chl-a^{-1} h^{-1} respectively at 48 hours as compared to non-treated free and immobilized cells. The comparison of nitrate reductase activity of free and immobilized cells of *Hapalosiphon* with and without Selenium treatment at 48 hours is given in Fig. 2.

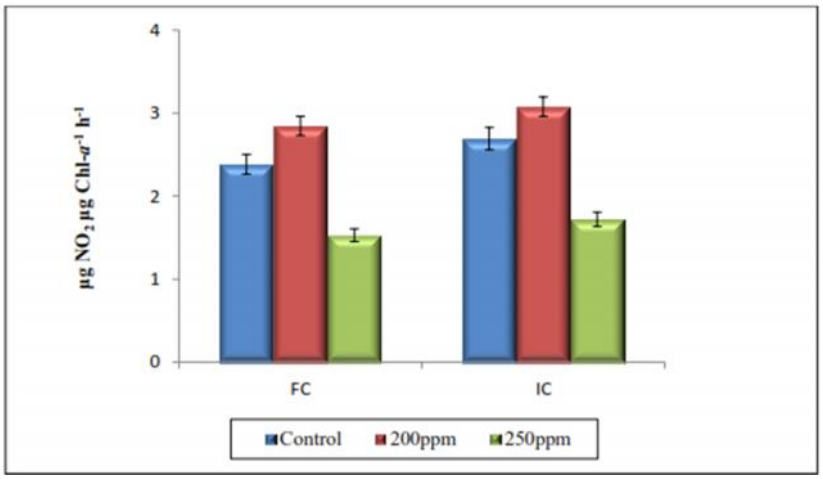

FIGURE 2. Comparison of nitrate reductase activity of free and immobilized cells of *Hapalosiphon* with and without Selenium treatment at 48 h.

TABLE 3. Effect of selenium on nitrogenase activity of *Hapalosiphon* sp. in free and immobilized cells condition at 48 hour.

Condition	Free Cell (FC ± S.D) *n* moles C_2H_4 µg Chl-*a* $^{-1}$ h^{-1}	Immobilized Cell (IC ± S.D) *n* moles C_2H_4 µg Chl-*a* $^{-1}$ h^{-1}
Control (without Se)	21.28 ± 3.09	26.07 ± 3.73
Sub lethal (with Se 200ppm)	22.48 ± 2.31	28.39 ± 3.80
Lethal (with Se 250ppm)	19.12 ± 2.09	21.36 ± 3.14

(Results mean ±SD, n=3)

Table-3 shows the nitrogenase activity in both Selenium treated and non-treated free and immobilized cells of *Hapalosiphon.* The nitrogenase activity studied in Selenium non-treated free and immobilized cells of *Hapalosiphon* was found to be 21.28 and 26.07 n moles C_2H_4 µg Chl-a^{-1} h^{-1} respectively at 48 hours. When the activities of free and immobilized cells were compared, the nitrogenase activity was found to be 22.5% higher in immobilized cells over the free cells. In case of 200 ppm Selenium treated free and immobilized cell, the activity was significantly increased to 22.48 and 28.39 n moles C_2H_4 µg Chl-a^{-1} h^{-1} respectively at 48 hours in comparison with Selenium non-treated free and immobilized cells. When 200 ppm Selenium treated free and immobilized cells were compared, the nitrogenase activity was found 26.2% higher in immobilized cells over the Selenium treated free cells. Whereas, in the case of 250 ppm Selenium treated free and immobilized cells, there was decrease in the nitrogenase activity to 19.12 and 21.36 n moles C_2H_4 µg Chl-a^{-1} h^{-1} respectively at 48 hours as compared to non-treated free and immobilized cells. The comparison of nitrogenase activity of free and immobilized cells of *Hapalosiphon* with and without Selenium treatment at 48 hours is given in Fig. 3.

Uptake study of selenium metal showed the higher uptake of selenium by immobilization (Fig-4). The study revealed that higher growth, nitrate reductase and nitrogenase activity was noted with immobilized cells compared to free cells.

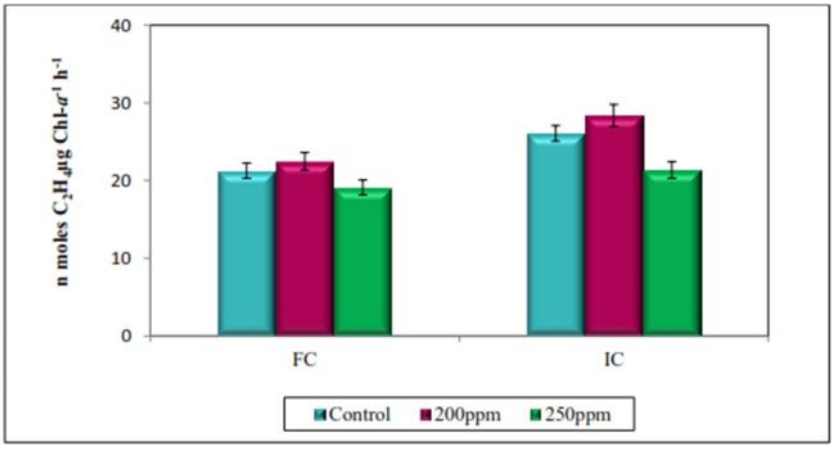

FIGURE 3: Comparison of nitrogenase activity of free and immobilized cells of *Hapalosiphon* with and without Selenium treatment at 48 h.

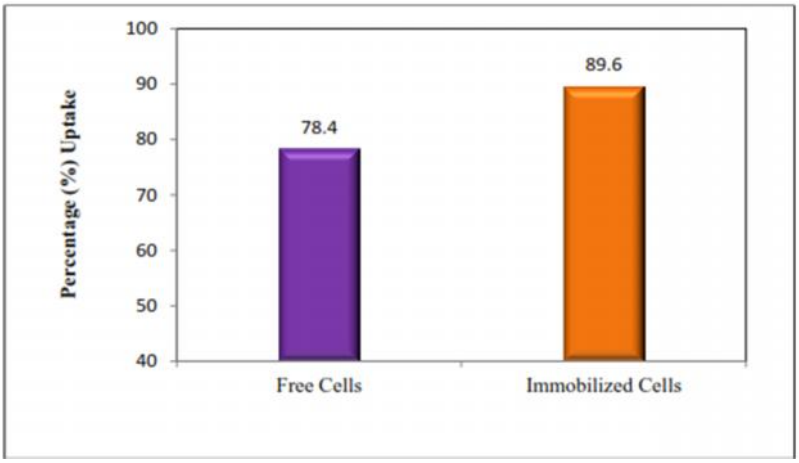

FIGURE 4. Uptake of Selenium metal added to the medium as lethal doses after 8 days of growth of free and immobilized cells of *Hapalosiphon*

DISCUSSION

The observation in the present study clearly indicate that immobilization has significant protective effect on growth of *Hapalosiphon* against the toxicity of Selenium at sub-lethal concentration when compare to free cells. The mechanism of metal toxicity to cyanobacteria in free cells condition is not fully understand but several 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, chlorophyll biosynthetic pathway and other processes related to it, and thereby reducing the growth. In contrast to this, observed protection due to calcium alginate immobilization on Chlorophyll-*a* content (growth was measured by extracting Chl-*a*) at sub-lethal concentration i.e. 200 ppm Selenium could be due to the metal binding property of alginate that stop selenium from entering the cells and causing detrimental effect. The chlorophyll content of immobilized cells generally has been found to be higher than that of free cells (Robinson *et al.,* 1985; Bailliez *et al.,* 1986; Abdel Hameed, 2002) probably, because self-shading and subsequent reduction in incident light in the immobilized state results in a promotion of photosynthesis and pigment synthesis (Hameed and Ebrahim, 2007). Alginate is a mixture of polyguluronic acid and polymannuronic acid which has abundant hydroxyl groups which bind the metal ions and prevent them from entering into the cells in full concentration thereby protecting against the decrease of chlorophyll-*a* content as observed in free cells caused by Selenium affecting the Chlorophyll-*a* synthesis pathway (Rai and Mallick, 1992; Banerjee and Mishra, 2002; Banerjee *et al.,* 2004). Basically, immobilization is known to bring about increase in growth by producing direct contact between cells and medium, thus increasing the surface area available for various cellular reactions (Tandeau and Houmard, 1993; Banerjee and Mishra, 2002; Banerjee *et al.,* 2004).Since the algal beads form a mono layer on the surface of the medium incident light utilization for photosynthesis is optimized in contrast to a situation in a free flowing culture where light penetration is not uniform due to density of the algal cells. This in turn reduces photosynthetic rates. The increase in nitrate reductase activity and nitrogenase activity in selenium treated free as well as immobilized cells at sublethal dose could be due to the fact that Selenium is of metabolic importance in cyanobacteria and algae, being involved as part of the 21st amino acid- selenocysteine present in many enzymes, selenoamino acids and proteins, essential to all living organisms (Gouget *et al.,* 2005; Banerjee *et al.,* 2008; Germ *et al.,* 2009). Nutritional essentiality of Selenium derives from the fact that several microbial enzymes are Selenium dependent and involves Selenium containing amino acids analogue to cysteine, selenocysteine. Maximum enzyme activity in sublethal doses is probably because this concentration of selenium does not affect the enzymes of the chlorophyll biosynthetic pathway allowing chlorophyll to be synthesized and permitting the photosynthesis to occur and supply of reductant and ATP required for enzyme activity will continue to give the observed results.

On the other hand, the increase in enzyme activity of selenium treated free cells was significantly less when compared to the immobilized cells. This could be because of the mitigating effect of immobilization to Selenium toxicity compared to free cells suggesting that Selenium may have been restricted from entering the cells in toxic concentration by metal binding ligands in alginate, thereby protecting against the decrease of enzyme activities as observed in free cells. Also, immobilization bring about increase in enzyme activity may be due to change in the cellular behaviour 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 (Marsac and Houmard, 1993; Banerjee and Mishra, 2002). The uptake of metal was higher in immobilized condition in comparison to free cells, due to the presence of abundant hydroxyl groups in the alginate, which bind the metal ions. Similar effects were observed by Brun *et al.,* 1998; Gloaguen *et al.,* 1996; Ye *et al.,* 1997.

CONCLUSION

It is clear from the present study that selenium may reaches the rice field ecosystems via irrigation or percolation, but the presence of naturally immobilized mat of *Hapalosiphon* can mitigate and tolerate the higher level of selenium. Therefore they could be playing a very important role in protecting from toxic effects of Selenium and may serve as one of the most promising and effective cyanobacterial strain for application in the rice field ecosystem as biofertilizer even in Selenium affected areas. This study also point out to the fact that *Hapalosiphon* cyanobacterial addition to the selenium contaminated soils is an effective bioremediation process, with great biotechnological importance.

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