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RESEARCH ARTICLE

Biochemical and Enzymatic investigation of *Westiellopsis prolifica* Janet in response to mercury stress

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Abstract

Cyanobacterial species are a large group of organisms largely used for the removal of heavy metals present in the various ecosystems of environment. In the present work we report the toxic effect of mercury on *Westiellopsis prolifica* Janet. BG-11 medium with different concentrations of Hg^{+2} was used to observe the effect on the organism. Effect of Hg^{+2} on the organismwas studied by estimating the growth, metabolic and enzymatic activities recorded after sixteen days. An increase in heavy metal concentration caused a decrease in growth along with a tremendous reduction in metabolites like carbohydrates and proteins. Also, a fall in activity of assimilating enzymes was observed with the rise in Hg^{+2} concentrations. Whereas, the amount of free amino acids and phenol increased at lower concentration but showed a decrease with increasing concentrations of Hg^{+2}. The changes in the DNA content were also observed when determined by Agarose Gel Electrophoresis. The experimental conditions used in the present work are simple and have low operational cost. Thus, *Westiellopsis prolifica* Janet can be used as a bioremediation tool for heavy metal contamination.

INTRODUCTION

The continuous discharge of heavy metals like, Hg, Cd, Cr, Cu, Ni, Sn, Pb, etc. into the aquatic environment, through industrial, domestic and agricultural activities has significantly contaminated our aquatic resources. Although many of the heavy metals are a trace requirement for most of the living organisms, a large dosage of them could adversely affect the enzymes and cellular membranes, disrupt biochemical pathways, etc and thereby render the organisms prone to death. Circulation of these harmful heavy metals within the food chain by transfer to higher trophic levels has begun to reveal a lot many inevitable consequences. These up comings have alerting us about the alarming rate at which the environment is being polluted.

Mercury is one of the most toxic pollutant in the environment. It is released into the environment from a variety of sources, both natural and anthropogenic (Rice et al, 1973) It is impossible to separate out current levels of mercury in the environment as either anthropogenic or natural, but several experts have estimated that humans have doubled or tripled the amount of mercury released into the environment (Whittan B. A., 1970).

The quick paced mercury pollution has resulted in the need for quick, effective, economic and ecofriendly methods for their clean up. This can be accomplished by chemical, physical or biological methods. However, chemical and physical processes require a large amount of energy input and chemicals and thus are not cost effective (El-Enany A.

E et al, 2000). Besides, it produce a considerable quantity of reactive chemical species as secondary wastes which would only contribute to further pollution. This leaves us with only one option- the biological method.Microbial biomass has been used for removal of heavy metals. The use of microbial biomass is cost effective for industrial wastewater treatment (Akthar et al 1995)Toxicological investigations was carried out by Singh and Singh, of the impact of inorganic mercury (Hg^{2+}) and methylmercury (CH_3Hg^+) in terms of growth, NH_4^+ uptake & in vivo glutamine synthetase (transferase) activity in the diazotrophic cyanobacterium *Nostoc calcicola* Breb. Photoautotrophic growth of the cyanobacterium was extremely sensitive to mercury compounds, CH_3Hg^+ being 2.5 times more toxic than Hg^{2+} (Singh and Singh, 1992).

Effect of the carbamate insecticide, Sevin (50% w/v) was examined on survival, growth and nitrogen fixation of filamentous blue-green alga, *Westiellopsis prolifica* by Adhikary et al. Lower concentration of the insecticide (10µg/ml) increased survival, growth and nitrogen fixation while higher concentrations showed an inhibitory effect (Adhikary et al., 1984).

But, before applying microorganisms (or any other biological agent) as a means of bioremediation, the effect of the mercury on these agents, their response to presence of high doses of mercury, and at last, their capability to remediate contaminated sites have to be assessed, and if required, modified (by biotechnological tools), in order to obtain maximum remediation.

For this reason, in the present study, the effect of mercury, one of the many heavy metals posing a threat, is being evaluated on a cyanobacterium species namely, *Westeillopsis prolifica*, so that in future, based on further studies and standardisation, it can be used as a successful tool for bioremediation of heavy metal- polluted aquatic environments.

In order to determine the changes occuring in the cyanobacterial cell metabolites in response to mercury, following were the objectives which were framed in mind:

☛ To investigate the biochemical changes such as Carbohydrates, Proteins, Amino acids and Phenols, of *Westeillopsis prolifica* to different doses of mercury.

☛To enumerate the variations in enzyme activity in response to different doses of mercury. Nitrate Reductase and Glutamate Synthetase were examined.

☛ To enumerate the changes in the DNA content using Agarose Gel Electrophoresis.

MATERIALS AND METHODS

Cyanobacterial strain

Westiellopsis prolifica was the strain used for these studies. W. prolifica Janet were obtained from Centre for Conservation and Utilization of Blue-Green Algae, IARI, New Delhi and were grown hotoautotrophically in nitrogen free BG 11 medium (Rippka, 1979) within controlled temperatures (25±2°C) under 3000 lux light with the photoperiod of 14: 10 hours.

Culture conditions

Cultures were grown and maintained in BG11media [17.65 mM $NaNO_3$; 0.18 mM $K_2HPO_4.3H_2O$; 0.30 mM $MgSO_4.7H_2O$; 0.25 mM $CaCl_2.2H_2O$; 0.03 mM citric acid ; 0.03 mM ferric ammonium citrate; 0.19 mM Na_2CO_3 ; 0.003 mM EDTA (Na_2 salt); 0.05 mM H_3BO_3 ; 9.15 mM $MnCL_2.4H_2O$; 0.77 mM $ZnSO_4.7H_2O$; 1.61 mM $Na_2MoO_4.2H_2O$; 0.37 mM $CuSO_4.5H_2O$; 0.17 mM $Co(NO_3)_2.6H_2O$] (Oberholster et al., 2005) at 25 ± 1°Cunder fluorescent illumination of 30 $\mu em^{-2}s^{-1}$ for 14:10 hours light: dark period. The medium was sterilized by autoclaving at 15 lbs for 20 minutes. The phosphate source (K_2HPO_4. $3H_2O$) was separately autoclaved and added to the medium immediately after autoclaving under aseptic conditions.

Heavy metal treatment

Mercury was used as the heavy metal in the form of mercuric chloride (Hg^{+2}). In order to determine the LC_{50} (in terms of chlorophyll content) of Hg^{+2} on *Westiellopsis prolifica*, the species was subjected to different concentrations of the heavy metal. Stock solution of Hg^{+2} of concentration 1mg/ml was prepared. From the stock, BG11media of different concentrations were prepared viz. 1 ppm to 10 ppm, from which 20 ml was taken into previously autoclaved sugar tubes and 2 ml well mixed, homogenized algal culture was inoculated and incubated for five days. After the incubation period, the total chlorophyll content and growth were estimated. Graphically, 3 ppm was determined as LC_{50}. Accordingly, the treatments were finalized as 2, 4 and 6 ppm.

Growth measurement

Growth of culture was determined by directly taking absorbance at 650 nm in a spectrocolorimter (Bausch and Lomb spectronic 20 spectrocolorimter) (Bagchi et al., 1985).

Metabolite estimation

Carbohydrates were assayed quantitatively as per Roe (1995). Total Proteins were determined as described by lowry et al (1951). Free amino acids were estimated by the method of Lee and Takahasi (1966), whereas phenols were measured according to Malick and Singh (1980).

Enzyme assays

The estimationof in vivo nitrate reductase activity was measured by method of Sempruch at al. (2008) and glutamate synthetase activity was done by Pamiljans at al. (1962).

Molecular techniques

Molecular diagnosis were performed by extracting DNA using phenol-chloroform method.The cultures were spinned down at 5000 rpm for 20min (20°C) and total genomic DNA was extracted from the cells using phenol–chloroform based method as prevously described by (Adams, 1988) and precipitated from t he supernatant by the addition of ice -cold absolute ethanol . Precipitated DNA was centrifuged at 15,000 rpm for 10 min at 4°C, and the pellet was suspended in minimal volume of TE buffer (10mM Tris, 1mM EDTA; pH 7.6).

Statistical analysis

The Correlation matrix as well as Test of Significance (Student't' test) of different variables of Westeillopsis prolifica to different concentrations of Hg^{+2} was prepared.

RESULTS

Lethal concentration (LC50 (in terms of chlorophyll content))

The LC_{50} in terms of chlorophyll content of *W. prolifica* to Hg^{+2} was found out in order to decide the heavy metal treatments for estimating the response.

Fig. 1 shows, that the LC_{50} (in terms of chlorophyll content) for Hg^{+2} was determined as 4 ppm. Thus, the concentration of heavy metal for different doses was decided as 2, 4 and 6 ppm for all the experiments.

Growth

Fig 2 shows that, the growth increases with an increase in time, despite the presence of the heavy metal. Although, the growth in treated *W. prolifica* is less when compared to the control. Also, it is evident that at higher concentrations of Hg^{+2}, the growth is less. The growth of *W. prolifica* varied from 0.005 to 0.062 (O.D. at 650 nm) in the medium. There was a gradual growth reduction of 44%, 60 % and 73% in 2ppm, 4ppm and 6ppm respectively, by the end of 15^{th} day. The highest growth (0.035) was observed in 2 ppm treatment by the end of 15^{th} day.

Estimation of metabolites

The biochemical response of *W. prolifica* to different doses of Hg^{+2} are shown below in **figures 3 to 6.** The variations caused in the physico-chemical activities of *w. Prolifica* by Hg^{+2} had its impact on the biochemical composition of the organism, so, the total carbohydrate, protein, amino acids and phenols were estimated.

(a) Total Carbohydrates

The carbohydrate content of W. *prolifica* increased from 33.2 µg/ml^{-20}to 100.5 µg/ml^{-20} as shown in **Fig.3**. A fall in the carbohydrate content of 86.92%, 89% and 98.8%, treated with 2, 4 and 6 ppm Hg^{+2} was observed after the 15^{th} day. The highest amount of carbohydrate content (13.15 µg/ml^{-20}) was found in treatment with 2 ppm Hg^{+2}whereas, the lowest amount (1.18 µg/ml^{-20}) was recorded in 6 ppm with maximum reduction.

(b) <u>Total Protein</u>

The protein content of W. *prolifica* increased from 75 µg/ml^{-20}to 182 µg/ml^{-20} as shown in **Fig.4**. A fall in the protein content of 78%, 93% and 98%, treated with 2, 4 and 6 ppm Hg^{+2} was observed after the 15^{th} day. The highest amount of protein content (39 µg/ml^{-20}) was found in treatment with 2 ppm Hg^{+2} whereas; the lowest amount (2.7 µg/ml^{-20}) was recorded in 6 ppm with maximum reduction.

(c) <u>Total Free Amino Acids</u>

As seen in the **Fig.5 t**he amino acid content in the cyanobacteria increased till the first 9 days from 4 µg/ml^{-20} to 256 µg/ml^{-20} after which, there was a rapid decline to 65 µg/ml^{-20}. The same pattern was observed in case of treated cyanobacteria. At the end of 15th day, there was 78.5%, 83% and 72% reduction in amino acid content at 2, 4 and 6 ppm respectively. at the end of the 15th day, the highest reduction (11 µg/ml^{-20})was observed in case of 4 ppm and lowest reduction (18 µg/ml^{-20})was seen in 6 ppm treatments.

(d) <u>Phenols</u>

As seen in the **Fig. 6**, the phenol content in the cyanobacteria increased till the first 12 days from 2.6 µg/ml^{-20} to 3.14 µg/ml^{-20} after which, there was a decline to 2.94 µg/ml^{-20}. The same pattern was observed in case of treated cyanobacteria. Although, in all cases, the phenol content exceeded the control. At the end of 15th day, there was 18%, 31% and 45% increase in phenol content at 2, 4 and 6 ppm respectively. At the end of the 15th day, the highest increase (5.32 µg/ml^{-20}) was observed in case of 6 ppm and lowest increase (3.6 µg/ml^{-20}) was seen in 2 ppm treatments.

Enzymes Activity

(a) Nitrate reductase activity:

The nitrate reductase activity (µg NO_2 mg^{-1} protein min^{-1}) of *W. prolifica*when supplemented with different doses of Hg^{+2} is represented in **Fig.7 .**The nitrate reductase activity ranged between 0.112 to 0.201 µg NO_2 mg^{-1} protein min^{-1}. The nitrate reductase activity was found to decrease with increase in concentration of Hg^{+2} and time. There was 90%, 95% and 96% inhibition in the enzyme activity at 2 ppm, 4ppm, and 6 ppm concentrations of Hg^{+2}, respectively. The Hg^{+2} toxicity to nitrate reductase was observed maximum at 6 ppm dose followed by 4 and 2 ppm Hg^{+2} concentrations.

(b) Glutamate synthetase activity:

The glutamate synthetase activity (µgPO4mg^{-1} protein min^{-1}) of *W. prolifica* supplemented with different doses of Hg^{+2} is represented in **Fig.8.** The glutamate synthetase activity ranged between toµg PO_4 mg^{-1} protein min^{-1}. The glutamate synthetase activity was inhibited by the effect of the Hg^{+2} in the concentration of 2 ppm, 4 ppm, and 6 ppm by 92%, 94% and 98.6% respectively. The glutamate synthetase activity was found to decrease with increase in concentration of Hg^{+2} and time. The Hg^{+2} toxicity (inhibition) to glutamate synthetase was observed maximum at 6 ppm dose followed by 4 and 2 ppm Hg^{+2} concentrations.

DNA extraction:

The presence of bands in the **Fig. 9** shows the presence of DNA in the samples. The cyanobacterium was treated with different concentrations of Hg+2 and the changes in the DNA content were determined by Agarose Gel Electrophoresis. The size of the band elucidates roughly the amount of DNA. As seen in the figure, an increase is obtained in the DNA content by treatment with 2 and 6 ppm Hg+2, while in case of 4 ppm, the content of DNA is seen to be decreasing. The increase in 2 and 6 ppm treated *W. prolifica* is more than in the untreated one. A decrease in the DNA content was noticed with time.

Statistical analysis

Test of Significance (Student't' test) of different variables of *W. prolifica* to different concentrations of Hg^{+2} was studied. As seen in **Table:1**, the highest 't' value found was for Phenol at 6 ppm (9.766), and the lowest for glutamate synthetase at 6 ppm (0.00063).

Table 2 reveals a significant positive correlation (r = 0.699 to 0.994) between all the parameters i.e. carotenoids, phycobilins, carbohydrates, proteins, phenols, amino acids, nitrate reductase and glutamate synthetase treated with different concentrations (2, 4 and 6 ppm) of Hg^{+2}.

TABLES

Table:1:Test of Significance (Student't' test) of different variables of*Westeillopsis prolifica* to different concentrations of Hg^{+2}.

Sr.no.	Variables	2 ppm	4 ppm	6 ppm
		Mercury(Hg^{+2})		

1.	**Carbohydrate**	**t =**	**0.030**	**0.0270**	**0.0261**
2.	**Protein**	**t =**	**0.0142**	**0.0110**	**0.0039**
3.	**Amino acids**	**t =**	**0.0108**	**0.0117**	**0.0043**
4.	**Phenol**	**t =**	**0.0028**	**0.0010**	**9.766**
5.	**Nitrate Reductase**	**t =**	**0.0058**	**0.00187**	**0.0014**
6.	**Glutamate Synthetase**	**t =**	**0.0078**	**0.0030**	**0.00063**

Where, p< = Level of signific

Table:2: Correlation matrix of different variables of *Westeillopsis prolifica* to different concentrations of Hg^{+2}.

	1	**2**	**3**	**4**	**5**	**6**
1	**1.000**	**0.945**	**0.882**	**0.811**	**0.870**	**0.936**
2	**1.000**	**1.000**	**0.962**	**0.886**	**0.966**	**0.938**
3	**1.000**	**1.000**	**1.000**	**0.826**	**0.985**	**0.971**
4	**1.000**	**1.000**	**1.000**	**1.000**	**0.904**	**0.779**
5	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**0.941**
6	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**	**1.000**

Where,
1: Carbohydrates; 2: Proteins; 3: Amino acids; 4: Phenols; 5: Nitrate reductase; 6: Glutamate synthetase.

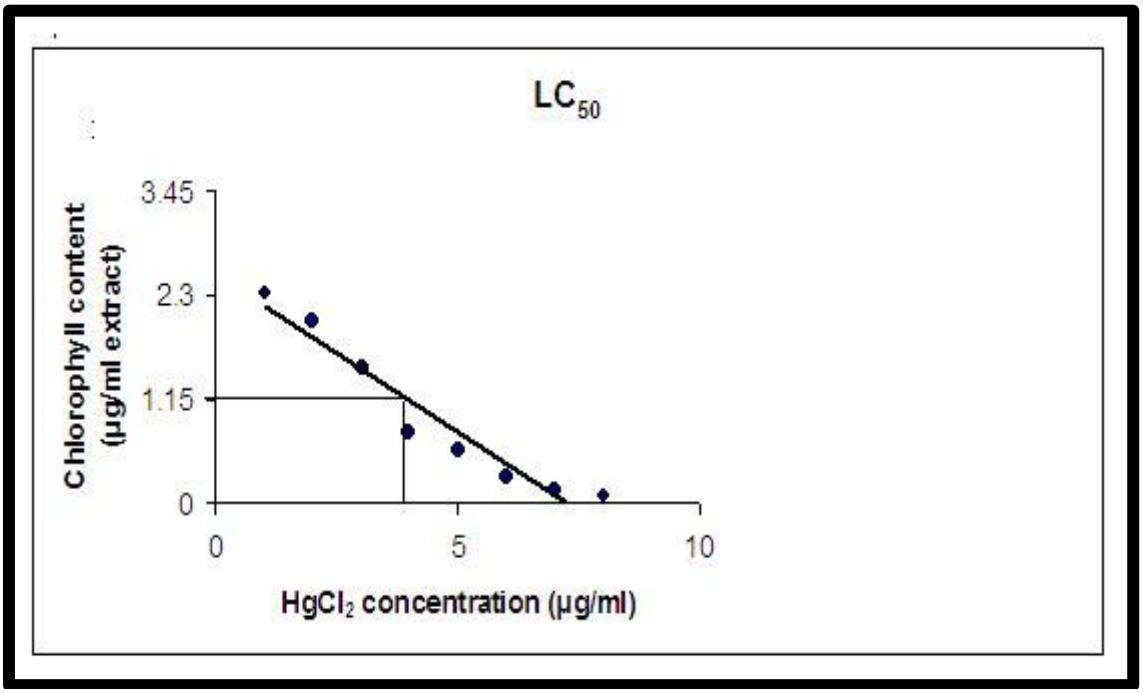

Figure 1.LC_{50} (in terms of chlorophyll content).

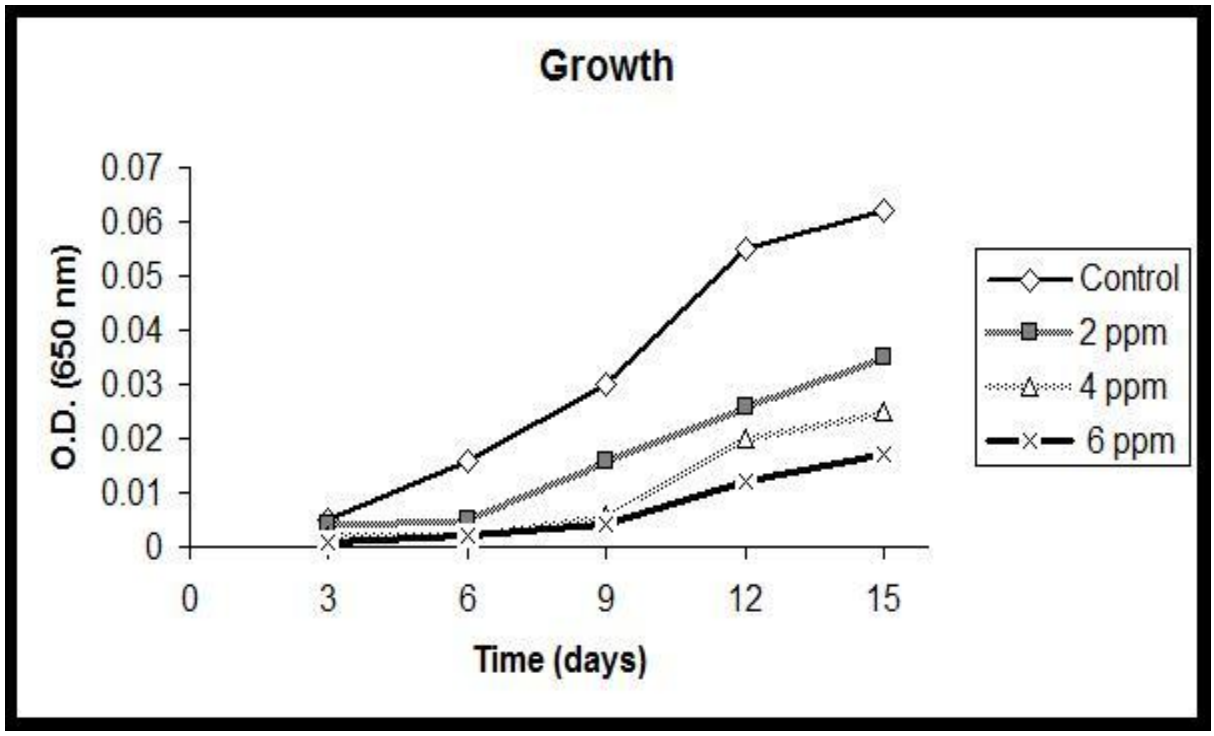

Figure 2 Growth pattern of *W. prolifica* as observed during an incubation period of 15 days with Hg^{+2}.

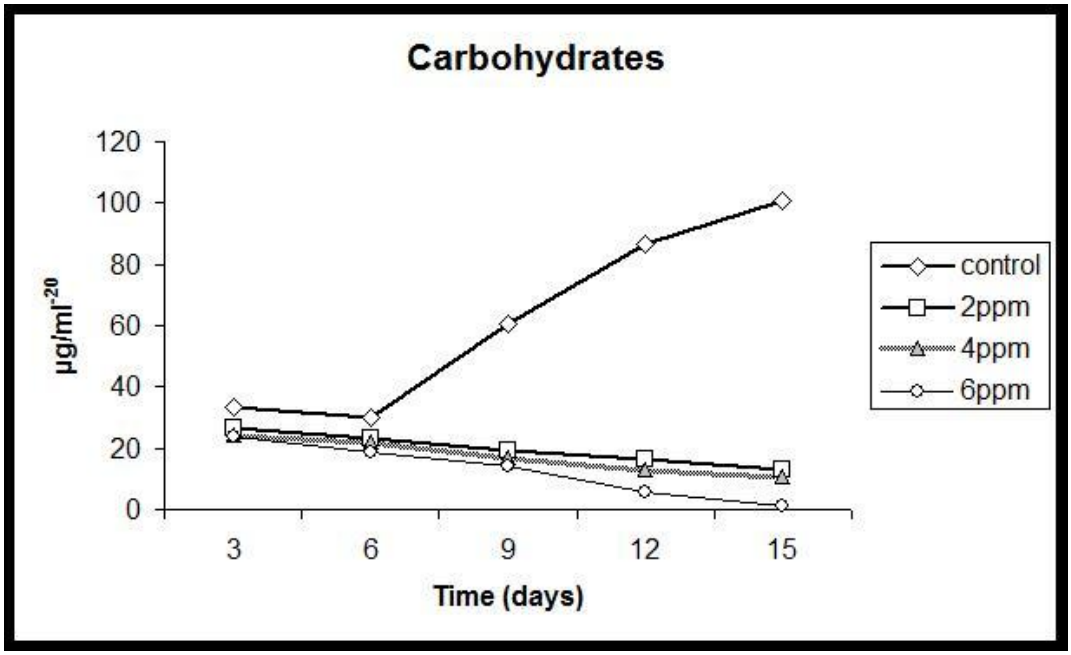

Figure 3 The reduction of carbohydrate content, in response to addition of different concentrations of Hg^{+2}, during an incubation period of 15 days.

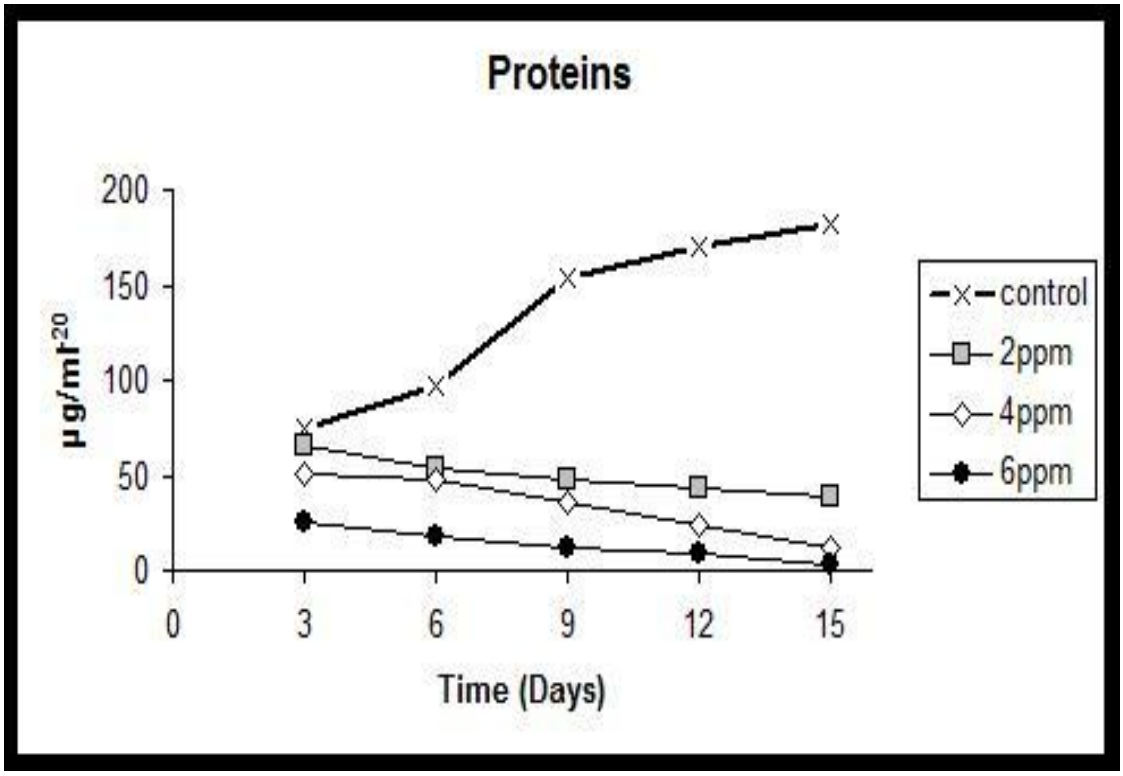

Figure 4 The reduction of protein content, in response to addition of different concentrations of Hg^{+2}, during an incubation period of 15 da

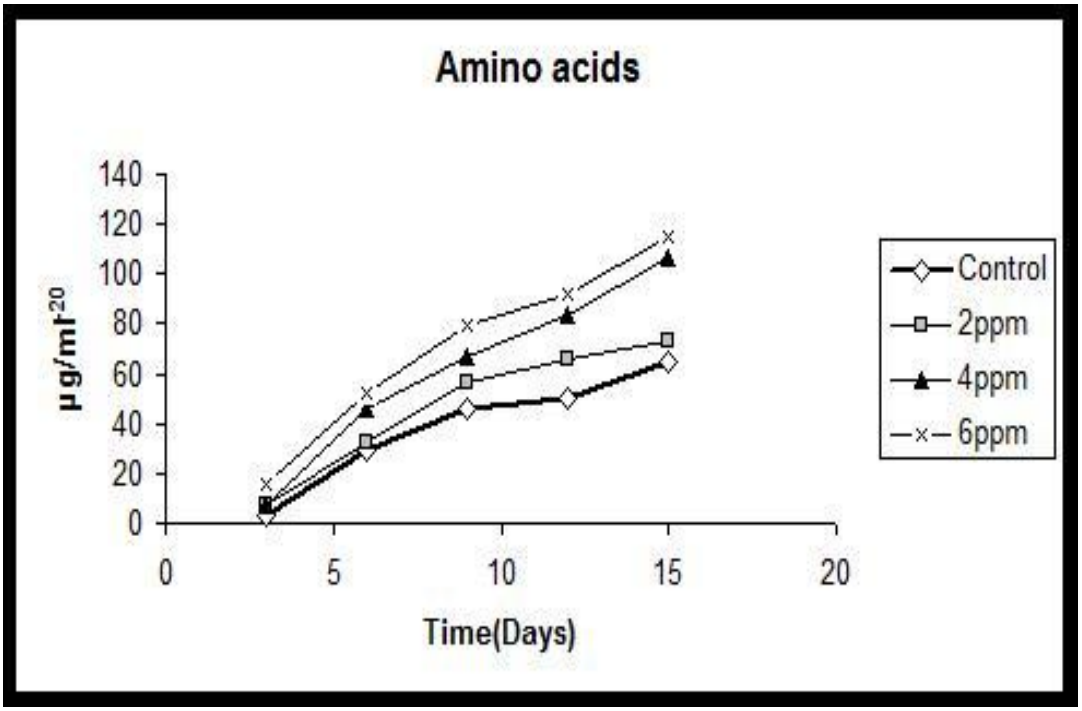

Figure 5 The reduction of amino acid content, in response to addition of different concentrations of Hg^{+2}, during an incubation period of 15 days.

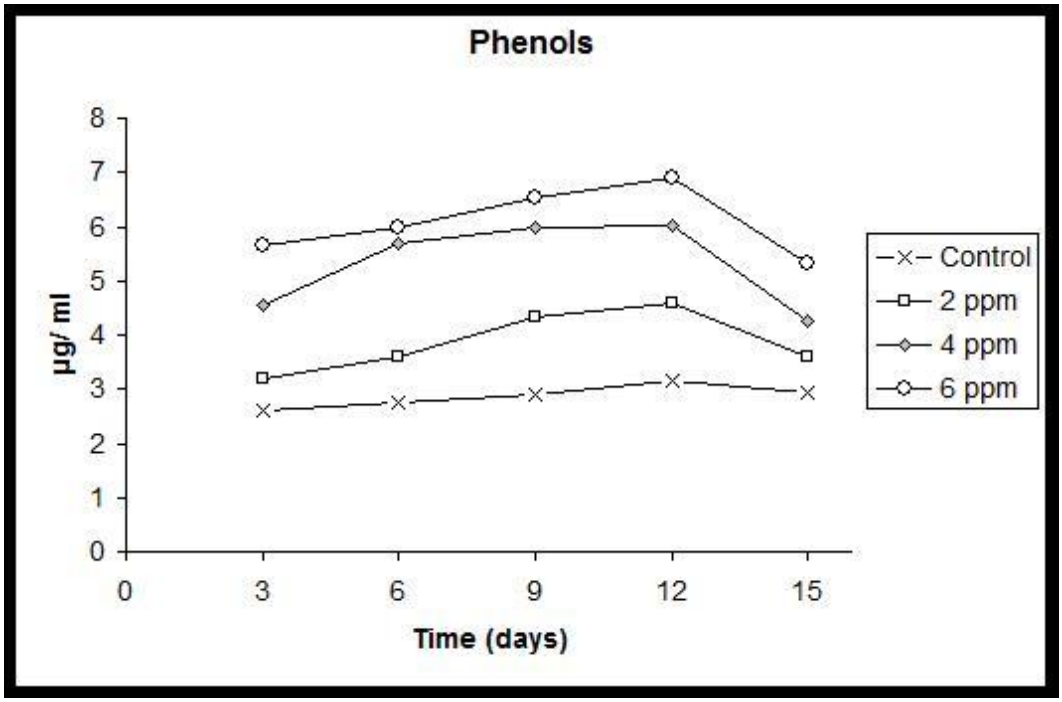

Figure 6 The increase of phenol content, in response to addition of different concentrations of Hg^{+2}, during an incubation period of 15 days.

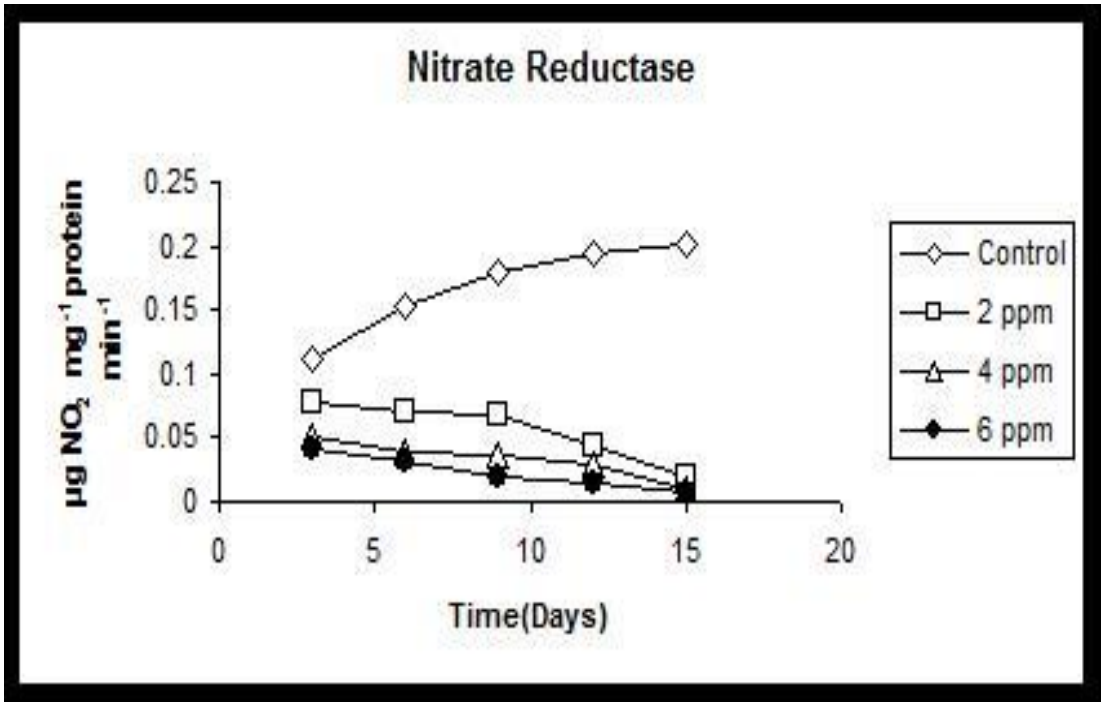

Figure 7 The inhibition in the nitrate reductase activity, in response to addition of different concentrations of Hg^{+2}, during an incubation period of 15 days.

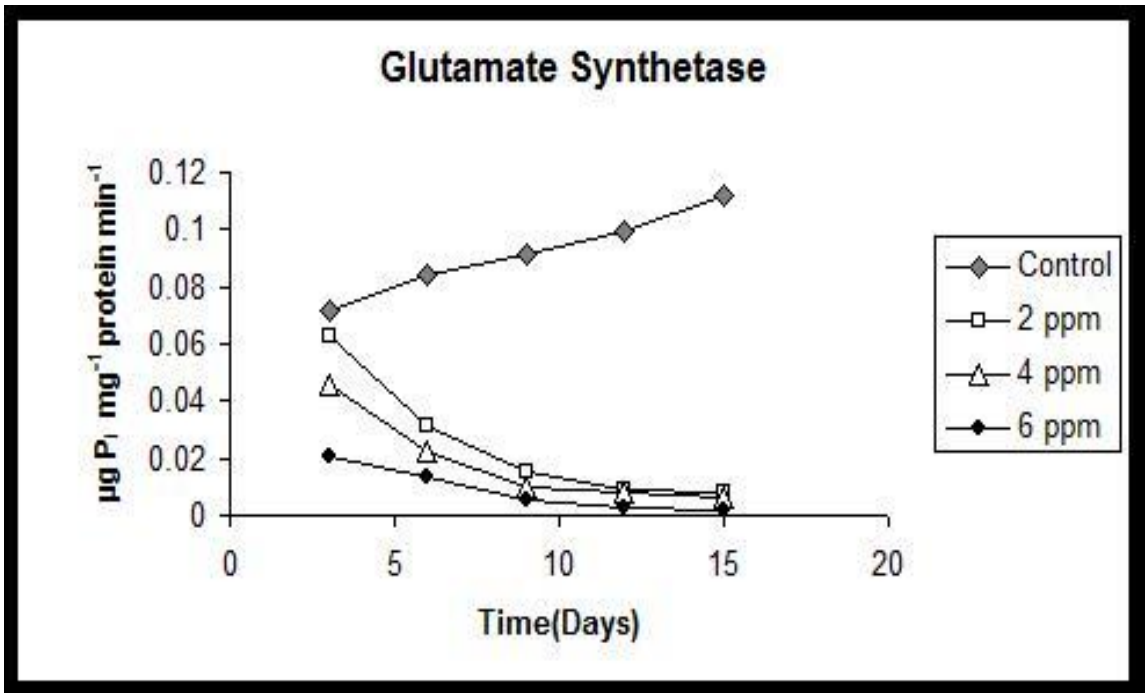

Figure 8 The inhibition in the glutamate synthetase activity, in response to addition of different concentrations of Hg^{+2}, during an incubation period of 15 days.

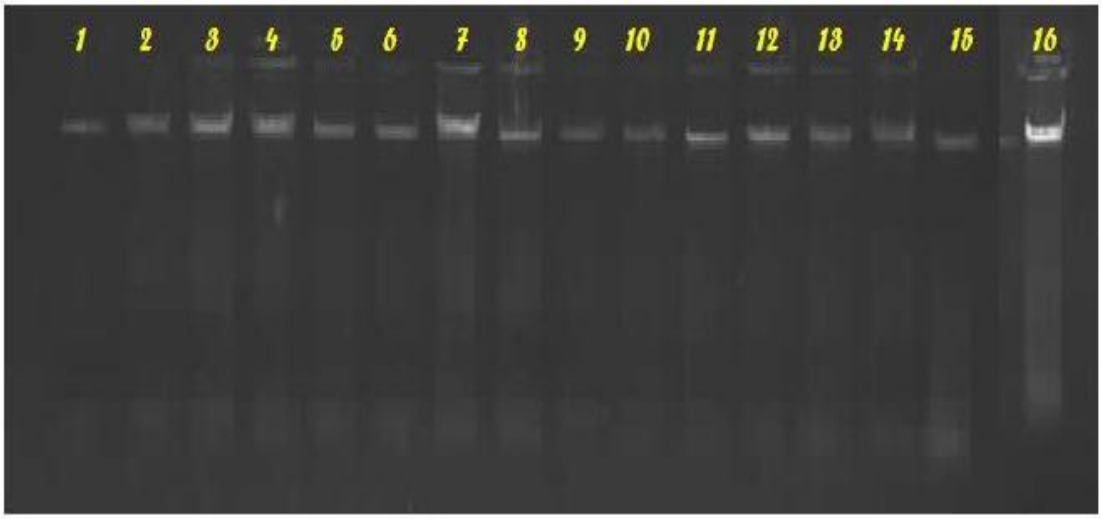

Figure 9. An Agarose gel used to observe the presence of DNA and effect of Hg^{+2} on the DNA content.

Lane 1, 2: DNA of untreated *W. prolifica* after an incubation of 3 days.
Lane 3, 4: DNA of *W. prolifica* treated with 2 ppm Hg^{+2} after an incubation of 3 days.
Lane 5, 6: DNA of *W. prolifica* treated with 4 ppm Hg^{+2} after an incubation of 3 days.
Lane 7, 8: DNA of *W. prolifica* treated with 6 ppm Hg^{+2} after an incubation of 3 days.
Lane 9, 10: DNA of *W. prolifica* treated with 2 ppm Hg^{+2}after an incubation of 15days.

Lane 11, 12: DNA of *W. prolifica* treated with 4 ppm Hg^{+2}after an incubation of 15 days.
Lane 13, 14: DNA of *W. prolifica* treated with 6 ppm Hg^{+2}after an incubation of 15 days.
Lane 15, 16: DNA of untreated*W. prolifica* after an incubation of 15 days.

DISCUSSION

From the graph **Fig. 1,** obtained for finding the **LC_{50}**, it was found out that the LC_{50} value of Hg^{+2} is 4 ppm for *Westeillopsis prolifica*, so the effect of various concentrations of mercury were done in a range of 2 to 6 ppm Hg^{+2}.

From the pattern observed in the **Fig. 2**, it is evident that the **growth** of the *W. prolifica*, although increases in the presence of Hg^{+2}, it is less than that in control. Similar results were obtained by Mallick and Rai (1990) in their demonstration of effect of Copper as well as by Dubey and Rai (1990) in their studies involving Chromium & tin.

Carbohydrate content of the treated *W. prolifica* with Hg^{+2} resulted in a decrease in the amount with time as well as increasing concentration of Hg^{+2} as shown in **fig. 3**. These results corroborate with the results of Filippis and Pallaghy (1976) who worked on a *Chlorella* sp. to see the effects of methylmercury treatment.

The differentHg^{+2} treatment doses to the *W.prolifica*showed a decrease in the **Protein** content as shown in the **fig.4** which is also seen in the work done by Mishra and Nanda (1997) which showed the protein content of the algae decreased significantly with increasing time and waste soil concentration.

Amino acid content of the Hg^{+2} treated *W.prolifica,* as shown in **fig.5,** increases with time and concentration. Similar work was done by Rai et al. (1981) with a contradictory finding about decrease in the amino acid content in cyanbacteria sp. like *Chlorella* treated with mercuric chloride.

Phenol content of the Hg^{+2} treated *W. prolifica,* as shown in **fig. 6,** increases with time and concentration of heavy metal. The same response is seen in the work done by Nirmal et al (1991).

The effects of the Hg^{+2} on the **Nitrate Reductase** and **Glutamate synthetase** activity of *W.prolifica* are shown in the **fig. 7** and **8.** The results revealed that there is inhibition of activity of both the enzymes which is also agreed by Mallick and Rai (1990) and Dubey and Rai (1987).

DNA content of the Hg^{+2} treated *W. prolifica* showed a decrease with time. These results are parallel with the ones obtained by Mishra and Nanda (1997) in their work on mercury and *W. prolifica*. Although, there was an increase in 2 and 6 ppm treatments a decrease was seen in 4 ppm treatment. The increase of DNA in 2 and 6 ppm was even larger than in control after the incubation period of 3days.

The results obtained above, were analysed by using Student's't' test and Correlation coefficient (r). These **statistical analysis** were also performed by Rai et al. (1994) while working with *Chlorella vulgaris* sp. to see the effect of Cu and Ni on growth, mineral uptake, and photosynthesis and enzyme activities at different pH values.
Our findings clearly demonstrated that heavy metal concentrations affecting cyanobacterial DNA and causing lethality to these microbes might be due to irreversible DNA damage. Friedberg et al.,2006 suggested that induction of DNA damage by a series of complex biochemical reactions as a result of chemicals, ionizing radiation, etc. tend to generate chain of single as well as double strand breaks and single strand gaps (Kumar et al., 2013)

CONCLUSION

The study aimed at evaluating the metabolic fate of Hg^{+2} on *Westiellopsis prolifica* have enlightened us about the important alterations taking place at cellular levels. Various parameters such as carbohydrate, proteins, amino acids, phenols, nitrate reductase, glutamate synthetase and DNA, of the cyanobacterium were evaluated in response to treatments. The present study concludes with some important results about *Westiellopsis prolifica* that would in future help in determining the areas where it can be used efficiently. The present study revealed that with increasing concentrations of Hg^{+2}, the content of amino acids and phenols of the *Westiellopsis prolifica* also increased. The treated *Westiellopsis prolifica* with 2 and 6 ppm Hg^{+2} showed an increase in the DNA content than 4 ppm Hg^{+2}, which decreased with time. On the other hand, other parameters like, carbohydrates and proteins, showed a decline in their content with increasing Hg^{+2}concentration. There was an inhibition in the nitrate reductase as well as

glutamate synthetase activities with an increase in heavy metal concentrations. The statistical studies concluded that the correlation between amino acids and nitrate reductase is highest whereas, phenols and glutamate synthetase shows lowest correlation. The 't' value for phenol (6 ppm) and glutamate synthetase (6 ppm) show the highest and the lowest values, respectively. Thus, Cyanobacterial species have been considered as candidates for use in bioremediation processes due to their potential of Bioaccumulation and Biosorption of heavy metals. But, proper studies are necessary for their practical use.

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