RESEARCH ARTICLE

EFFECT ON BIOCHEMICAL PARAMETERS OF CYANOBACTERIUM ANABAENA SP UNDER LEAD STRESS.

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Abstract

Effect of lead on Anabaena sp at five different concentrations (0.1, 0.5, 1, 1.5 and 2 ppm) were examined. Parameters such as growth response, pigment content, total protein content, and membrane damage, photosynthetic efficiency of PS II, SOD activity and heterocyst frequency were evaluated in laboratory conditions under control and treated conditions. Growth rate in all treated samples were much slower as compared to the control and the rate further decreased as the concentration of lead and days of incubation increased. The heavy metal toxicity is best observed by changes in growth of the algae. Protein content, photosynthetic pigment content (chl-a and car) and photosynthetic efficiency in terms of Fv/Fm of PSII. MDA accumulation in the alga in the treated sample is much higher as compared to control and the accumulation gradually increased as the day of exposure and concentration of heavy metal increased. SOD activity increased with increase in lead concentration and was observed maximum in 2 ppm as compared to control. Heterocyst frequency also decreased with increase in lead concentration and found to be minimum in treated sample.

Introduction:

Heavy metal causes serious environmental pollution. Contamination of the aquatic environment by heavy metals has been a subject of much concern in the recent years. It has become a worldwide phenomenon and their levels vary depending upon natural and anthropogenic pollution (Isani et al., 2009). With the rapid development of many industries (mining, surface finishing, energy and fuel producing, fertilizer, pesticides, metallurgy, iron and steel, electroplating, electrolysis, electro-osmosis, leather, photography, electric appliances manufacturing, metal surface treating) and aerospace and atomic energy wastes containing metals are directly or indirectly discharged into the environment causing serious environmental pollution and threatening human life (Gavrilescu, 2004; Malkoc and Nuhoglu, 2005; and Kumar et al., 2009). Several physico-chemical treatments like ion exchange (Volesky, 1994), chemical precipitation, electrochemical treatment (Atkinson et al., 1998), membrane technologies, adsorption or activated carbon etc are being used for the removal of heavy metals ions from aqueous wastes (Volesky, 2001). But these treatments are costly and end-product is usually high concentrated metal-loaded sludge, which is difficult to be disposed of (Atkinson et al., 1998). In contrast phytoremediation emerged as a good option (Gupta et al., 2000, Pavanant et al., 2006; Vijayaraghavan et al., 2006) and use of algal biomass as a biosorbent has emerged as an attractive, economical and effective bioremediating agent due to certain added advantages over others

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Lead is one of the major heavy metal used in pipes, drains, and soldering materials and mostly it is used in batteries. Apart from the natural weathering processes, the other lead contamination factor of the environment are mining, smelting activities, paints (Malik, 2004), gasoline, explosives and disposal of municipal sewage sludges (Ryan and Chaney, 1994). Exposure to lead produces anemia, liver and kidney damage, brain damage and ultimately death (Henick-Kling and Stoewasnd, 1993). Lead is a toxic metal with no biological function (Allen, 1984). Lead is found to be more toxic metal than nickel, copper and zinc as reported in Anabaenaasp (Hemlata and Fatma, 2009). Studies have also shown that lead inhibits metabolic processes such as nitrogen assimilation, photosynthesis, respiration, water uptake and transcription (Krupa et al., 1993). Lead ions (Pb\(^{2+}\)) can intensify processes of ROS production leading to oxidative stress (Cuypersset et al., 1999; Foyer et al., 1997). These processes destructively effect cell structure and metabolism which are mutually connected with each other, by decreasing the efficiency of oxidation-reduction enzymes or the electron transport systems in the cell (Stroinski and Kozlowska, 1997). Many genera of cyanobacteria are reported to be used to remove heavy metals e.g. *Tolypothrixstenus*, *Calothrixprietina* show high ability to remove mercury, *Scytomenaschlenidii*, *Anabaena cylindric*al, *A. torulosa* have high cadmium removal efficiency at 96-98%. *Gloeocapsa* sp., *Nostoc*paludosum, *N. piscinale*, *N. punctiformae*, *N. commune*, *Oscillatoriaagardhii*, *Phormidiummolle* and *Tolypothrix* remove 90-96% of lead. *Aphanothecechalyptica* and *Spirulinaplatus*could remove only 22% and 35% of lead respectively from battery factory wastewater (Kitjahnah and Incharoensakdi, 1992) and (Incharoensakdi and Kitjahnah, 1998). As blue green algae has much more potential for heavy metal removal from contaminated sites, in this study *Anabaena sp* isolated from local industrial water bodies was chosen to study the effect of lead on some biochemical parameters.

**Material and Methods:-**

**Maintenance of culture:-**

Test algae *Anabaena sp* were obtained from Cyanolab, School of Life Sciences, Sambalpur University. These samples were grown in nitrogen free BG-11 medium (Stanier et al., 1971). Flask capacity of 250 ml was used as algal culture medium under laboratory condition. Axenic culture in log-phase were inoculated in experiment and maintained under controlled conditions of light (7.5 W/m\(^2\)) and temperature (26±0.5°C) inside a culture room. For toxicity studies analytical grade of Pb(NO\(_3\))^2 was used as test solutions at various concentrations. For each concentration of Pb(NO\(_3\))^2 and control three replicates were taken. All the parameters were measured at three days interval till 15\(^{th}\) day of exposure.
Measurement of Growth:-
Measurement of growth was performed using light scattering technique by monitoring absorbance at 760 nm in a UV-Visible spectrophotometer (Shimadzu model UV-150-02) at 3 days intervals.

Estimation of Protein:-
Protein quantification was done following the method of Lowry et al., (1951) and absorbance was recorded at 750 nm against appropriate blank. Standard curve was prepared by taking different concentrations of bovine serum albumin (BSA). The protein content was calculated as µg of protein/ ml of algal suspension.

Estimation of Chlorophyll:-
Chlorophyll content was determined following the methodology of Mackinney(1941). The absorbance of the clear extract was measured at 660 nm in a UV-visible spectrophotometer (Shimadzu, model UV-150-02).

Estimation of Carotenoid:-
Carotenoids were estimated as per the method of Jensen(1978). Algal samples were harvested by homogenization and pellets were suspended in 3 ml of 80% chilled acetone. Then subjected to repeated thawing and freezing followed by centrifugation and supernatant collected. The process was repeated till the supernatant became colorless. The absorbance of the final volume was recorded at 470 nm.

Measurement of MDA Accumulation:-
The amount of malondialdehyde, a product of thylakoid lipid peroxidation was estimated according to Du and Bramlage (1992). Thiobarbituric acid (0.5% in 20% TCA) was added to equal volume of algal suspension and kept in a water bath at 95°C for 25 minutes and then centrifuged at 3000 rpm for 5 minutes for clarification. Absorbance of the clear solution was measured at 532 nm and corrected for non-specific turbidity by subtracting absorbance at 600 nm. The amount of accumulated MDA was estimated by using an absorption coefficient of 155 mM/ml.

Measurement of Photosynthetic Efficiency:-
Photosynthetic efficiency of algal suspension in terms of chlorophyll fluorescence was measured in a Plant Efficiency Analyzer (Handy PEA, Hansatech Instruments, Norfolk, UK). The algal suspension was dark-adapted for 20 minutes before the measurements and then Fo, Fm and Fv/Fm were analysed by the Handy PEA. The calculation of the rate for competing energy dissipation pathways in the sample of dark adapted (Fo) and light saturated (Fm) conditions had shown that maximal fluorescence (Fv/Fm) is directly proportional to the quantum efficiency of PS-II.

Assay of Superoxide Dismutase (SOD) Activity:-
The SOD activity was measured by the method of Dhindsa et al., 1981. The cyanobacterial cells were harvested by centrifugation and homogenized in 2 ml of 0.5 M Phosphate buffer (pH 7.5). The homogenate was centrifuged at 15,000 rpm at 4°C for 10 min and the supernatant obtained was used as enzyme extract. All the steps in the preparation of enzyme extract were carried out at 0-4°C. SOD activity in the supernatant was assayed by its ability to inhibit photochemical reduction of nitro blue tetrazolium (NBT). The test tube containing assay mixture 1.5ml reaction buffer (0.1M Phosphate buffer pH 7.8 + 0.5M Phosphate buffer pH 7.5), 0.2ml of 200 mM methionine, 0.1 ml of 1 M Na2CO3, 0.1 ml of 2.25 mM NBT, 0.1 ml of 3mM EDTA, 0.1 ml of 60 μM Riboflavin and 0.1 ml enzyme extract. Riboflavin was added last and the tubes were shaken and placed for 50 min below a light bank consisting of 15W fluorescent lamps. The tubes were kept in light for 10 minutes and then kept in darkness covered with a black cloth. The absorbance of the reaction mixture was read at 560nm. A non-irradiated reaction mixture which did not develop colour, served as control.

Heterocyst Frequency:-
Heterocyst frequency was determined by counting the number of heterocyst per hundred vegetative cells in at least 20-25 healthy and equal length filaments at different locus as well as within the filament taken at different concentration by the formula given below.

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\text{Heterocyst frequency} = \frac{\text{Total number of heterocyst}}{\text{Total number of vegetative cells}} \times 100
\]

Results:-
The heavy metal toxicity is better observed by changes in growth conditions. Gradually decrease in growth was observed with increase in the lead (Pb^{2+}) concentration and days of inoculation as compared to control (Fig.1). The
total protein content of *Anabaena sp* under Pb\(^{+2}\) treated and control condition also showed the similar trend (Fig.2). As the Pb\(^{+2}\) concentration increases, the total protein accumulation rate decreases.

Chlorophyll *a* (Fig.3) and carotenoid accumulation (Fig.4.) and loss follow the same trend as observed in growth curve. The negative effect of Pb\(^{+2}\) on Chlo and Car content was noticed clearly and the effect increased by the increasing concentration.

Measurement of MDA accumulation (Fig.5) showed a linear increase in control as well as in all concentrations of Pb\(^{+2}\) treated samples. The level gradually increased as the time and concentration of heavy metal increased. However, control sample showed minimum peroxidation value throughout the experimental period of 15 days.

The data on the measurement of Fv/Fm during the laboratory incubation of the samples (Fig.6) show similar kinetics like that of the photosynthetic pigment and protein accumulation and loss of *Anabaena sp* in control and treated samples. As the concentration of lead increased, photosynthetic efficiency decreased. Lead particularly at higher concentration may directly or indirectly cause damage to D1 protein of PS II leading to photo inhibition (Bhattacharyya et al., 2011). Kinetics of SOD activity (Fig.7) follows kinetic almost same pattern of MDA accumulation. A significant increase in SOD activity were observed as the concentration of lead increased. The heterocyst frequency (Fig. 8) decreases with increase in lead concentration, higher concentration were found inhibitory showing complete death of the cyanobacterium with no sign of heterocyst.

![Graph](image)

**Fig 1:** Effect of lead on Growth of Anabaena sp.
Fig 2: Effect of lead on total protein content of *Anabaena sp*.

Fig 3: Effect of lead on Chl content of *Anabaena sp*.
Fig 4: Effect of lead on carotenoid content of Anabaena sp.

Fig 5: Effect of lead on MDA accumulation of Anabaena sp.
Fig 6: Effect of lead on photosynthetic efficiency of *Anabaena sp*

Fig 7: Effect of lead on SOD activity of *Anabaena sp*
Discussion:-
Unlike higher plants, cyanobacteria can adopt to nutritional stress and other environmental changes quite readily (Reuter & Muller, 1993). Toxicity effect of different concentrations of lead and cadmium like damaged and reduced numbers of chloroplasts, disintegrated cell wall and death, decrease in growth and total chlorophyll content with increase in exposure time and concentration in filamentous green algae Cladophorafracta has been reported by Lamia et al., 2005. Another study was reported by El-Sheeket al., 2005 on toxicity effect of copper, manganese, iron and zinc on Anabaena subcylindrica and Nostocmuscorum.

In this study the reduction in growth (fig.1) could be due to inhibition of normal cell division by the metal, as reported in Dunaliellatertioclecta exposed to mercury and Chlorella vulgaris exposed to copper, mercury and cadmium (Rosko and Rachlin, 1977). However, there is increase in pigment content at low concentration of Pb$^{+2}$ also agrees with the findings of Rath (1984), Sahu (1987) and Shaw (1987). They reported increased growth rate, increase in pigment content, photosynthetic rate, respiration rate and enzyme activity at low concentration of mercurial compounds on Westellopsisprolifica. Rana et al., 2010 also reported that growth rate decreased in Lyngbyasp with increase in lead concentration.

Loss in protein content was observed (fig.2) as the time of incubation and concentration of lead increased. This finding supports the results reported by Ybarra and Webb, 1999; El-Enany and Issa, 2000, on the protein content of cyanobacteria. Low level of protein in treated samples could be due to stimulation of non-specific proteases causing degradation of macromolecules (Anand and Subramanium, 1997). This decrease in protein content may also be correlated with the accumulation and loss of phycobiliprotein which constitute up to 50% of total soluble cellular protein (Grossman et al., 1993, MacColl, 1998). Heavy metal concentration showed gradual decrease in protein content with increase of metal concentration in Anabaenaflos-aquae (Surosz and Palinska, 2004).

De Filippiset al., 1981 reported that the reduction in chlorophyll a content is a common symptom of heavy metal toxicity. Carotenoid plays an important role in photoprotection of chlorophyll (Deo and Biswal, 2001) by quenching the triplet Chlorophyll and scavenging the singlet oxygen and other reactive oxygen species (ROS) (Choudhury and Behera, 2001). Edwin, 1997; Moreland, 1980; Bhunia et al., 1991 suggested that the loss of carotenoid accumulation may be due to the inhibition of carotenoid biosynthesis pathway leading to chlorosis of the organism or may be due
to degradation membrane component of the thylakoid (Allen, 1984). In the present work, the low level of chlorophyll (fig.3) and carotenoid content (fig.4) in lead treated samples supports the observations of Das and Adhikary (1996), Sikha and Singh (2004) and Xia (2005).

One of the deleterious effects induced by heavy metal exposure in plants, algae and cyanobacteria is lipid peroxidation, which can directly causes biomembrane deterioration. Malonaldehyde (MDA) is regarded as a reliable indicator of oxidative stress (Demiral and Turkan, 2005). Lipid peroxidation expressed as MDA accumulation was reported to be high under heavy metal stress (Gallego et al., 1996; Cho and Park 2000; Shah et al., 2001). In cyanobacteria, the lipids present in the thylakoid membranes contain a high percentage of polyunsaturated fatty acid (PUFA) residues and are thus most susceptible to peroxidation (Halliwell 1999; Bandopadhyay et al., 1999). Production of ROS during stress condition in cyanobacteria have been reported (Hideg and Vaas, 1996) which followed a series of damaging reactions with biological molecules resulting in enhanced production of MDA in treated samples. Free radical formation occurs due to strong inhibition of PS II. These active oxygen species (O\(^2\), H\(_2\)O\(_2\), OH, \(^1\)O\(_2\)) causes severe toxicological problems and results in peroxidation of membrane lipids and general cellular oxidation. The present result is also in agreement with the above results and also with the reports of (Choudhury et al., 2007; Fatma et al., 2007) on Spirulina platensis and Westellopsis prolifica.

Photosynthetic efficiency of PS II can be measured by the ratio of Fv/Fm. It is known that photo inhibition occurs when the rate of excitation energy captured exceeds the rate of consumption in photosynthetic reactions (Osmond, 1981; Powles, 1984). Photo inhibition in terms of Fv/Flm has been found both in higher plants (Panda et al., 2006; Rodrigues et al., 2007) as well as in algae (Ying and Hader, 2002; Xia, 2005). The primary site of photo inhibition is the reaction centre (D1 protein) of PS II (Demming and Bjorkman, 1987). Photo inhibition is manifested as a decrease in oxygen evolution (Krause, 1988) and photochemical efficiency (Falk and Samuelsson, 1992). Photosynthetic functions also have been found to be affected directly or indirectly by heavy metals since thylakoids lamelle of cyanobacterialcells contain all chlorophylls and carotenoids, which are disassembled in the presence of heavy metals (Baszynski and Krupa, 1995; Prasad et al., 2002). Decrease in Fv/Fm ratio in the present investigation (fig.6) supports the earlier findings.

Pb\(^{2+}\) ions can intensify processes of ROS production leading to oxidative stress (Cuypers et al., 1999). Among the four major active oxygen species super oxide radicals O\(_2\), H\(_2\)O\(_2\), hydroxyl radicals are most active and destructive. SOD and catalase (Asada 1994)activity enhances under a variety of stressful condition like Cu, Al, Mn, Fe, Zn, Pb toxicities (Prasad, 1994) for maintenance of overall defense system of cell subjected to oxidative damage (Slooten et al., 1995). In our findings increased SOD activity (fig.7) was observed with increase in metal concentration and day of incubation.

Heterocyst frequency count is a measure of the rate of nitrogen fixation by BGA (William et al., 1970). Among the filamentous BGA, the ability to fix nitrogen under aerobic conditions is strictly correlated with the ability to form heterocyst (Stewart, 1969). In the present investigation, the heterocyst frequency (fig.8) decreased at higher metal concentration. This could be either due to the inhibited synthesis of proteins involved in heterocyst differentiation or due to degradation of pre-existing heterocyst. The increased in heterocyst frequency may be due to impaired nitrogen fixation due to lack of functional nitrogenase enzyme (Orus and Marco, 1991).

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