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Original Research Paper

Toxicity of Lead on Biochemical Changes of Nitrogen Fixing Cyanobacteria, *Aulosira fertilissima* Ghose

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ABSTRACT

The study was aimed to evaluate biochemical fate of *Aulosira fertilissima* when treated with different doses of $PbNO_3$ which is the one of the sources of heavy metal of lead. Moreover, cyanobacteria, a group of prokaryotes, symbiotic, N_2 fixing organism, ubiquitous in distribution and used as a biofertilizer in the paddy fields. The heavy metals not only destroy organisms but also kill the non-target cyanobacteria in paddy fields. Therefore, in the current study an attempt has been made to study acute toxicity of $PbNO_3$ on biochemical changes like pigment contents, chlorophyll-*a*, *b*, total, phycobilins and carotenoids, metabolites- carbohydrates, proteins and phenols, and enzyme activity-protease and nitrate reductase of nitrogen fixing cyanobacteria *Aulosira fertilissima* Ghose when grown in BG11 media. Retardation of chlorophyll-*a, b*, total, phycobilin and carotenoids was observed in all the treatments of $PbNO_3$ when compared with control as the days progressed. On the other hand, metabolites like carbohydrates, proteins and phenols also showed decrease in their content after 96 hours treatment of different doses. However, the present study revealed that there is a rise in protein content in 6 ppm, and phenol content in 12.0 ppm and 22.0 ppm. Besides, there was an inhibition in the nitrate reductase and protease activities with an increase in $PbNO_3$ doses. Further, cyanobacterial species, *Aulosira fertilissima* could be considered for bioremediation processes due to their potentiality to tolerate up to 6.00 ppm of $PbNO_3$ dose without any adverse effect, but proper studies are necessary for their practical reuse and development of heavy metal resistant strains.

INTRODUCTION

Cyanobacteria (BGA) are aerobic photoautotrophs, requiring only oxygen, light and inorganic substances. These blue green algae are well distributed over land and water, in some cases where vegetation cannot exist, and gram-negative gliding bacteria. In environmental samples cyanobacteria are easily recognized by light microscopy. If illuminated with green light, they will show bright red auto fluorescence. This is due to the presence of phycobili-proteins and chlorophyll-*a*. Some photosynthetic filamentous cyanobacteria are capable of forming specialized structures called heterocysts. Heterocysts solve the problem of performing plant-like photosynthesis (which produces oxygen) and at the same time fixing N_2 to ammonia (a process that involves enzymes that are inactivated by O_2) due to presence of nitrogenase enzyme. Nitrogenase reduce N_2 to NH_4^+ and releases H_2. A high amount of glutamine synthetase (GS) and low amount of glutamine oxo-glutarate aminotransferase (GOGAT) is present in heterocyst. Glutamine reacts with NH_4^+ and forms two molecules of glutamate, one-molecule returns to heterocyst and second molecule takes part in production of other metabolites. These organisms are directly or indirectly affected by number of pollutants including heavy metals in the environment.

Lead is one of the worst pollutants in the atmosphere, occurs in the form of $PbNO_3$. It is a major constituent of the lead-acid battery used extensively in cars, used as a colouring element in ceramic glazes, form glazing bars for stained glass and other multi-lit windows, candles to treat the wick to ensure a longer, more even burn, as a coolant. Recent literature shows that heavy metals adversely affect various biochemical process of nitrogen fixing and non-nitrogen fixing cyanobacteria. Chen et al. (2006) conducted a greenhouse pot experiment to evaluate the impact of different concentrations of lead acetate in soil microbial biomass and community structure during growth of Chinese cabbage (*Brassica chinensis*) in two different soils. Chaudhary et al. (2006) expressed that the effect of lead, copper and zinc over a concentration gradient of 0.05-0.20 mg/L on proline, malondialdehyde (MDA) and superoxide dismutase (SOD) in the cyanobacterium *Spirulina platensis*-S5. Despite a reduction in growth of the test microorganism, its MDA, SOD and proline contents increased under the heavy metal stress, corresponding to the concentration of the metal ion in the culture medium. Sode et al. (1997) showed Cu^{2+} induced the activity of a temperate marine cyanophage, ms-1 to *Synechococcus* sp. NKBG 042902. This induction was specific to Cu^{2+} and dependent upon Cu^{2+} concentration. Cr, Pb, Co and Zn were not effective as inducers. Certain heavy metals like cadmium, nickel, mercury and chromium are known to inhibit the growth, pigment synthesis, nutrient uptake, nitrogen fixation and photosynthesis in different species of cyanobacteria *Anabaena inaequalis, Anabaena doliolum* and *Nostoc muscorum* (Rai et al. 1990).

Nirmal Kumar (1991), Nirmal Kumar & Rana (1991), Nirmal Kumar & Rita Kumar (2002) and Niraml Kumar et al. (2007) explored different pesticides like isoproturon, bavestine, aldrin and fluchloralin, and industrial effluents at various doses on *Westialliopsis prolifica*, *Anabaena* spp. and *Nostoc muscorum*. The study showed gradual increase of chlorophyll-*a*, carotenoids, carbohydrates, protein and phenol of cyanobacteria at lower dose of treatments. However, the higher treatments were found to be inhibitory. As these organisms not only fix atmospheric nitrogen, but also improve the yield and biomass of paddy field, these heavy metals affect pigment production, metabolites, enzyme activity, protein synthesis, RNA synthesis and DNA synthesis. However, no attempts have been made to study acute toxicity of lead on biochemical changes such as pigment production-chlorophylls, phycobilins and carotenoid, metabolic variations like carbohydrates, proteins and phenols, and enzyme activity in terms of protease and nitrate reductase of nitrogen fixing cyanobacteria, *Aulosira fertilissima*. Therefore, the present investigation has been undertaken.

MATERIALS AND METHODS

The species of cyanobacteria, *Aulosira fertilissima* was made axenic by treating with streptomycin and benzyl penicillin. Growth of cyanobacterial isolates was not affected when exposed to 62.5 ppm of benzyl penicillin and 31 ppm of streptomycin, and this concentration of antibiotic was effective in preventing bacterial contamination. Cultures were periodically checked for bacterial contamination with the bacterial broth. $PbNO_3$ was used as the heavy metal and determined the LC_{50} in terms of chlorophyll content of *Aulosira fertilissima*, by which the species was subjected to different concentration of $PbNO_3$. Finally, three concentrations were selected, i.e., 6.00, 12.00 and 22.00 ppm for the acute toxicity studies of $PbNO_3$ in response to biochemical variations of *A. fertilissima.* This strain was used for the experimental purpose and grown in BG_{11} media without nitrogen source. The treated cultures were incubated and grown photoautotrophically in a culture room, maintained at 22±2°C and illumination maintained with white fluorescent tubes providing an approximate intensity of 2000 lux with 16 hour photoperiod and 8 hour dark period.

The biochemical parameters were estimated to determine the effect of $PbNO_3$ on this cyanobacteria. Estimation of chlorophylls, phycobilin pigment, carbohydrates, proteins, phenols, nitrate reductase and protease activities was made using the standard methods (Thimmaya 1999). The results of the analysis have been reported per 20 mL culture (mL^{-20}). The Student (*t*) test and correlation coefficient of different parameters with respect to different variables were done.

RESULTS

Chlorophylls are essential component for photosynthesis and occur as green pigments in all photosynthetic organisms. The chlorophyll-*a* content of *A. fertilissima* varied from 0.115 to 0.89 μg mL^{-20} (μg/20 mL culture). The retardation of chlorophyll-*a* content was observed with increasing concentrations when compared with control (Fig. 1a). There was linear fall of chlorophyll-*a* in 12 ppm followed by 22 ppm dose which was sharply declined by 63 percent, 84 percent and 88 percent at 6 ppm, 12 ppm and 22 ppm, respectively, by the end of 96 hours treatment. The chlorophyll-*b* content of *A. fertilissima* varied from 0.563 to 0.725 μg mL^{-20}. There was retardation of chlorophyll-*b* content with increasing concentration of $PbNO_3$ as the time period progressed (Fig. 1b). However, there was a gradual fall of chlorophyll-*b* content in 6.0 ppm at 48 hours and then it was found to rise. The control showed linear raise in chl-*b* content as time period passed. However, chl-*b* content reduced by 9 percent, 12 percent and 26 percent at 6.0 ppm, 12.0 ppm and 22.0 ppm respectively, by the end of 96 hours treatment. Total chlorophyll content of *A. fertilissima* varied from 0.905 to 1.534 μg mL^{-20}. An increase in chlorophyll content in control as time period progress was achieved (Fig. 1c). After 96 hours of treatment, in all cases chlorophyll-*b* of *A. fertilissima* was declined when compared with control. The reduction changes observed after 96 hours treatment were 75 percent, 79 percent and 94 percent at different concentrations of $PbNO_3$ in 6.0 ppm, 12.0 ppm and 22.0 ppm, respectively. Phycobilins such as phycoerythrobilin and phycocyanobilin are the antenna pigments in cyanobacteria. These are accessory light harvesting pigments present in photosynthetic organisms. Phycobilin, which are water soluble pigments dominated by phycocyanin, content of *A. fertilissima* varied from 0.182 to 0.398 μg mL^{-20}. Phycobilin content reduced as time period progressed in all the doses but the values were lowered when compared with control (Fig. 1d). The percent reduction, observed after 96 hours treatment, was 2 percent, 33 percent and 55 percent at different concentrations of $PbNO_3$ in 6.0 ppm, 12.0 ppm and 22.0 ppm respectively.

Carotenoids are the secondary light absorbing pigments (also called as accessory pigments). They may be yellow, red or purple. Total carotenoid content of *A. fertilissima* varied from 1.28 to 3.93 μg mL^{-20}. The control showed gradual raise in carotenoid content as time period passed (Fig. 1 e). The carotenoid content was reduced by 24 percent, 38 percent and 68 percent at the end of 96 hours treatment in 6.0 ppm, 12.0 ppm and 22.0 ppm, respectively. The lowest amount of carotenoid (1.28 μg mL^{-20}) was observed in 22 ppm. The biochemical response of *A. fertilissima* to different doses of $PbNO_3$ is shown in Figs. 2(a,b,c) and 3(a,b). The variation caused by $PbNO_3$ on *A. fertilissima* and its impact on the biochemical composition of total carbohydrates, proteins and phenols of the organism was estimated.

Carbohydrates are the most abundant biomolecules present in all the living organisms, and content in *A. fertilissima* ranged from 9.98 to 13.48 μg mL^{-20}. There was a gradual fall of carbohydrate content when treated with 6.0 ppm, 12.0 ppm and 22.0 ppm observed after 96 hours treatment (Fig. 2a). The highest amount of carbohydrate content (13.48 μg mL^{-20}) was found in untreatment followed by 6.0 ppm of $PbNO_3$ treatment, whereas, lowest amount of carbohydrate content (3.67 μg mL^{-}

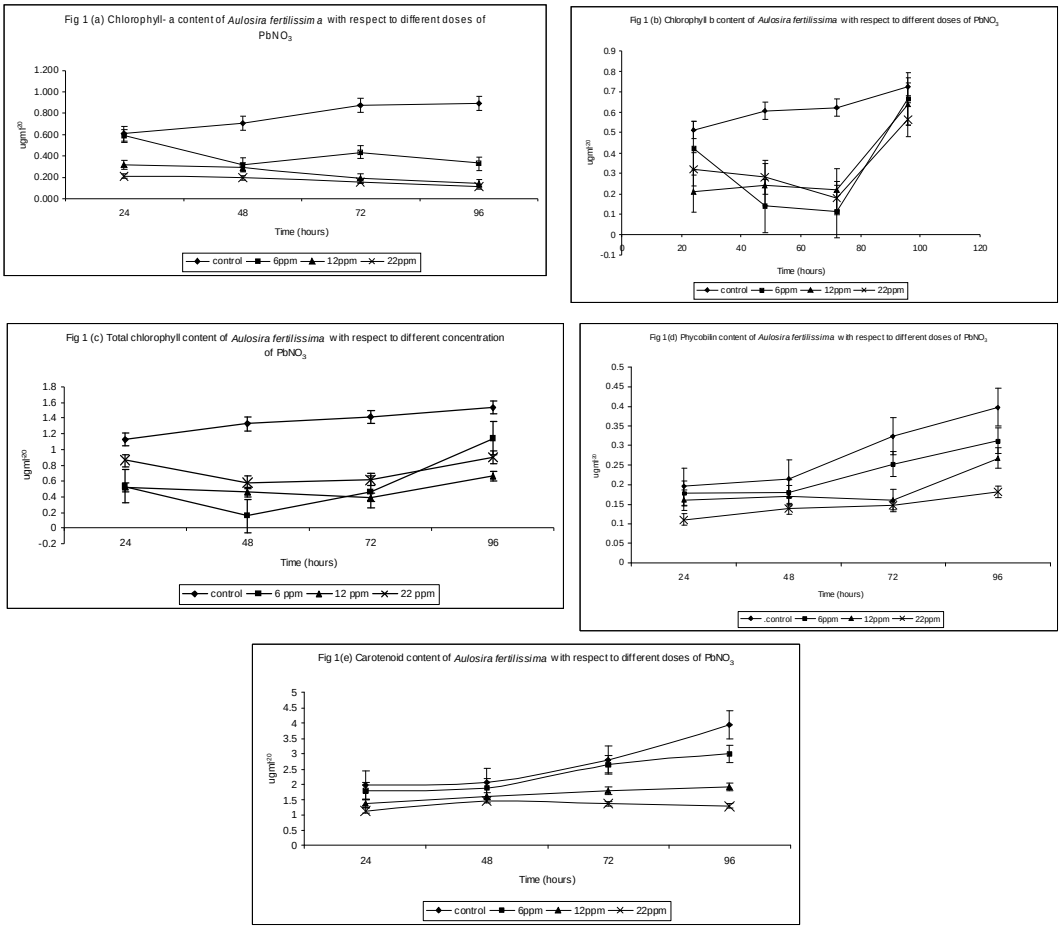

Fig.1 a to e: Pigment contents of *A. fertilissima* in response to addition of different concentration of $PbNO_3$ during the period of 96 hours.

^{20}) was observed at 22.0 ppm after 24 hours. The drop of carbohydrate concentration was by 13 percent, 24 percent and 26 percent at 6.0 ppm, 12.0 ppm and 22.0 ppm after 96 hours treatment.

Proteins are the macromolecules formed by the peptide bond formation between amino acids. They have innumerable functions in cells like structural, regulatory, catalytic (enzymes), etc., and have four levels of protein structural organization. The total protein content of *A. fertilissima* ranged between 98.67 and 121 µg mL^{-20}. There was rise in protein content at 6.0 ppm, however, reduction was observed in doses of 12.0 ppm and 22.0 ppm till 96 hours treatment (Fig. 2 b). At the end of 96 hours treatment at 6.0 ppm there was greater amount of protein than that of the untreated culture but 12.0 ppm and 22.0 ppm showed a gradual fall by 6 percent and 9 percent, respectively.

Phenols are the aromatic metabolites which trigger various biochemical processes of the organisms. They consist of a hydroxide group which is widespread in photosynthetic organisms. The phenol content of *A. fertilissima* varied from 2.266 to 10.045 µg mL^{-20}. Highest amount of protein was showed in 22.0 ppm and 12.0 ppm treated cultures than that of control and 6.0 ppm doses

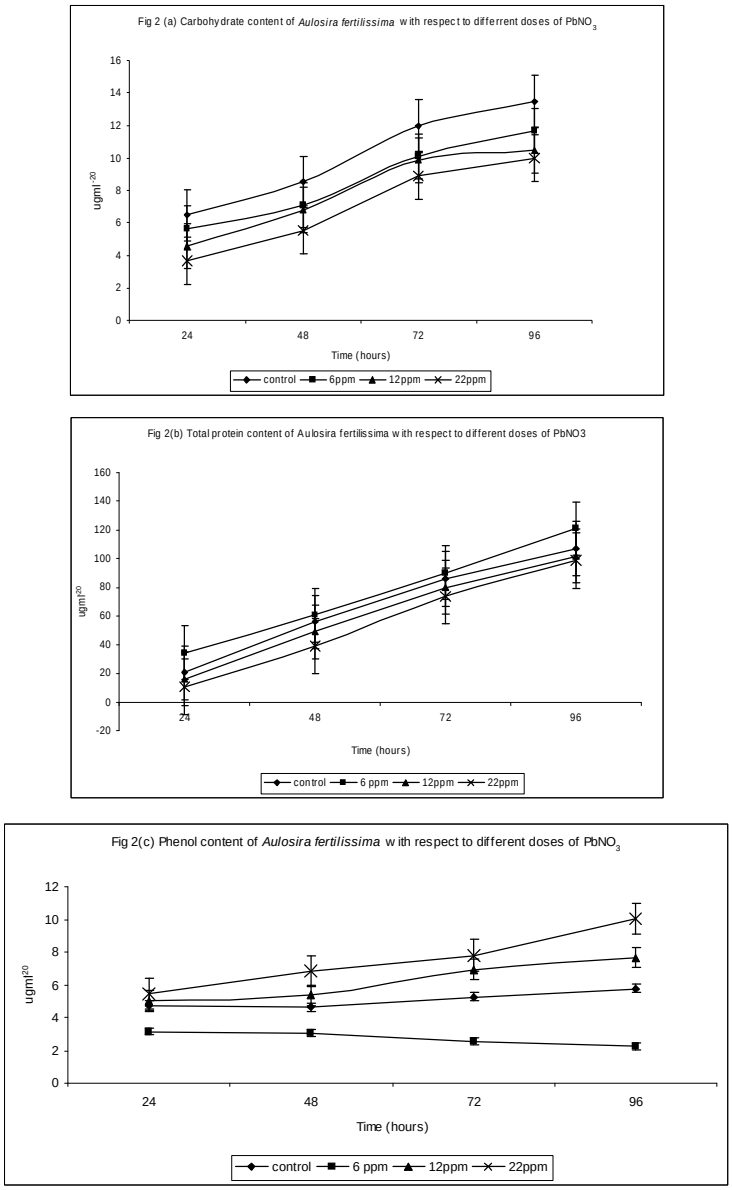

Fig. 2a to c: The biochemical contents of *A. fertilissima* in response to addition of different concentrations of $PbNO_3$ during the period of 96 hours.

(Fig. 2 c). While in case of 6.0 ppm, lower amount phenol content was observed than that of the unprocessed culture. After 96 hours treatment, highest amount of phenol content (10.045 µg mL^{-20}) was achieved at 22.0 ppm treatment.

The nitrate reductase (NR) is one of the key enzymes involved in fixation of atmospheric nitrogen. This enzyme is sensitive to light, temperature and oxygen. NR activity of *A. fertilissima* varied

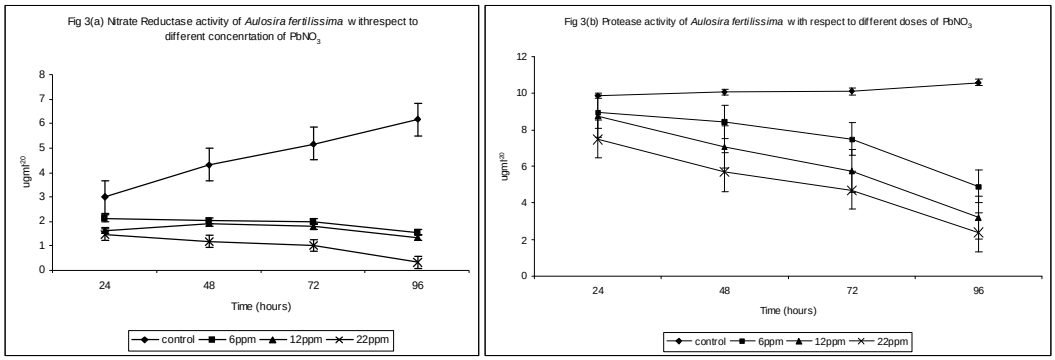

Fig. 3 a, b: The biochemical contents of *A. fertilissima* in response to addition of different concentrations of $PbNO_3$ during the period of 96 hours.

from 0.32 to 1.16 µg mL^{-20}. The nitrate reductase activity was found to decline with increased concentration of $PbNO_3$ and as the time progresses (Fig. 3.a). The $PbNO_3$ toxicity to nitrate reductase was observed maximum at 22.0 ppm dose followed by 12.0 and 6.0 ppm treatments by the retardation of 75 percent, 79 percent and 95 percent at 6.0 ppm, 12.0 ppm and 22.0 ppm concentration. The protease activity of *A. fertilissima* fluctuated from 9.85 to 10.6 µg mL^{-20}. The protease activity was found to decrease with increase in concentration of $PbNO_3$ and progress of time. The $PbNO_3$ toxicity to protease was observed maximum at 22.0 ppm dose followed by 12.0 ppm and 6.0 ppm (Fig. 3b). A drop of protease activity was observed by 54 percent, 70 percent and 78 percent at 6.0 ppm, 12.0 ppm and 22.0 ppm doses, respectively.

The Student's (*t*) test was performed between all the variables by the end of 96 hours treatment with different concentrations of $PbNO_3$. The variables ranged between 0.024 and 0.053 with respect to 6.0 ppm of $PbNO_3$ concentration, in 12.0 ppm concentration from 0.0152 to 0.037, and in 22.0 ppm concentration between 0.015 to 0.017. ANOVA (analysis of variance) showed that proteins and phenols were less significant with other parameters like chlorophyll-*a, b*, total chlorophyll, phycobilin, carotenoids, carbohydrates, nitrate reductase and protease.

DISCUSSION

It is substantiated from the above observations that chlorophyll-*a, b* and total chlorophyll content of *A. fertilissima* have gradual fall with the increasing concentration of $PbNO_3$ when compared to the control. Similar observations were also made by Surosz & Palinska (2005) while working with different heavy metals on *Anabaena flos-aquae*, and also found reduction of chlorophyll content in cells treated with copper, which may be due to displacement of magnesium atom of chlorophyll molecules by the heavy metal (Wu & Lorenzen 1984). 100µmol/L of copper reduced chlorophyll content of *Spirodela polyrhiza*, but lower nickel concentration of 25µmol/L stimulated chlorophyll biosynthesis as detected by Pandey et al. (1999). Besides, Padhy (1985) pointed out that phycobilin content of the treated cyanobateria, *W. prolifica* with different concentrations of metals resulted in lower amount with both increased time as well as doses. Similar results were also achieved in current study when *A. fertilissima* treated with various doses of $PbNO_3$. Reduction in chlorophyll content and phycobilin was also observed by Rai et al. (1990), Rai & Abraham (1993 and 1995) when using *Anabaena torulosa, Plectonema boryanum, Anabaena doliolum* and other species of cyanobacteria.

Carotenoid content of treated *A. fertilissima* with different concentrations of $PbNO_3$ resulted in gradual fall with both the progress of time and increased doses. Ira Bhatt (2006) and Nirmal Kumar et al. (2007) also achieved similar observations and found the reduction of carotenoid during bioremediation of nutrients and the toxic meal from, Amul, GSFC and Atlas Dye Industrial effluents by *Westielliopsis prolifica, Nostoc punctiformis* and *A. fertilissima*. Reduction in chlorophyll indicated the inhibition of growth. Veneela & Verma (2006) studied the influence of two metals, copper and cadmium on the growth and ultra structures of cyanobacterium *Anabaena flos-aquae* grown at three different temperatures, 10°C, 20°C, and 30°C. The highest concentration of chlorophyll-*a* was observed at 20°C and the lowest at 10°C. Both the toxic metal ions, Cu^{2+} and Cd^{2+}, inhibited growth of the tested cyanobacterium. Chlorophyll-*a* concentration decreased with the increase of metal concentration. A 50 percent decrease in the growth of *A. flos-aquae* population, compared with the control, was reached at 0.61 mg/L cadmium and at 0.35 mg/L copper (at 20°C). Growth of *Aulosira fertilissima* was suppressed by the endrin at 10 ppm in rice fields and UV-B and lead as found by Ahmed & Venkataraman (1973) and Rai et al. (1995) respectively, which is corroborated in current study that 12.00 and 20.00 ppm dose retarded the growth of *A. fertilissima*.

Padhy (1985) stated that the retardation of carbohydrate content might be due to the interference of chemicals with the photosynthesis process. Mallick & Rai (1992) and Kapoor & Arora (1996) showed metal induced inhibition on carbohydrate content and photosynthesis. Kapoor & Arora (1998) explained that total carbohydrate content was increased when aluminium concentration mixed into the medium up to 40 ppm on the *Anabaena doliodum* and *Nostoc muscorum*. In the present study too, the carbohydrate content of the treated *A. fertilissima* with $PbNO_3$ resulted in diminution. Protein content of treated *A. fertilissima* was depleted with the progress of time in 12.0 and 22.0 ppm treated cultures. The interruption of protein synthesis might be due to inhibition of enzymes and structural protein essential for growth stated by Kapoor & Arora (1996) by metals in effluent. Thiel (1990) has emphasized the decrease in protein content in starved cells of *Anabaena variabilis*.

Phenol content of $PbNO_3$ treated *A. fertilissima* shooted up with the progress of time and increased concentration of heavy metal. The similar observations were also made by Nirmal Kumar (1991) when treated with a herbicide N, N-dimethyl, N-isopropyle phenyle urea on *Nostoc muscorum*. Uma et al. (2005) also substantiated that increase in phenol could be due to conversion of other metabolites as protectants in organisms during stress or drought conditions. The activity of nitrate reductase with different doses of $PbNO_3$ on treated *A. fertilissima* revealed inhibition of activity of enzyme, which also corroborated with findings of Timothy et al. (1989) and Mallick & Rai (1990). The fall of protease activity of *A. fertilissima* with progress of time and doses in the present study was coincided with the observations made by Mallick & Rai (1994) on kinetic studies of mineral uptake and enzyme activities of *A. doliolum* under metal stress.

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