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Responses of cyanobacterium *Anabaena doliolum* during nickel stress

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Abstract: *Growth and biochemical responses of heterocystous nitrogen fixing cyanobacteria Anabaena doliolum were studied upon exposure to various concentrations of nickel (0.1 to 100 μM) for duration of upto 4 days, in view of its tolerance. The growth of the cyanobacterium was increased in terms of cell density (O.D), heterocyst frequency, chlorophyll-a, carotenoid and c-phycocynin upto moderate exposure (10 μM for 96 hr of Ni treatment). Protein and total non protein thiol were measured as stress responsive metabolites and frequency of heterocyst and spores were observed in responses to Ni. The present study demonstrated that the tested cyanobacterium exhibited dose specific responses of metal towards studied different morphological, physiological and biochemical parameters.*

Key words: *Bioremediation, Cyanobacterium, Nickel*
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Introduction

Heavy metals including nickel (Ni) have been released into the environment over a long period of time through various anthropogenic activities. Burning of fossil fuels and residual oils, coal mine spoils, sewage sludge, production of Ni-Cd batteries are the primary source of Ni and exhibit toxic effect on biota (El-Enany and Issa, 2000). Average yearly emission of Ni was $55,650 \times 10^3$ kg in atmosphere, 113×10^6 kg in aquatic ecosystem and 416.5×10^6 kg in soil in 1983-84 (Nriagu and Pacyna, 1988). Nickel ion has been shown to be an essential micronutrient for many microorganisms as it plays important role in four microbial enzymatic activities (Thauer *et al.,* 1980; Welch, 1995). In plants, it is required in trace amount for various physiological processes. (Kupper *et al.,* 1996) but, at higher concentrations it is toxic to plants (Srivastava *et al.,* 2005). Algae play an important role in maintaining the equilibrium of aquatic ecosystem (Campanella *et al.,* 2000). They are often used in phytotoxicity test for environmental monitoring (Boswell *et al.,* 2002). Nickel is known to be accumulated by algae (Skarr *et al.,* 1974; Trollope and Evans, 1976) and its deleterious effect on phytoplankton community has been well established (Stratton and Corke, 1979; Rai and Raizada, 1985, 1986; Rai *et al.,* 1981, 2000) along with myriad of different types of laboratory studies (Singh *et al.,* 1999; Rangsayatorn *et al.,* 2002). Blue green algae (BGA) are diverse group of photosynthetic prokaryotes many of which have remarkable affinity for heavy metals (Rai *et al.,* 2000) and are growing frequently in metal contaminated sites. These algae are known to fix atmospheric nitrogen and convert insoluble phosphorous into soluble phosphate (Irisarri *et al.,* 2001). They are also used as a bioremediater of heavy metal in aquatic bodies (Gamila and Nagalaa, 1999; Nakanishi *et al.,* 2004). The BGA and green alga (GA) are able to bind large amounts of metals due to the presence of mucilaginous sheath (Wang *et al.,* 1998; Tien, 2002). Although, studies pertaining to Ni stress on growth, carbon fixation, nutrient uptake and enzymatic activity have been conducted on *Anabaena doliolum* under different metal stress (Mallick and Rai, 1990), the impacts on photosynthetic pigments, protein, non-protein thiol and morphological abnormalities have not been studied under Ni stress. Therefore, the present study aimed to investigate the responces of *Anabaena doliolum* under low to high concentrations of Ni with a view to explore the phytoremediation capacity of this high biomass cyanobacterium.

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Materials and Methods

The filamentous cultures of heterocystous cyanobacteria *Anabaena doliolum* Bardwaja was obtained from Botany Department, Banaras Hindu University Varanasi. *A. doliolum* was grown in BG-11 medium (150 μmole m^{-2} s^{-1} light intensity and 14/10 hr photoperiod at 26±2°C). The pH of the medium was adjusted to 7.5 by 4 mM Tris HCl buffer. The cyanobacterium was sub cultured weekly into fresh medium and only the cultures in the logarithmic phase were used for toxicity test. Stock solution (1000 μM) of Ni was prepared by using, nickel chloride ($NiCl_2.6H_2O$) salt. Various concentrations (0.1, 1, 10, 50 and 100 μM) of Ni were prepared by diluting the stock with BG-11 medium and filtered through a millipore membrane filter (0.45 mm). The media was autoclaved before culturing BGA. The cyanobacteria was harvested after 24, 48 and 96 hr and used for the determination of various parameters. Survival and growth of the cyanobacterium was measured as cell density at 700 nm. For biomass estimation cyanobacterium were centrifuged at 10,000× g for 10 min, pelleted and blotted to remove excess water and biomass was taken on fresh weight basis (fresh weight) using a Sartorius electronic

Table - 1: Effect of Ni on biomass and cell density of *Anabaena doliolum* during different time duration

Ni (μM)	Biomass (g fresh wt.)			Cell density (at 700 nm)		
	24 hr	48 hr	96 hr	24 hr	48 hr	96 hr
00	0.376 ± 0.040^{c}	0.397 ± 0.027^{c}	0.436 ± 0.017^{a}	0.252 ± 0.053^{b}	0.357 ± 0.056^{c}	0.380 ± 0.060^{b}
0.1	0.595 ± 0.007^{a}	0.600 ± 0.020^{a}	0.398 ± 0.036^{a}	0.273 ± 0.009^{a}	0.348 ± 0.045^{c}	0.430 ± 0.021^{a}
1.0	0.516 ± 0.071^{b}	0.490 ± 0.086^{b}	0.314 ± 0.031^{a}	0.292 ± 0.016^{a}	0.393 ± 0.063^{b}	0.473 ± 0.047^{a}
10	0.507 ± 0.010^{b}	0.419 ± 0.028^{b}	0.071 ± 0.014^{b}	0.300 ± 0.029^{a}	0.420 ± 0.045^{a}	0.444 ± 0.056^{a}
50	0.456 ± 0.016^{b}	0.226 ± 0.010^{d}	0.035 ± 0.013^{b}	0.256 ± 0.016^{b}	0.372 ± 0.010^{b}	0.462 ± 0.058^{a}
100	0.400 ± 0.022^{c}	0.217 ± 0.009^{d}	0.030 ± 0.029^{b}	0.210 ± 0.026^{c}	0.316 ± 0.026^{a}	0.462 ± 0.058^{a}

All the values are means of triplicates ± S.D. ANOVA significant at $p<0.01$, Different letters indicate significantly different values (DMRT, $p<0.05$)

Table - 2: Effect of different concentrations of Ni on heterocyst frequency, initiation of spores, and total spore frequency of *A. doliolum*

Ni (μM)	Heterocyst frequency (%)	Spore initiation (Days)	Total spore frequency (%)
00	5.6	18	90
0.1	6.6	20	50
1.0	6.6	25	60
10	8.0	26	80
50	7.3	27	65

Values are means of triplicates

balance. Heterocyst frequency was determined by counting the number of heterocyst per hundred vegetative cells in at least 20-25 healthy and equal length filaments by the formula given below.

$$\text{Heterocyst frequency (\%)} = \frac{\text{Total number of heterocyst}}{\text{Total number of vegetative cells}} \times 100$$

The spore formation was calculated by the following formulae:

$$\text{Spore frequency (\%)} = \frac{\text{Total number of spores}}{\text{Total number of vegetative cells}} \times 100$$

Chlorophyll *a* was extracted in 100% chilled methanol by taking treated and untreated algal biomass, after centrifugation at 10,000× g for 10 min at 4°C, absorbance of the supernatant was read at 665 and 663 nm on a Labomed, INC.UVD-3200 spectrophotometer and chlorophyll content was calculated following the method of Thomas *et al.* (1999). Carotenoid content in the extracts were calculated by the formula given by Duxbury and Yentsch (1956). The phycobilin pigment *vis-a-vis c*-phycocynin was estimated using the method of Bennett and Bogorad (1971), The *c*-phycocynin was extracted in distilled water through freezing and thawing of samples over night and centrifuged at 10,000x g for 10 min at 4°C. The absorbance of the supernatant was recorded at 615 and 652 nm wavelength.

For metal estimation centrifuged and pelleted cyanobacteria samples were kept in oven at 80°C for 48 hr. Dried and powered samples were weighed and digested in concentrated HNO_3: $HClO_4$ ($_{V/V}$ 3:1) at 80°C. Digested samples were analyzed on flame atomic absorption spectrophotometer (GBC Avanta). Level of protein was estimated following the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Non-protein thiols (NP-SH) were measured following the method of Ellman (1959) using glutathione as standard. The photomicrographs were taken with Nikon microscope SZ1450. All the experiments were repeated thrice taking three replicates in each case. To confirm the validity of the data ANOVA ($p<0.01$) was performed and significant differences in metal accumulation in cyanobacteria species were tested by Duncan's multiple range test (DMRT, $p\leq0.05$) following Gomez and Gomez (1984).

Results and Discussion

The effect of Ni toxicity on biomass and cell density of *Anabaena doliolum* is shown in Table 1. The biomass and cell density exhibited differential responses. Ni stimulated the biomass upto 100 μM exposure at 24 hr while the maximum biomass was observed after 48 hr at 0.1 μM. At 96 hr exposure biomass significantly decreased. The density of cell was increased up to 50 μM at 24 hr in comparison to untreated cyanobacterial cultures however the maximum level was observed at 1 μM Ni after 96 hr.

The effects of Ni toxicity on chlorophyll *a* content of *A. doliolum* against different concentration gradient have been presented in Fig. 1A. A concentration and duration dependent response was obtained under Ni stress. It increased up to 10 μM Ni exposure up to 96 hr while, higher concentrations (>10 μM) proved to be inhibitory.

Similar to the photosynthetic pigments the carotenoid content of *A. doliolum* was increased upto 10 μM after 96 hr (Fig.1B). The increase in carotenoid content could be considered as a mechanism to reduce the Ni toxicity through quenching free oxygen radicals. Similar observation has been reported by Mallick and Rai (1990) under Ni stress.

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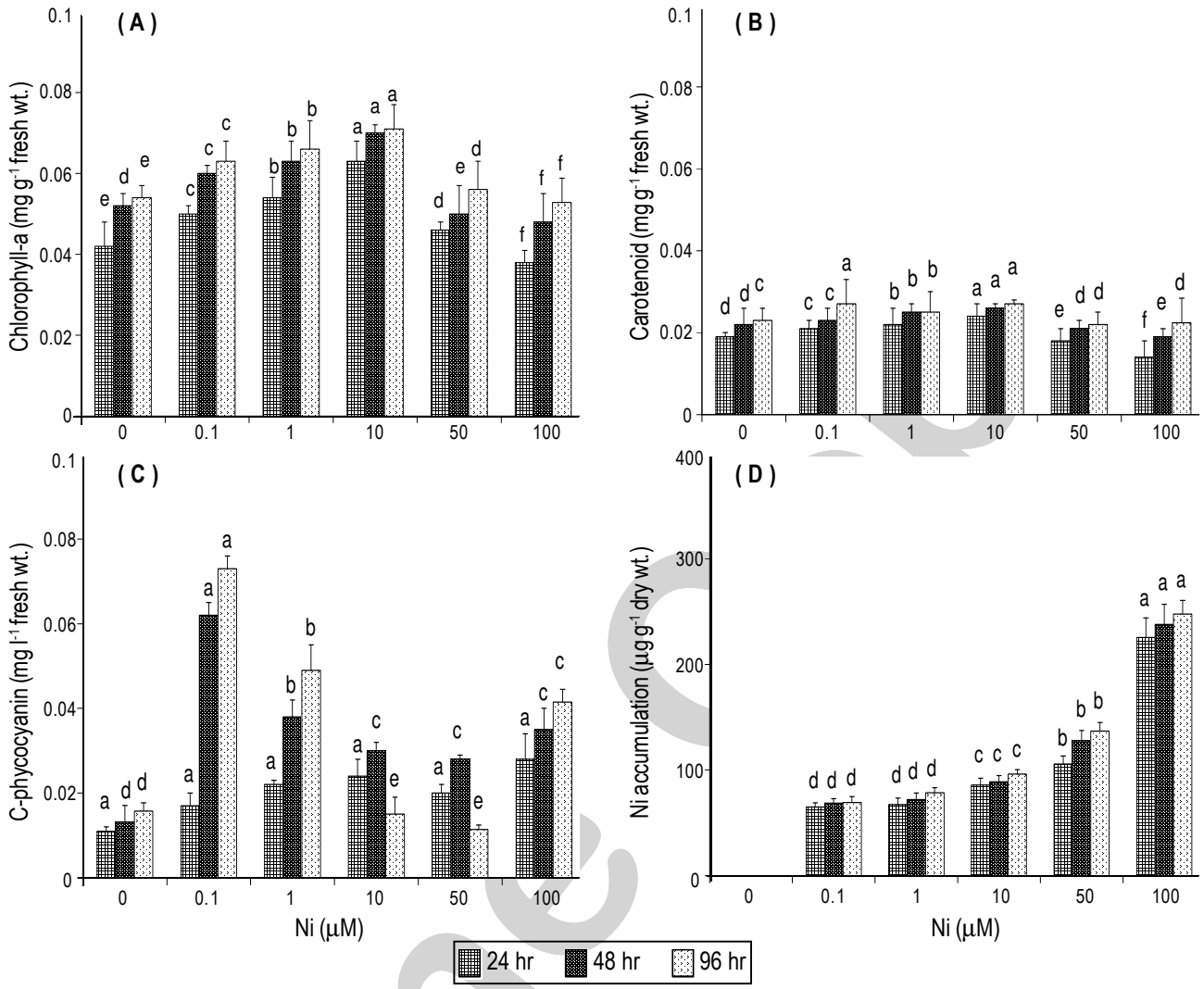

Fig. 1: Effect of Ni concentration on *A. doliolum* during exposure period: (A) Chlorophyll a, (B) Carotenoid, (C) C-phycocyanin and (D) Ni accumulation. All the values are mean of triplicate ± SD. ANOVA significant at $p<0.01$. Different letters indicate significantly different values (DMRT, $p<0.05$)

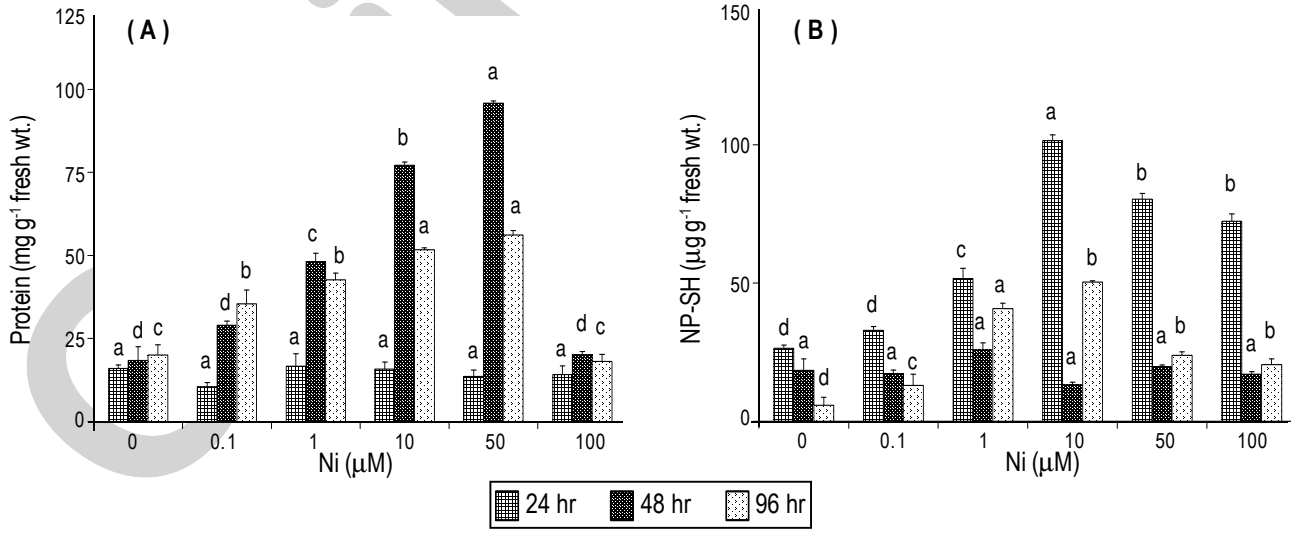

Fig. 2: Effect of Ni concentration on *A. doliolum* during exposure period: (A) Protein (B) NP-SH. All the values are mean of triplicate ±SD. ANOVA significant at $p<0.01$. Different letters indicate significantly different values (DMRT, $p<0.05$)

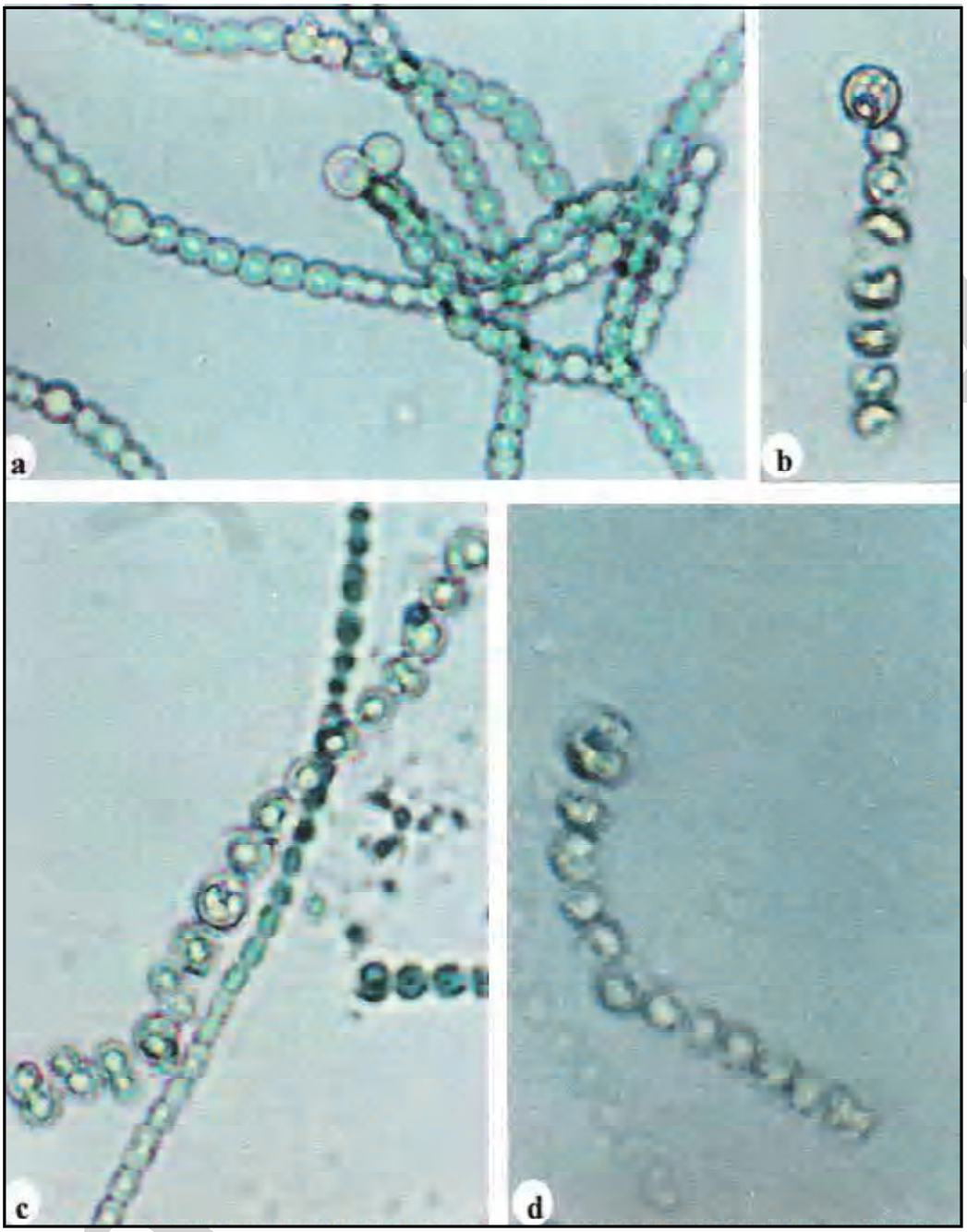

Fig. 3: Electron microscopic structure of *A. doliolum* (a) Normal filament of *A. doliolum,* (b) Enlarged heterocyst and spores of *A. doliolum* exposed to 10 μM Ni, (c) Spores and vegetative cells of *A. doliolum* exposed to 1 μM Ni after 25 days exposure, (d) Enlarge size of heterocyst of *A. doliolum* exposed to 50 μM Ni. H= Heterocyst; V= Vegetative cells; S= Spores

The effect of different concentration of Ni on *c*-phycocyanin of *A. doliolum* is shown in Fig. 1C. The level of *c*-phycocyanin increased up to 1 μM, till 96 hr while upto 50 μM, till 48 hr, in comparison to control. At higher concentration and duration *i.e.* 50 μM, till 96 hr the level of *c*-phycocyanin decreased, however, interestingly at 100 μl concentration its level increased at all durations.

The amount of Ni accumulated by *A. doliolum* at different concentration is shown in Fig. 1D. The Ni accumulation in the cyanobacterium is increased in a concentration and duration dependent manner throughout the experiment. The cyanobacterium accumulated 247.6 μg g^{-1} dry wt. Ni after 96 hr of growth at 100 μM Ni concentration.

The effect of different concentration of Ni on protein content of *A. doliolum* is shown in Fig. 2A. The protein content of the cyanobacterium increased in concentration and duration dependent manner up to 50 μM till 96 hr then declined, however it did not declined below control levels at any exposure concentration and duration. The induction in protein may be due to the synthesis of metal binding proteins, the metallothioneines helping the cyanobacterium in chelation of toxic Ni ions. The increase in protein content might also be attributed to increased nitrogen fixation due to increased heterocyst frequency. Similar increase in protein content is also observed by Lim *et al.* (2006). The effect of different concentration of Ni on NP-SH is shown in Fig. 2B. NP-SH content of cyanobacterium was found stimulatory at all the concentration and time duration. The maximum level of NP-SH was observed at 24 hr with highest level being 101.55 μg g^{-1} fresh wt. at 10 μM concentration.

The results obtained during present observation indicate that lower concentration of Ni can stimulate growth of cyanobacteria by acting as growth promoter and may increase nitrogen fixation with increase in heterocyst frequency. However, some times Ni may delay the sporulation there by checking growth and related process of cyanobacterium.

The effect of different Ni concentrations on heterocyst frequency and spore initiation has been demonstrated in Table 2. The spore formation was initiated after 18, 20, 25, 26 and 27 days upon various Ni exposures. The data revealed that the, heterocyst frequency and sporulation in cyanobacterium was affected differently. The heterocyst frequency of the cyanobacterium increased with increasing Ni concentration upto 50 μM. However, higher concentrations were found inhibitory showing complete death of the cyanobacterium with no sign of heterocyst. Such results could be ascribed either due to the inhibited synthesis of proteins involved in heterocyst differentiation or due to degradation of pre-existing heterocyst. Time of sporulation was extended from 18 days to 26 days upon Ni exposures. Fig. 3 shows the photomicrograph of *A. doliolum* growing under control condition and in the medium supplemented with different concentration of Ni. Fig. 3a shows the exponentially growing filaments, showing vegetative cells and heterocyst. While Fig. 3b shows enlarged heterocyst and spores at 10 μM Ni concentration. It is interesting to note that Ni concentration of 1 μM resulted into 50% vegetative cells and rest cells were converted into spores after 25 days (an extended period for sporulation, Fig. 3c). High concentrations of Ni (50 μM) resulted into transformation of all the vegetative cells into spores. Large size of heterocyst was observed during the study of Fig. 3d. Such an observation could be taken as an adaptive strategy to ambient toxicity during Ni stress. Enhancement of growth could be linked with increase in heterocyst frequencies. Heterocyst is actual site of N_2 fixation under aerobic condition (Stewart *et al.,* 1969) and spores are generally formed under adverse conditions. The increase in heterocyst frequency may be due to impaired N_2 fixation, due to the lack of functional nitrogenase enzyme (Orus and Marco, 1991). Thus the cyanobacterium *A. doliolum* exhibits a varied biochemical response against different concentration of Ni toxicity.

It may be concluded from the present study that the exponentially growing cells of *A. doliolum* were able to tolerate Ni up to 10 μM concentration probably through production of thiolic peptides and metal binding proteins. The cyanobacterium may be used as a phytoremediation tool for the removal of Ni from the moderately contaminated waste water. As it is a high biomass producing strain it can be conveniently separated from the solution by filtration and can be used at pilot scale.

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