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Study of Physiological and Biochemical Alterations in Cyanobacterium under Organic Stress

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ABSTRACT

Combining nitrogen fixation with other bacterial metabolic capabilities to convert the waste carbon into useful raw materials would be a highly desirable further enhancement to the process. To study the possibility of further enhancement of the value proposition of waste remediation from cost-neutrality to profitability, we can use nitrogen-fixing or non nitrogen fixing cyanobacteria that also naturally accumulate effluent from remediating materials. A filamentous heterocystous cyanobacterium, *Nostoc muscorum,* unicellular-non heterocystous cyanobacterium, *Synechococcus* PCC 7942, filamentous non heterocystous cyanobacterium, *Spirulina platensis* and heterocystous filamentous cyanobacterium *Anabaena cylindrica,* have the ability to degrade industrial pollutants such as benzene, toluene and xylene. We have done the growth measurements and estimated the amount of pigments like chlorophyll-a, carotenoids, phycocyanin. We also have studied the accumulation of total peroxide radicals and lipid peroxidation due to accumulation of MDA and stress busters such as ascorbate, proline, glutathione etc under all the above stress. Total antioxidant activity of cyanobacteria is measured by TBARS assay. We have also studied the enzymes involved in the antioxidant mechanism like superoxide dismutase and peroxidase. The amount of pigments started decreasing as the concentration of the stress increased. But, the amount of ascorbate and proline and other antioxidant activities increased under stress. This suggests that cyanobacteria may be able to survive under organic stress. Biodegradation is increasingly being considered as a less expensive alternative to physical and chemical means of decomposing organic pollutants. Biosensors may be designed to indicate and estimate pollutants in natural wastes, using PS2 particles/ thylakoids/spheroplasts.

Key words: Cyanobacteria, chlorophyll a, carotenoids, proline, MDA, SOD

INTRODUCTION

Cyanobacteria, are a major group of bacteria that occur throughout the world (Al-Kahtani and Fathi, 2008). Mechanism of any adaptation in cyanobacteria has been elucidated in terms of osmoprotective compounds and maintenance of low internal contents of inorganic ions. These substances even in high concentration are compatible with cellular metabolism, it is assumed that they are able to protect macromolecules against denaturation and thus to improve their function in a cell environment of stress (Nagasathya and Thajuddin, 2008).

Cells exposed to stresses undergo changes in their metabolism in order to adapt with changes in their environment. Stress changes the morphological, physiological and biochemical responses of plants. It affects adversely growth and development (Amirjani, 2011) of cells. Antioxidants enzyme and organic osmolytes such as proline are known to occur widely in higher plants and normally accumulates in large quantities in response to environmental stresses (Heshmat, 2011). Stress alleviation in cyanobacteria has been known to be achieved through the production of stress proteins. They are known to synthesize a variety of proteins in response to various stresses (Karthikeyan and Gopalaswamy, 2009). All aerobic organisms are subject to oxygen toxicity that results from the formation of reactive intermediates such as hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and hydroxyl radical (OH^-). One of the main defensive mechanisms against these reactive intermediates is superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1), an enzyme that is ubiquitous in aerobic organisms including both eukaryotes and prokaryotes (Fridovich, 1976, Fridovich, 1979) Under stress conditions cyanobacterial pigments, i.e., chlorophyll *a*, carotenoids and phycocyanin and adversely affected. Furthermore, cells produce more peroxide radicals (Stahl and Sies, 2005). Toxicity by oxygen radicals has been suggested as a major cause of cancer, aging, heart disease and cellular injury in hepatic and extrahepatic organs (Troll and Weisner, 1985). There is compelling evidence that superoxide dismutases (SOD, EC 1$5$1$1) are essential for biological defence against the superoxide anion (Fridovich, 1983). Proline accumulates rapidly and more frequently than any other amino acid under unfavorable environmental conditions (Lutts *et al.*, 1999), especially in drought and salt stress on addition to heavy metal stress. It could be involved in stress resistance mechanisms by acting as an stressprotectant, thereby, facilitating osmoregulation, protection of enzymes and stabilization of cytosolic acidity (Saradhi and Saradhi, 1991). The role of proline is well documented in stress management (Schat *et al.*, 1997) as well as Rhizobium (Chien *et al.*, 1992; Singh *et al.*, 2001). The functions of glutathione in cyanobacterial cells have not been clarified and the enzymes and the genes involved in its biosynthesis in cyanobacteria have not been purified or cloned (Kaneko *et al.*, 1996).

MATERIALS AND METHODS

Plan of work mentioned in this study is the original idea of Dr. Shanthy sundaram and the whole work is done by Ms. Soumya K.K. under the guidance of Dr. Shanthy Sundaram at the Centre for Biotechnology, University of Allahabad, Allahabad, India during her thesis work 2007-2009.

Test organism and culture conditions: The cyanobacterium *Nostoc muscorum*, maintained in BG-11 medium, (Hughes *et al.*, 1958) and modified by Stanier and Cohen-Bazire (1977). *Synechococcus* PCC7942 is grown in BG-11 medium with nitrogen source sodium nitrate with pH 7.5 but *Anabaena cylindrica* was grown in Allen-Arnon = s media, Allen (1968) and *Spirulina platensis* was grown in Zaurouk=s medium (Vonshak, 1997) with pH 7.9 at 24+2°C under 72 Fmol photon m^{-2} sec^{-1} Photosynthetically Active Radiation (PAR) with a photoperiod of 14:10 h (light:dark). The cultures were shaken periodically were obtained from a the Algae Laboratoy, Botany Department, University of Allahabad, Allahabad.

Measurement of survival and growth: To measure the survival, cyanobacterial cells were treated with different concentrations of Benzene (300 FM), Toluene (300 FM), Xylene (300 FM),

pNP (400 FM). The lethal doses were determined against the control. Growth was estimated by using the equation suggested by Myers and Kratz (1955).

Estimation of chlorophyll a: Growth was also measured by extracting total chlorophyll a of the culture. The methanol extracted supernatant was estimated for cellular chlorophyll a by employing the standard extinction coefficient (13.42) for a solution of 1 mg mL^{-1} of chlorophyll a (Mackinney, 1941).

Estimation of carotenoids: For estimation of Carotenoids 96% acetone was used as a solvent (Hellebust and Craige, 1978). Absorbance of acetone extract was taken using 96% acetone as blank at 460 nm by UV Spectrophotometer.

Organic compounds induced alterations in antioxidant compounds: Organic compounds induced alterations in antioxidant status of *in vitro* cultured cells were experimented and observed. The following are the key parameters that have been studied, based on the status of antioxidant compounds and enzymes.

The treatment with organic compounds: Acclimated cells were obtained by successive cultivation (4-5 times) at increasing doses of BTEX and pNP upto 300 FM (in aqueous form) as described by Attaway and Schmidt (2002). Hereafter, this strain is referred to as the acclimated strain and the other as control strain.

Determination of ascorbate: Ascorbate was extracted from the pellets of 1 mL test samples with 5% w/v sulfosalicylic acid and the amount of ascorbate was determined in the supernatant obtained after centrifugation at 15,000 rpm for 10 min, using the method given by Oser (1979) and expressed as micromol ascorbate mg^{-1} protein.

Estimation of total proline content: Proline content in the cyanobacterial homogenate was measured according to Bates *et al.* (1973). The proline level is expressed as nmol mg^{-1} protein.

Estimation of Reactive Oxygen species (ROS): Total Peroxide radicals: Total amount of hydrogen peroxide radicals was estimated by Shonosuke (1976). The red color was measured at 480 nm.

Lipid peroxidation (MDA): The MDA concentration was estimated by the method of Heath and Packer (1968). MDA contents was determined using the coefficient.

Estimation of total antioxidant activity in cyanobacteria under stress

Total antioxidant activity: The assay of thiobarbituric acid reactive substances TBARS is summarized (Ohkawa *et al.*, 1979). Pink colored upper organic layer was removed for measurement of OD at 532 nm.

Determination of total glutathione (GSH): The total glutathione (GSH+2XGSSG) was measured by a modified method of Tietze (1969). The change in the optical density was followed at 412 nm and 25°C.

Assay of antioxidant enzymes

Superoxide dismutase activity: SOD (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium chloride (NBT) according to the method of Giannopolitis and Ries (1977) using a reaction mixture (3 mL). The blue color was measured at 560 nm.

Peroxidase activity: Peroxidase (EC 1.11.1.7) activity in a reaction mixture (3 mL) containing 16 mM H_2O_2, 10 mM pyrogallol and crude extract (650 Fg protein mL^{-1}) was determined spectrophotometrically according to the method of Gahagen *et al.* (1968) and the activity was measured as increase in optical density at 430 nm.

Statistical analysis: Data were statistically analyzed and the results were expressed as means ("SE) of 3 independent replicates.

RESULTS

Growth behavior of *Nostoc muscorum* under BTEX and pNP stress

Specific growth rate of cyanobacteria under organic stress: A continuous decline in the growth of control and acclimated cyanobacteria under different stresses, viz. Benzene, Toluene, Xylene and p-Nitrophenol, is showed in Fig. 1. Percentage of specific growth reduction and specific growth rate (Fig. 1) is also calculated.

The *Nostoc muscorum* cells registered appreciable (18.2%) reduction in specific growth rate at 200 μM Benzene, 36.6% reduction in 300 μM while growth inhibition by 200 μM Toluene (27.08%), 300 μM Toluene (36.37%) and 300 μM Xylene cells showed higher reduction 90.91% than 200 μM Toluene 63.64% and pNP reduction was mild. The 300 μM pNP showed 20.01% and 27.28% in 400 μM concentrations.

In *Synechococcus* PCC 7942, Xylene affected the most (Fig. 1). 200 μM showed 76.93% and 86.54% in 300 μM Xylene respectively while in 200 μM Benzene, 20.2% and in 300 μM Benzene showed 23.08%. Toluene also affected the cells. The specific growth rate in 200 μM toluene treated cells were higher (80.77%) than the 300 μM Toluene (75.96%). Para Nitrophenol showed mild effects. In 200 μM p-NP the reduction in growth was only 5.77% and 300 μM showed 33.46%.

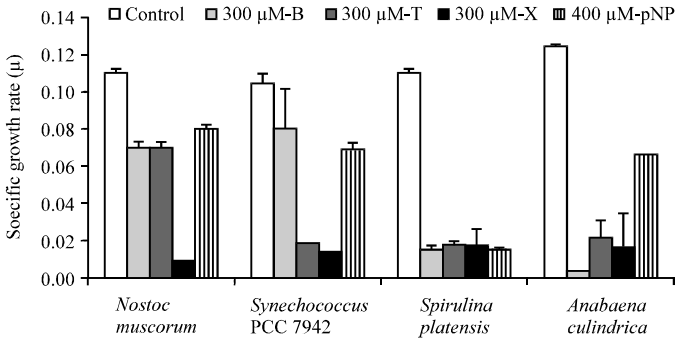

Fig. 1: The effect of Benzene, Toluene, Xylene and para Nitrophenol concentration on specific growth behavior in Cyanobacteria under organic stress. Error bars indicate SD of three replicates. p-value is lesser than 0.05

In *Spirulina platensis* higher concentration of p-NP affected the most. Benzene 200 μM concentration treatment showed 63.64% percentage of growth reduction and 300 μM Benzene showed 85.46%. The 200 μM Toluene showed 83.75% growth reduction and 83.64% in 300 μM Toluene. 10.91% of specific growth reduction was showed by 200 μM Xylene and 83.64% in 300 μM. In 200 μM the specific growth rate was 20.01 and 86.37% in 300 μM.

The higher concentration of Benzene affected the growth a lot in *Anabaena cylindrica* that is 96.94% in 300 μM Benzene and in 200 μM showed 43.55%. The 200 μM Toluene showed 36.3% and 300 μM showed 82.26%. Xylene also affected the growth of *A. cylindrica*. The 200 μM Xylene showed 39.52% and 300 μM Xylene showed 86.3%. p-NP showed little effect. The 200 μM p-NP showed 12.1% and 300 μM showed 46.78% specific growth reduction. Percentage of growth reduction is given in the Fig. 1.

Photosynthetic pigment analysis: Pigments were isolated from all the stress given in the growth. But the data is given only of the stress used for the experiments that is 300 FM concentrations of BTX and 400FM concentrations of pNP.

Chlorophyll a of *Nostoc muscorum* markedly reduced at doses of Benzene, Toluene, Xylene and para- Nitrophenol (Fig. 2a). The control showed 3.68 to 4.6 mg mL^{-1} Chl a under stress during the experiment. But the stressed cells showed 3.46 to 0.22 mg mL^{-1} Chl a in benzene, 3.32 to 0.47 mg mL^{-1} Chl a in Toluene and 3.5 to 0.09 mg mL^{-1} Chl a in Xylene. In para Nitrophenol the chl a was 3.84 to 2.82 mg mL^{-1} Chl a. The cell death was not found in p-NP. While, in the others, the stress was lethal to the pigments.

The inhibition rate of chl a was high on the 48th h of incubation. BTX showed 95, 88 and 98% reduction in chlorophyll a on the 168th h and pNP showed 31% of reduction. Figure 2a shows the amount of chlorophyll a isolated upto 168th h. The amount of Chlorophyll a of *Synechococcus* PCC. The 7942 markedly reduced at the doses of Benzene, Toluene, Xylene and para-Nitrophenol (Fig. 3a). Chl a was isolated continuously for 7 days in triplicates. The control showed increase in the amount by 3.24 to 4.02 mg mL^{-1} chl a. The benzene treated cells showed 3.08 to 0.76 mg mL^{-1} chl a, Toluene treated show 3.52 to. 68 mg mL^{-1} chl a, Xylene showed 3.37 to 1.01 mg mL^{-1} chl a, respectively. The pNP showed less reduction in chl a. It showed 3.41 to 3.93 mg mL^{-1} chl a.

The inhibition rate in *Synechococcus* PCC 7942 was high on the 48th h of incubation. The BTX showed 90, 98.8 and 86 reduction in chlorophyll a on the 481th h and pNP showed 4% of reduction. The Fig. 3a showed the amount of chlorophyll a isolated upto 168th h. The parentheses showed the decrease of chlorophyll a under organic stress.

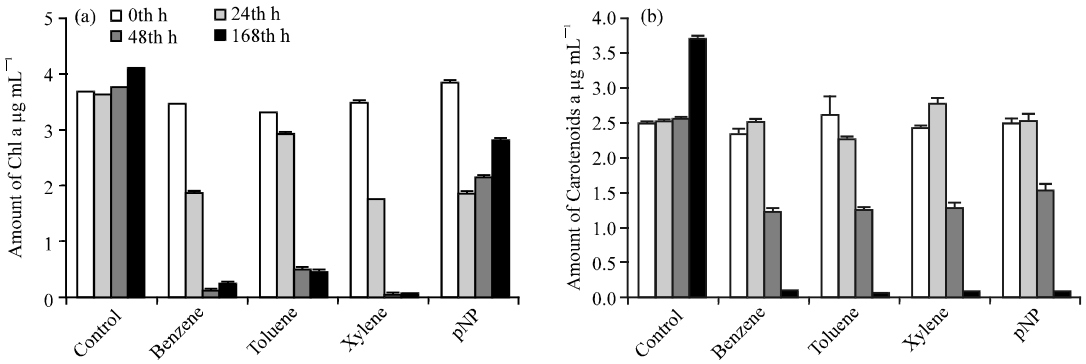

Fig. 2: Effects of Benzene, Toluene, Xylene and p-Nitrophenol treated *Nostoc muscorum* on (a) Chlorophyll-a and (b) carotenoids. Error bars indicate SD of three replicates

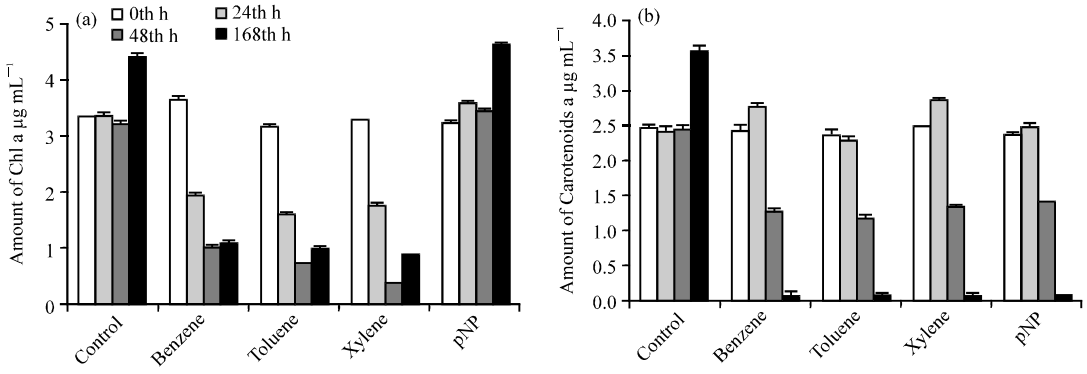

Fig. 3: Effects of Benzene, Toluene, Xylene and p-Nitrophenol treated *Synechococcus* PCC.7942 on (a) Chlorophyll-a and (b) Carotenoids. Error bars indicate SD of three replicates

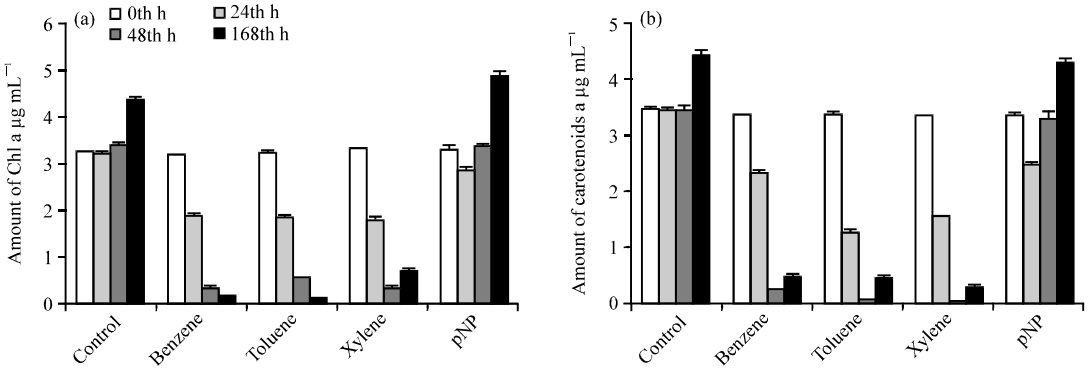

Fig. 4: Effects of Benzene, Toluene, Xylene and p-Nitrophenol treated *Spirulina platensis* on (a) Chlorophyll-a and (b)Carotenoids. Error bars indicate SD of three replicates

Chlorophyll a of *Spirulina platensis* showed significant difference in the amount of chl a during all the doses of Benzene, Toluene, Xylene and para-Nitrophenol (Fig. 4a). In the control chlorophyll was increased by 3.23 to 4.37 mg mL^{-1} Chl a. But the stressed cells showed 3.19 to 0.14 mg mL^{-1} Chl a in benzene, 3.2 to 0.09 mg mL^{-1} Chl a in Toluene and 3.302 to 0.68 mg mL^{-1} Chl a in Xylene. In para Nitrophenol the chl a was 3.2 mg mL^{-1} Chl a on the 0th day and on the seventh day it was 4.87 mg mL^{-1} Chl a. The cell death was not found in p-NP and the chlorophyll a amount was increased than the control. While others the stress was inhibited the pigments.

In *Spirulina platensis* the inhibition rate was high on the 168th h of incubation. BTEX showed 96.8, 98 and 84.4% reduction in chlorophyll a on the 168th h and pNP showed 11% of increase in chlorophyll a. The Fig. 4a shows the amount of chlorophyll a isolated upto 168th h.

In *Anabaena cylindrica,* chlorophyll a showed significant change in the amount of chlorophyll a during all the seven days, at the doses of Benzene, Toluene, Xylene and para-Nitrophenol (Fig. 5a). In the control chlorophyll was increased by 3.31 to 4.37 mg mL^{-1} Chl a. But the stressed cells showed 3.36 to 1.05 mg mL^{-1} Chl a in benzene, 3.15 to 0.95 mg mL^{-1} Chl a in Toluene and 3.26 to 0.85 mg mL^{-1} Chl a in Xylene. In para Nitrophenol the chl a was 3.22 mg mL^{-1} Chl a on the 0th day and on the seventh day it was 4.62 mg mL^{-1} Chl a. No cell death was found in p-NP and the chlorophyll a amount was increased than the control from the 24th h itself. While, in others, stress inhibited the pigments.

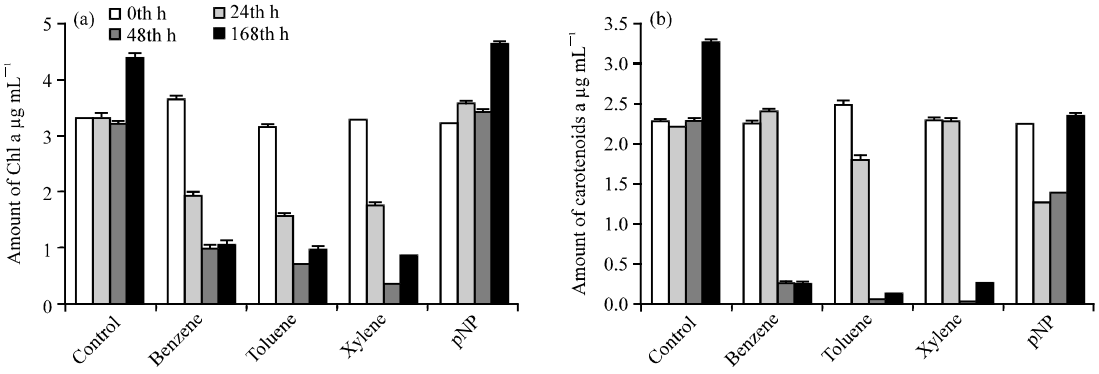

Fig. 5: (a, b) Effects of Benzene, Toluene, Xylene and p-Nitrophenol treated *Anabaena cylindrica* on Chlorophyll-a. Error bars indicate SD of three replicates

The inhibitory impact of different stresses on *Anabaena cylindrica* chlorophyll and was comparatively high in all the stress. The inhibition rate was high on the 48th h of incubation. BTEX showed 70, 79 and 90% reduction in chlorophyll a on the 48th h and pNP showed 7% of increase in chlorophyll a. The Fig. 5a shows the amount of chlorophyll a isolated upto 168th h. The parenthesis shows the decrease of chlorophyll a under organic stress.

Carotenoids of *Nostoc muscorum* showed significantly different than the chlorophyll a. The amount of Carotenoids were high in the 24th h and gone lesser thereafter. The amount of amount of carotenoids during all the seven day during the stresses of Benzene, Toluene, Xylene and para- Nitrophenol are showed (Fig. 2b). In the control carotenoids were increased by 2.51 mg mL^{-1} carotenoids to 3.68 mg mL^{-1} carotenoids. But the stressed cells showed 2.3 to 0.09 mg mL^{-1} carotenoids in benzene, 2.5 mg mL^{-1} carotenoids to 0.04 mg mL^{-1} carotenoids in Toluene and 2.4 mg mL^{-1} carotenoids to 0.68 mg mL^{-1} in Xylene. In para Nitrophenol the amount of carotenoids was 2.48 mg mL^{-1} carotenoids on the 0th day and on the seventh day it was 0.07 mg mL^{-1} carotenoids. The high amount of carotenoids was got on the 24th h of incubation on *Nostoc muscorum*. In benzene, Xylene and p-NP it was more than control. Later the BTX caused degradation of carotenoids as showed in the Fig. 2b. It showed 97.6, 98.9 and 98% reduction, respectively in carotenoids. On the 168th h pNP showed 98% of inhibition in carotenoids.

In *Synechococcus PCC.7942* carotenoids showed significantly different than the chlorophyll a. Carotenoids were higher the most in the 24th h and got degraded thereafter. The amount of carotenoids during all the seven day during the stresses of Benzene, Toluene, Xylene and para-Nitrophenol are measured (Fig. 3b). In the control, carotenoids were increased by 2.44 mg mL^{-1} carotenoids to 3.54 mg mL^{-1} carotenoids. But the stressed cells showed 2.42 to 0.07 mg mL^{-1} carotenoids in benzene, 2.37 to 0.08 mg mL^{-1} carotenoids in Toluene and 2.48 mg mL^{-1} carotenoids to 0.08 mg mL^{-1} in Xylene. In para Nitrophenol the amount of carotenoids was 2.35 mg mL^{-1} carotenoids on the 0th day and on the seventh day it was 0.08 mg mL^{-1} carotenoids. The higher amount of carotenoids was got on the 24th h of incubation of *Synechococcus* PCC7942. In benzene, Xylene and p-NP it was more than control. BTX caused degradation of carotenoids as showed in the Fig. 3b and it showed 98, 98 and 98% reduction respectively in carotenoids. By 168th h pNP showed 98% of inhibition in *Synechococcus* PCC7942.

In *Spirulina platensis* carotenoids was high in the 24th h but it was lesser than *N. muscorum* and *S. elongatus* PCC 7942 and got degraded thereafter. Control species showed comparatively

higher amount than the other species. The amount of carotenoids during all the seven day during the stresses of Benzene, Toluene, Xylene and para-Nitrophenol are measured (Fig. 4b). In the control, carotenoids were increased by 3.46 to 4.42 mg mL^{-1} carotenoids. But the stressed cells showed 3.35 to 0.46 mg mL^{-1} carotenoids in benzene, 3.35 to 0.45 mg mL^{-1} carotenoids in Toluene and 3.35 mg mL^{-1} carotenoids to 0.27 mg mL^{-1} in Xylene. In para Nitrophenol the amount of carotenoids was 3.36 mg mL^{-1} carotenoids on the 0th day and on the seventh day it was 4.3 mg mL^{-1} carotenoids. The lower amount of carotenoids was got on the 168th h of incubation. In p-NP carotenoids were mildly affected. BTX caused degradation of carotenoids as showed in the Fig. 4b. It showed 90, 90 and 94% reduction, respectively in carotenoids. After the 168th h pNP showed 3% of inhibition.

Anabaena cylindrica carotenoids was high in the 24th h and got degraded thereafter. The amount of carotenoids during all the seven day during the stresses of Benzene, Toluene, Xylene and para-Nitrophenol are measured (Fig. 5b). In the control, carotenoids were increased by 2.26 to 3.24 mg mL^{-1} carotenoids. But the stressed cells showed 2.24 to 0.24 mg mL^{-1} carotenoids in benzene, 2.46 to 0.13 mg mL^{-1} carotenoids in Toluene and 2.28 mg mL^{-1} carotenoids to 0.26 mg mL^{-1} in Xylene. In para Nitrophenol the amount of carotenoids was 2.22 mg mL^{-1} carotenoids on the 0th day and on the seventh day it was 2.34 mg mL^{-1} carotenoids. The inhibitory impact of different stresses on carotenoids was comparatively higher than the chlorophylls in BTEX. p-NP showed decreased in carotenoids after incubation. The lower amount of carotenoids was got on the 168th h of incubation. In p-NP carotenoids were mildly affected. BTX caused degradation of carotenoids as showed in the Fig. 5b and it is 92.6, 96 and 92% reduction, respectively in carotenoids on the 168th h. pNP showed 28% of inhibition. The Fig. 5b shows the amount of carotenoids isolated upto 168th h.

Antioxidant studies

Accumulation of ascorbate under organic stress: To investigate the regulation of ascorbate metabolism under stressful conditions we measured it in contrast to defense compound or surface protectant, antioxidant low molecular compound ascorbate declined (12%) only in xylene treated *N. muscorum* cells. In benzene treated cells showed an increase of 47% and toluene and p-NP showed 29 and 7% increase in ascorbate accumulation in cells according to the control. In *Synechococcus* PCC 7942 the increased accumulation of ascorbate was found in the p-NP treated cells (111%) and benzene (33%). Inhibition of accumulation of ascorbate was found in toluene (43%) and xylene (20%). According to the controls *S. platensis* showed maximum accumulation in toluen (95%) treated cells and in p-NP (34%). Benzene and Xylene inhibited the accumulation by 14 and 9%, respectively. In *A. cylindrica* 103% increase of ascorbate accumulation was found in benzene and 53% was found in pNP. Toluene (47%) and Xylene (27%) showed inhibited accumulation see (Fig. 6a, b).

Accumulation of proline under organic stress: The proline content increased drastically under organic stress conditions (Fig. 6a). It was maximum in presence of toluene (357%) and xylene (314%) in *Nostoc muscorum* and benezene showed 142% and p-NP showed 100% increase in accumulation of proline after 24 h incubation of organic stress. In all studied strains, proline content was higher than control. *Synechococcus* PCC7942 showed increase of 89, 644, 578% and 200% increase under BTX and p-NP stress. In *Spirulina platensis* the accumulation of proline was highest in Xylene (244%) and others showed 96, 196 and 172% increase. *Anabaena cylindrica* showed 363% increase of proline in toluene treated cells and others showed 85, 116 and 98% of increase in accumulation as given in the Fig. 6a.

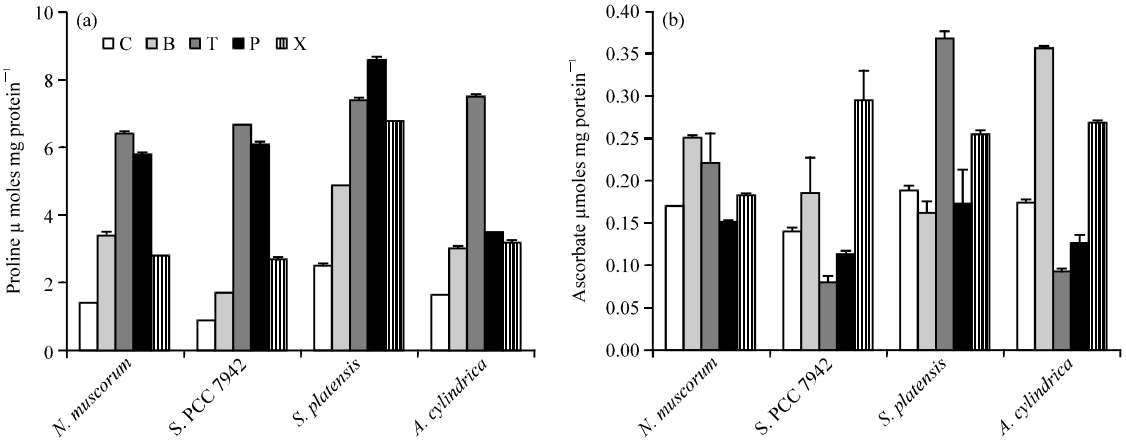

Fig. 6: (a, b) Accumulation of ascorbate and proline in cyanobacteria under organic stress

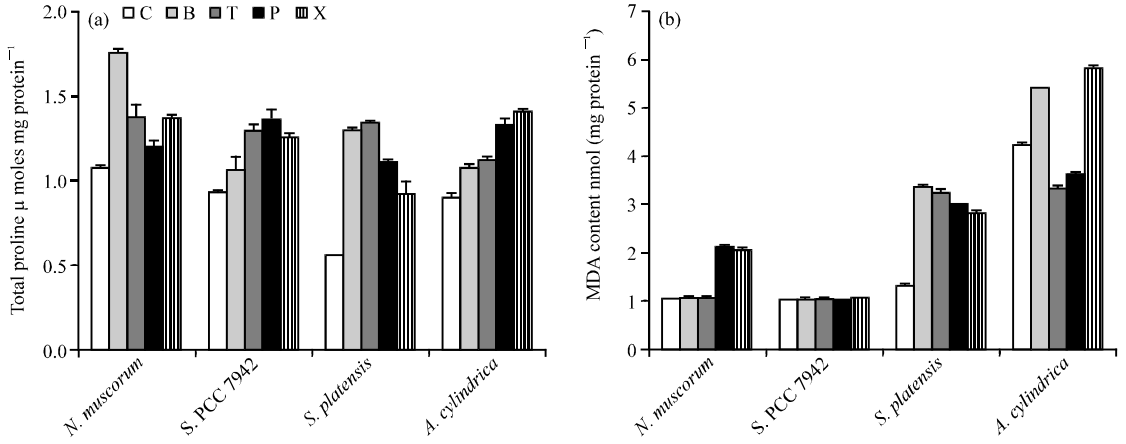

Fig. 7: (a, b)Total peroxide and Lipid peroxidation or MDA accumulation under organic stress in cyanobacteria

Accumulation of total peroxide radicals under organic stress: Total peroxide radicals are formed in resistance under stress see Fig. 7a. In *N.muscorum,* benzene treated cells showed the highest hydrogen peroxide molecules. In toluene, Xylene and p-NP showed 28, 12 and 28% production. In *Synechococcus* PCC 7942 the production of hydrogen peroxide radicals was 14, 40, 47 and 36 in BTX and p-NP, respectively. Out of the four species *S. platensis* showed the highest peroxide radicals and it was 132% in benzene treated, 140% in toluene treated and 99% in Xylene. P-NP showed 65% increased total peroxide. In *A.cylindrica* p-NP showed 55% of increased production of hydrogen peroxide radicals. BTX showed 19, 23 and 47%.

Lipid peroxidation: Lipid peroxidation (estimated as malondialdehyde) and a total peroxide radical accumulation was used as reliable markers of oxidative stress (Halliwell and Gutteridge, 1999) and was measured under organic stress in four cyanobacteria. Organic stress induced the formation of MDA indicating enhanced lipid peroxidation in cyanobacteria (Fig. 7b). Content of MDA increased approximately by 105 and 99% in Xylene and p-NP treated cells in *N. muscorum*.

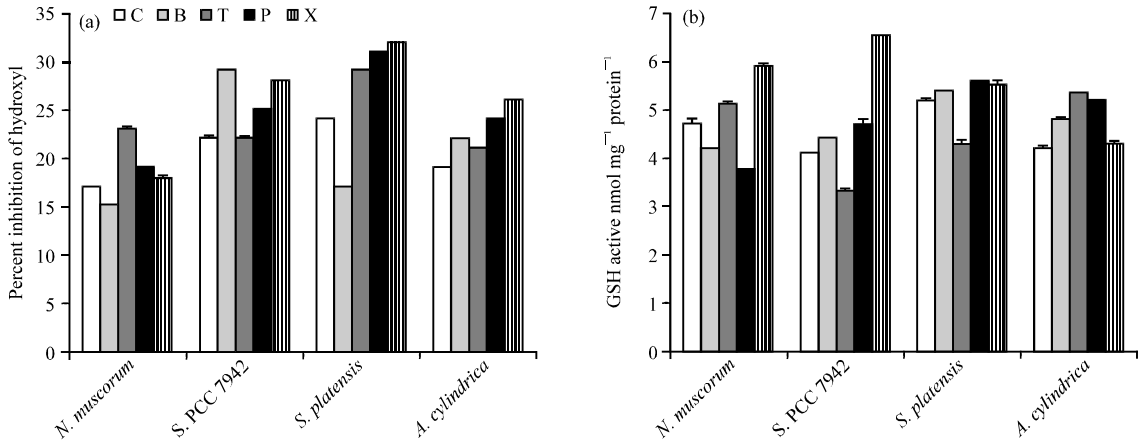

Fig. 8: (a,b)Percent inhibition of hydroxyl free radicals and glutothione accumulation under organic stress in cyanobacteria

Benzene and toluene treated cells showed only a 4 and 2% increase. *Synechococcus* PCC 7942 showed the lowest lipid peroxidation. It showed 0.39, 0.49, 1 and 3% in BTX and p-PNP. *Spirulina platensis* showed the highest MDA production showed 152, 242, 126 and 112% in BTX and p-NP respectively. In *A. cylindrica* enhanced lipid production was found in only benzene (28%) and p-NP (38%). Toluene (22%) and Xylene (15%) showed decreased MDA production see Fig. 7b.

Percent inhibition of hydroxyl free radicals: The free hydroxyl radicals react with the deoxyribose to produce deoxyribose propanol which reacts with thiobarbituric acid and forms red-colored complex. The amount of colored product is estimated by measuring the absorbance of visible light by the sample. Percent inhibition of hydroxyl radicals showed the antioxidant activity of cyanobacteria under organic stress see Fig. 8a. In *N. muscorum* toluene, Xylene and p-NP treated cells showed 35, 12 and 6% increase in inhibition of hydroxyl free radicals while benzene treated cells showed 12% decrease. *Synechococcus* PCC 7942 also showed increase in percent inhibition of hydroxyl free radicals. It showed 31, 14 and 27% in benzene, Xylene and p-NP treated cells, respectively. In toluene it was same as the control. *Spirulina platensis* showed increase in percent inhibition of hydroxyl free radicals in toluene (21%), Xylene (29%) and p-NP (33%). Benzene showed 30% decrease in percent inhibition of hydroxyl free radicals. In *A.cylindrica* all the organic treated cells showed increase in percent inhibition of hydroxyl free radicals, like benzene showed 16%, toluene 11%, Xylene showed 26% and p-NP showed 37% p-NP.

Glutathione: Glutathione activity also increased under organic stress. In *N. muscorum,* benzene (11%) and xylene (20%) showed decrease in glutathione accumulation. But toluene (8%) and p-NP (24%) showed increase in glutathione accumulation. In *Synechococcus* PCC 7942 showed decrease in glutathione in benzene (20%) and xylene (32%). In toluene (35%) and p-NP (35%) showed increase in glutathione activity. In *S. platensis* benzene showed 2% increase, toluene showed 84% increase and p-NP showed 46% increase in glutathione activity but Xylene treated cells showed 22% decrease in activity. See Fig. 8b for the results of glutathione activity under stress. In *A. cylindrica* we found that all the stress had improved glutathione activity compared to the controls. Benzene, toluene, Xylene and p-NP showed 14, 27, 24 and 2% of increase in glutathione activity.

Table 1: Effect of organic stress on the activity of superoxide dismutase enzyme in cyanobacteria after 24 h of treatments

	Organisms exposure (SOD unit mg protein $^{-1}$)			
Organic stress	*N. muscorum*	*Synechococcus* PCC 7942	*S. platensis*	*A. cylindrica*
Control	8.88±0.02	9.10±0.04	7.21±0.03	5.26±0.07
Benzene	8.90±0.06(0)	7.24±0.00(-20)	7.32±0.09(2)	11.80±0.07(124)
Toluene	6.13±0.00(-31)	12.33±0.00(35)	13.30±0.00(84)	10.36±0.03(97)
Xylene	10.21±0.07(15)	6.23±0.09(-32)	5.60±0.00(-22)	7.22±0.04(37)
p-NP	6.90±0.00(-93)	12.33±0.01(35)	10.52±0.00(46)	11.29±0.04(114)

Means±SE. Values in parenthesis are (%) increase. All the treatments are significantly different ($p<0.01$, $*p<0.05$) from control (Student's t-test)

Table 2: Effect of organic stress on peroxidase activity in cyanobacteria after 24 h of treatments

	Organisms exposure (Peroxidase (Change in OD 430 nm (mg protein)$^{-1}$) min^{-1})			
Organic stress	*N. muscorum*	*Synechococcus* PCC 7942	*S. platensis*	*A. cylindrica*
Control	1.23±0.02	0.986±0.00	1.16±0.00	1.72±0.00
Benzene	1.97±0.00(60)	1.22±0.03(24)	1.41±0.01(22)	1.92±0.02(12)
Toluene	1.13±0.00(-8)	1.33±0.00(35)	1.37±0.00(18)	1.46±0.03(-15)
Xylene	1.8±0.00(46)	2.71±0.00(175)	2.1±0.00(81)	1.92±0.04(12)
p-NP	2.64±0.05(115)	2.347±0.01(138)	2.52±0.00(117)	2.29±0.03(33)

Means±SE. Values in parenthesis are (%) increase. All the treatments are significantly different ($p<0.01$, $*p<0.05$) from control (Student's t-test)

Superoxide dismutase activity: In *N. muscorum* superoxide dismutase activity was increased in only xylene (15%) treated cells, benzene treated cells showed as same as the control. Toluene (31%) and p-NP (93%) showed decreased activity. *Synechococcus* PCC 7942 showed enhanced SOD activity in toluene (35%) and p-NP (35%) and benzene (20%) and Xylene (32 %) showed decreased SOD activity. In *S. platensis xylene* showed 22% decreased activity but the others showed 2, 84 and 46% enhanced SOD activity. *A. cylindrica* all the treated cells showed enhanced activity. The 124, 97, 37 and 114% enhanced SOD activity was showed by benzene, toluene xylene and para Nitrophenol respectively as shown in the Table 1.

Peroxidase activity under organic stress: The results depicted in the Table 2 showed the change in Peroxidase activity under the stress. In *N. muscorum* highest activity was found in para Nitrophenol and it showed 115%, benzene showed 60% and xylene showed 46% increased Peroxidase activity. *Synechococcus* PCC 7942 showed 24, 35, 175 and 138% enhanced activity under BTX and p-NP stress. In *S. platensis* it was 22, 18, 81 and 117% in BTX and p-NP, respectively. *A. cylindrica* showed 12% increased activity in benzene treated cells, 12 and 33% in xylene and p-NP treated cells. But, toluene showed 15% decrease in the peroxidase activity.

DISCUSSIONS

The abiotic stresses (Benzene, Toluene, Xylene and para Nitrophenol) generated significant reduction in growth and metabolic rates in all the cyanobacteria (*Nostoc muscorum*, *Synechococcus*

PCC7942, *Spirulina platensis* and *Anabaena cylindrica*) which were used. But reduction was higher in the all most all except the p-NP treated cells (Fig. 1). After 7-15 days all the treated cells started growing on the same media in addition of organic compounds. This may be because the induced changes enabled the cells to regulate alterations in physiological variables. The strains might be due to damage of the cellular constituents or inactivation of vital processes such as nutrient uptake, enzyme activities and photosynthesis as observed by Altamirano *et al.* (2000) in *Ulva rigida*.

The present study confirms the adverse effect of organic stress on pigments (Fig. 2-5) like chlorophyll a, carotenoids and phycocyanins. The reduction in chlorophyll content due to different stresses may be the result of inhibition of chlorophyll biosynthesis brought about by inhibition of α-aminolevulinic acid dehydrogenase and protochlorophyllide reductase (Ouzounidou, 1995). This increased Car content might diminish the amount of photons available for the absorption by Chl a by shadowing and thus, irradiance can act as secondary stress factor. In cyanobacteria, phycobiliproteins (PBPs) that are attached to the stromal surface of thylakoid membranes serve as the primary light-harvesting antenna for PS2. The composition and function of PBPs in cyanobacteria changed in response to stress conditions. Although, all cyanobacteria are photoautotrophic, many can utilize simple Dissolved Organic Carbon (DOC) compounds for heterotrophic growth or for mixotrophic growth in the light (Khoja and Whitton, 1971; Fogg *et al.*, 1973; Sahu and Adhikary, 1982; Al-Hasan *et al.*, 2001). All environmental stresses are affecting the production of active oxygen species in plants, causing oxidative stress (Smirnoff, 1993; Hendry, 1994; Bartosz, 1997). The balance between the production of activated oxygen species and the quenching activity of antioxidant is disturbed, which, often, results in oxidative damage (Del-Rio *et al.*, 1991; Smirnoff, 1993). Among the four major active oxygen species (superoxide radical $O^{-}{}_{2}$, hydrogen peroxide H_2O_2, hydroxyl radical OH^{-} and singlet oxygen ${}_1O^2$) H_2O_2 and the hydroxyl radical (Fig. 8) are most active, toxic and destructive (Smirnoff, 1993). Under normal circumstances, concentration of oxygen radicals remain low because of the activity of protective enzymes, including superoxide dismutase, catalase and ascorbate peroxidase (Asada, 1984) but under the stress conditions imposed by physical, chemical and biological pollutants, this balance may get disturbed, causing enhancement of detrimental processes (Kumar *et al.*, 2008).

The antioxidant activities are the mechanism of the cell to survive under the stress (Table1, 2). So the maximum activities are found in the 24th h of incubation with stress in all the four species. After 24th day these activities are found to be decreased. These active oxygen species undergo deleterious reactions and cause oxidative stress in the cellular systems (Prasad *et al.*, 2005). Thus, the deleterious effects of organic stress on four strains of cyanobacterium were correlated by estimating the rate of lipid peroxidation, superoxide radical and H_2O_2 production and status of antioxidants. Results showed in Fig. 6-8 and Table 1 and 2 demonstrate the increased antioxidant enzymes activity, superoxide radical and total peroxide content, lipid peroxidation, free hydroxyl radicals in the cells.

We hypothesize that in cyanobacteria under organic stress the universal osmoprotectant proline, small molecular weight compounds like ascorbate, glutathione, peroxide and all free radicals plays significant role in resistance development against organic stress in addition to SOD, APX, CAT antioxidant enzymes. These compounds cause severe toxicological problems and results in peroxidation of membrane lipids and general cellular oxidation. Increases in ascorbate, proline and MDA contents with organic stress are indicative of a correlation between free radical

generation and proline accumulation (Fig. 6-8). This is also in agreement with the earlier reports on *Spirulina platensis* and *Westellopis prolifica* (Choudhary *et al.*, 2007; Fatma *et al.*, 2007; Prasad *et al.*, 2005; Chris *et al.*, 2008; Kumar *et al.*, 2008).

The growth responses of these cyanobacteria to under stress showed (Fig. 1) considerable differences probably due to different degree of damage caused by it directly or indirectly on DNA, proteins and photosynthetic apparatus. Very high accumulation of cellular proline (above 100% of the total amino acid pool under stress as compared to just 5% under the normal condition) has been reported earlier in many higher plant species due toincreased synthesis and decreased degradation under variety of stress conditions such as water, salt, drought and heavy metal (Bates *et al.*, 1973; Bohnert and Jensen, 1996; Delauney and Verma, 1993). Although, the actual reason behind the accumulation of proline (presumably by way of synthesis from glutamic acid) is yet to be known, in plants or plant parts exposed to stress, it could probably be due to a decrease in the activity of electron transport system (Venekemp, 1989).

It is suggested that the enhanced total peroxide production is may be due to increased cellular metabolism rate to combat or may be produced as resultant of protection against the cells for the stress.

CONCLUSION

Nostoc muscorum, Synechococcus PCC7942, *Spirulina platensis* and *Anabaena cylindrica* have varying tolerance potential to organic compounds and the order of tolerance was almost same in all the strains. These observations are based on 3 μM mL^{-1} tested concentration where the percentage increases in absorbance growth curve. During present investigation we have successfully attempted our proposed hypothesis and achieved expected results. We found that the antioxidant enzymes were more active under stress as improved hydroxyl radical scavenging activity. Even though the organic compounds caused lethal effects the cyanobacteria survived in that stressful conditions. These antioxidant compounds may help them to survive. Oxidative stress and resistance is being imparted through antioxidant enzymes as well as compounds like proline, ascorbate, glutathione etc. Enhanced production of these compounds being produced during the stress conditions which helps in degradation of organic compounds and to combat adverse conditions and can be utilized in the cosmetic and medical industry. The metabolic characteristics of cyanobacteria in organic waste-treatment systems in order to gain a better understanding of their physiological contributions during biological waste-treatment. This in turn would provide some valuable insight into the role of cyanobacteria in the microbial community as well as their potential impact on waste-treatment efficiency.

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