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A N I N T E R N A T I O N A L J O U R N A L

Biochemical Composition of Seven Species of Cyanobacteria Isolated from Different Aquatic Habitats of Western Ghats, Southern India

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ABSTRACT

The aim of this work was to study the biochemical constituents of seven species of cyanobacteria namely, Calothrix fusca, Gloeocapsa livida, Lyngbya limnetica *and* Scytonema bohneri *isolated from Panekal sulfur spring. The species namely,* Oscillatoria acuminata *from petrochemical refinery,* O. calcuttensis *from dairy effluent and* O. foreaui *from a sewage drain located in the Western Ghats of Southern India under laboratory culture conditions. The biochemical constituents were analyzed in terms of total carbohydrates, total protein, total free amino acid, total lipid, fatty acid and mineral contents. The analysis showed that maximum amount of total carbohydrate in* S. bohneri *(28.4% dry weight) and minimum in* O. foreaui *(8.0% of dry weight). Maximum amount of total protein and total free amino acid were in* O. foreaui *(7% of dry weight).* O. calcuttensis *showed higher amount of total lipid (20% dry weight). A total of 12 types of fatty acids were detected among which lauric acid was in highest quantity in all the seven species. Among the polyunsaturated fatty acids, oleic acid was present in all the species ranging from 1.68 to 3.89%.* O. foreaui *showed highest quantities of copper, manganese, ferrous and zinc. Nickel was maximum in* S. bohneri *(11.05 $\mu g mL^{-1}$).* O. acuminata *showed highest quantity of magnesium (21.050 mg g^{-1}) and it was least in* O. foreaui *(12.812 mg g^{-1}).*

Key words: cyanobacteria, polluted waters, sulfur spring, biochemical constituents

INTRODUCTION

Cyanobacteria are one of the useful organisms widely used in food industries and in few biotechnological applications (Venkataraman and Becker, 1985; Fatma et al., 1994; Thajuddin and Subramanian, 2005; Rastogi and Sinha, 2009). They store reserve food materials which can be used as the source of pigments, lipids, vitamins, proteins and certain secondary metabolites (Tan, 2007; Cardozo et al., 2007). Cyanobacterial protein has received worldwide attention for either as food supplement or as an alternative source of food. Some species of *Anabaena, Nostoc* and *Spirulina* are consumed as food due to their high protein and fibre content (Anusuya et al., 1981; Anupama, 2000). A large number of marine nitrogen fixing cyanobacteria serve as complete aquaculture feed source due to their nutritional quality and non toxic property. They are also rich

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in vitamins and amino acids (Ciferri and Tiboni, 1985; Borowitzka, 1988). They contain significant quantity of lipids and fatty acids. Mahajan and Kamat (1995) reported that *Spirulina platensis* accumulated large amount of gamma linolenic acid. Fatty acid profile is used as an effective taxonomic tool for many species (Kruger et al., 1995; Li and Watanabe, 2004). In addition, minerals like zinc, magnesium and selenium are reported in some species (Jensen and Ginsberg, 2000). Due to their ability to accumulate heavy metals either by bioaccumulation or biosorption, cyanobacteria are useful for heavy metal removal from the polluted waters (Karna et al., 1999).

At present, effort is made on large-scale production of their metabolites for therapeutic use by culturing them under controlled laboratory conditions. In this work, the total carbohydrates, total proteins, total amino acids, total lipids, fatty acid profile and mineral content in the seven species isolated from the study area located at Western Ghats of Karnataka, Southern India were studied.

MATERIALS AND METHODS

Sampling locations

Cyanobacterial samples were collected in the Western Ghats region of Dakshina Kannada district of Karnataka, Southern India in three consecutive seasons (2003 – 04, 2004- 05, and 2005- 06). The samples were taken from the following areas: four species of cyanobacteria namely, *Calothrix fusca*, *Gloeocapsa livida*, *Lyngbya limnetica* and *Scytonema bohneri* were isolated from Panekal sulfur spring; O*scillatoria acuminata* was isolated from petrochemical effluents; O*scillatoria calcuttensis* from dairy effluents and *O. foreaui* from sewage water of a municipal drain situated at the outskirts of Mangalore city.

Isolation of cyanobacteria

Water samples were collected in five liter plastic cans. The method of isolation and purification of cyanobacteria were according to Ferris and Hirsch (1991). A sample was shaken to suspend the sediment and then triplicate aliquots were removed and diluted with 100 ml of sterile distilled water and was filtered using Millipore filters. The filters were placed onto the Petri plates containing BG-11 medium (Stanier et al., 1971). The plates were incubated for 15 days and were microscopically examined for the growth of cultures. Individual species were picked aseptically, sub-cultured in 500 mL Erlenmeyer flasks and incubated under continuous illumination (2000 lux) at 28 ± 2^0C with 14:10 h light: dark regime. The BG-11 medium without combined nitrogen source was used for the isolation and maintenance of *Calothrix fusca* and *Scytonema bohneri.* After 30 days, the cultures were harvested for the analysis of biochemical constituents.

Biochemical analysis

Total carbohydrate

For total carbohydrate, 100 mg of dried, pre-weighed cyanobacterial sample was hydrolyzed with 2.5N hydrochloric acid at 100ºC for one hour to prepare the extract. The total carbohydrate was determined according to Dubois et al. (1956). For this, 0.2 ml sample of the extract was transferred to an assay tube to which 1.0 ml of 5% phenol and 5.0 mL of concentrated sulphuric acid was added, with the tubes placed in water bath maintained at 25^0C for 30 minutes. The samples were then analysed at 490 nm using UV - visible spectrophotometer (Systronics India Ltd.) against the blank and compared with the standard glucose solution (concentration from 10 to 100 µg mL^{-1}).

Total protein

The total protein was determined according to Lowry's method (Lowry et al., 1951). The cyanobacterial proteins were precipitated by hot 6% trichloro acetic acid, extracted with 4.5 ml hot (55^0C) alkaline reagent (i.e., 2% alkaline Na_2CO_3 and $CuSO_4$ – Na - K tartarate solution) for 3 min; the filtrate was collected and the volume was made up to 5.0 ml with alkaline reagent. About 0.5 ml of Folin-Ciocalteau was added and mixed rapidly and allowed to stand for 10 min. at room temperature. After the color development, spectrophotometric readings at 750 nm were determined and compared with the results from solutions of bovine serum albumin standards in concentration from 40 to 200 µg mL^{-1}.

Total free amino acid

For amino acid analysis, 50 mg of the dried sample and 10 mL of 80% ethanol were used to prepare an extract. Total free amino acid content was estimated by Moore and Stein's ninhydrin reaction (Moore and Stein, 1948). For this, 1.0 ml of extract was transferred to the assay tube. Then,

3.8 mL of Ninhydrin reagent was added and tubes were heated in a boiling water bath for 12 min.. The samples were then analysed at 570 nm using UV - visible spectrophotometer (Systronics India Ltd.) against the blank and compared with the standard leucine solution which ranged between 10 and 100 µg mL^{-1}.

Total lipid and fatty acid

Total lipid content was evaluated using Folch's method (Folch et al., 1957) and fatty acids were determined by gas chromatography (Miller and Berger, 1985). The total lipids of the dried samples (about 100 mg) were extracted with chloroform - methanol solvent (2:1, v/v) and the filtrate was transferred to a pre-weighed bottle. It was dried in an evaporator and final weight was taken. The difference between the initial weight and final weight gave the total lipid content. The total lipids were dissolved in 1.0 ml of chloroform - methanol (2:1, v/v) for the analysis of fatty acids. Fatty acids were determined by gas chromatographic technique (Miller and Berger, 1985).

Analysis of mineral content

For the analysis of metallic elements, 0.5 g of the dried cyanobacterial mass and 5.0 ml of 1.0N nitric acid were used to prepare an extract. The mineral concentrations of the sample extract were determined by atomic absorption spectrophotometry (GBC AVANTA, Germany) as described by Poppiti and Sellers (1994).

Statistics

The results are expressed as the mean ± SD of three experiments.

RESULTS

The cellular constituents of the selected species of cyanobacteria expressed in percentage dry weight are given in Fig.1. These were 19.5, 18.0, 18.4 and 28.4% for *C. fusca, G. livida, L limnetica* and *S. bohneri* respectively; the percentage of total protein were 1.6, 1.8, 3.1 and 0.7% respectively; the total free amino acid content were between 1.7 – 5.3% and the total lipids were between 10.5 and 14.5%. Of the three species of *Oscillatoria* isolated from different effluent waters, *O. acuminata* showed 14.0% of total carbohydrate and total lipids; *O. foreaui* showed 8.0% of total carbohydrate and total lipids. The total protein was 6.9% and total amino acid content was 1.8% and 7%, respectively, of *Oscillatoria acuminata* and *O. foreaui. O. calcuttensis* isolated from dairy effluents showed 9.6, 2.2, 20.0 and 2.1% of total carbohydrate, total protein, total lipids and total free amino acids, respectively.

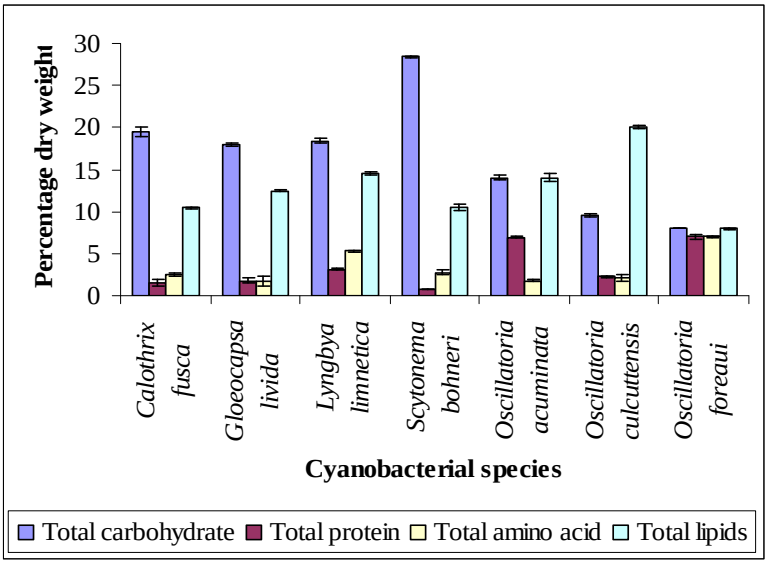

Figure 1 - Cellular constituents of cyanobacterial species isolated from different aquatic habitats expressed in % of dry weight. Bars indicate the standard deviation.

The fatty acid profile is presented in Table 1. Of the 13 different fatty acids analyzed, a total of 12 types of fatty acids were detected. These species appeared to synthesize seven types of saturated fatty acids ($C_{8:0}$, $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{20:0}$) and five types of polyunsaturated fatty acids (PUFA) ($C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$, and $C_{22:1}$) of which lauric acid ($C_{12:0}$) was present in the highest quantities in all the seven species. *G. livida* and *O. foreaui* showed high lauric acid content, i.e., 45.3 and 44.53%, respectively. The other predominant fatty acid recorded was myristic acid ($C_{14:0}$). The arachidic acid ($C_{20:0}$) and erucic acid ($C_{22:1}$) were present only in *L. limnetica* (0.25 and 0.33%, respectively). Caprylic acid ($C_{8:0}$) and capric acid ($C_{10:0}$) content was maximum in *C. fusca* but minimum in *L limnetica.* Of the polyunsaturated fatty acids, oleic acid ($C_{18:1}$) was present in all the species that ranged between 1.68 and 3.89 %. However, linoleic acid ($C_{18:2}$) was detected in all the species, except *S. bohneri* and *G. livida*. But linolenic acid ($C_{18:3}$) was present only in *O. foreaui* isolated from sewage with 2.28 %.

Table 1 - Fatty acid composition* (in %) of seven species of cyanobacteria isolated from different aquatic habitats of the Western Ghats, Karnataka.

Fatty acid profile	Sulfur spring water				Petrochemical effluent	Sewage water	Dairy effluent
	Lyngbya limnetica	*Calothrix fusca*	*Scytonema bonheri*	*Gloeocapsa livida*	*Oscillatoria calcuttensis*	*Oscillatoria acuminata*	*Oscillatoria foreaui*
$C_{8:0}$ Caprylic acid	3.36±0.7	10.45±1.2	9.58±0.5	8.10±0.1	8.13±2.3	7.59±0.8	7.45±0.3
$C_{10:0}$ Capric acid	1.99±1.2	6.85±0.6	5.99±0.3	5.86±0.5	5.11±0.5	4.66±1.9	5.79±0.6
$C_{12:0}$ Lauric acid	17.17±0.9	41.39±0.2	41.40±0.4	45.30±0.6	35.29±0.3	33.33±0.3	44.53±0.6
$C_{14:0}$ Myristic acid	5.32±2.1	11.92±0.4	10.78±0.7	16.77±0.9	10.42±1.2	10.58±0.7	10.65±0.8
$C_{16:0}$ Palmitic acid	6.09±0.2	10.04±1.3	8.33±0.3	4.69±2.0	7.46±0.5	8.35±0.2	7.17±1.1
$C_{16:1}$ Palmitoleic acid	2.48±0.5	Nil	Nil	Nil	0.27±1.5	Nil	Nil
$C_{18:0}$ Stearic acid	1.60±1.5	1.65±0.8	1.88±0.5	Nil	1.64±1.7	Nil	1.66±0.5
$C_{18:1}$ Oleic acid	3.10±1.1	2.47±0.7	2.09±1.9	2.91±1.0	3.72±0.7	3.89±2.2	1.68±2.3
$C_{18:2}$ Linoleic acid	1.56±0.5	1.14±0.4	Nil	Nil	2.18±0.3	2.94±1.4	1.98±1.5
$C_{18:3}$ Linolenic acid	Nil	Nil	Nil	Nil	Nil	Nil	2.28±0.6
$C_{20:0}$ Arachidic acid	0.25±0.1	Nil	Nil	Nil	Nil	Nil	Nil
$C_{22:1}$ Erucic acid	0.33±0.4	Nil	Nil	Nil	Nil	Nil	Nil
$C_{24:0}$ Lignoceric acid	Nil	Nil	Nil	Nil	Nil	Nil	Nil

*Values are the means of three replicates ± standard error.

The seven metallic elements namely, magnesium, copper, manganese, ferrous, zinc, nickel and lead analyzed by the atomic absorption spectrophotometer indicated that lead was absent in all the species and magnesium was found in highest concentration, followed by ferrous; nickel was recorded in least concentration (Table 2). *O. foreaui* showed highest concentration of copper, manganese, ferrous and zinc (57.65, 539.20, 6402, and 211.20 $\mu g mL^{-1}$, respectively). The concentration of nickel was maximum in *S. bohneri* (11.05 $\mu g mL^{1}$). The highest concentration of magnesium was in *O. acuminata* (21.05 mg g^{-1}) and it was least in *O. foreaui* (12.812 mg g^{-1}).

Table 2 - Mineral composition*(in μgmL^{-1}) of seven species of cyanobacteria isolated from different aquatic habitats of the Western Ghats of Karnataka.

Source	Species	Cu	Mn	Fe	Zn	Ni	Mg	Pb
Sulfur spring	*Calothrix fusca*	38.90±0.8	204.80±0.6	4779.00±0.3	94.00±0.9	13.20±2.2	13,350.00±0.0	Nil
	Gloeocapsa livida	29.00±3.0	344.40±0.3	4641.00±0.8	137.50±0.4	13.00±3.2	18,494.00±0.6	Nil
	Lyngbya limnetica	31.20±2.2	379.60±0.5	5455.70±0.2	134.10±1.1	2.20±0.7	18,650.00±0.5	Nil
	Scytonema bohneri	42.96±1.3	211.50±2.0	3367.00±1.0	102.60±2.2	11.05±0.1	13,190.00±1.4	Nil
Petrochemical effluent	*Oscillatoria acuminata*	15.20±0.6	161.90±3.3	3365.00±3.2	53.00±0.3	5.40±0.0	21,050.00±0.1	Nil
Dairy effluent	*Oscillatoria calcuttensis*	13.60±1.5	255.70±1.0	2237.50±1.2	80.00±0.1	5.80±2.2	18,600.00±0.1	Nil
Sewage water	*Oscillatoria foreaui*	57.65±1.9	539.20±0.2	6402.00±0.5	211.20±0.2	9.37±1.2	12,812.00±1.0	Nil

*Values are the means of three replicates ± standard error.

DISCUSSION

Carbohydrate, protein, amino acids and lipids

The analysis showed that maximum amount of total carbohydrate was found in *S. bohneri* (28.4% dry weight) and minimum in *O. foreaui* (8% of dry weight). The carbohydrates formed a major component of these species, except *O. calcuttensis* in which the total lipid was higher (20% dry weight). Four species of cyanobacteria from sulfur spring showed high concentration of carbohydrates than others.

The influence of effluents on the biochemical composition of cyanobacteria has been studied by Manoharan and Subramanian (1992). They conducted an experiment to see the influence of paper mill effluent on the physiology and biochemistry of the *O. pseudogeminata* var. *unigranulata* and found that total carbohydrate content of *Oscillatoria* of paper mill effluents showed more than two-fold increase in its level from that of the control. But total free amino acid, total protein and total lipid content was less with unsterilized as well as sterilized effluents. Hosmani and Anitha (1998) reported similar type of results for carbohydrate and protein contents of *Microcystis aeruginosa*, (84.44 and 22.0 mg mL^{-1} of carbohydrates and protein, respectively) The investigation carried out by Walach (1987) indicated that carbohydrate synthesis increased with decreased nitrogen availability under constant carbon availability.

Besides food value, extracellular and intracellular carbohydrates of cyanobacteria are involved in some other properties. The cellular carbohydrate content serve to facilitate the buoyancy changes in the bloom forming *M. aeruginosa* reported by Kromkamp and Mur (1984). Another characteristic feature of cyanobacteria is their ability to secrete carbohydrate and protein extracellularly and their potential role in metal removal and in food and package industries (Shah et al., 2000). Kawaguchi and Decho (2000) and Kawaguchi et al. (2003) reported the extracellular polymeric secretion by *Schizothrix* sp., *Synechocystis* sp. and *Oscillatoria* sp. found in the Exuma Cays and Highborne cay in the Bahamas, which contained acidic polysaccharides and proteins. Growth promoting and inhibiting effect of carbohydrates secreted as extracellular substances by some species of cyanobacteria was reported by Safonova and Reisser (2005).

In the present study, the maximum amount of total protein and total free amino acid was found in *O. foreaui* (7% of dry weight). *O. calcuttensis* showed higher concentration of total lipid (20% of dry weight). The total percentage of protein was less when compared to other cyanobacterial strains. The four strains of *Spirulina* were analyzed for their protein and total lipid contents by Fatma et al. (1994) and values obtained were within the range of 43 – 55% and 2.7 - 6.8%, respectively. The protein content was highest and lipid content was lowest when compared to the present study.

Shashikumar and Madhyastha (2002) reported a total of 18.4% amino acid content in the *Synechococcus aquatilis,* isolated from an estuary which was very high compared to the values of the present study, whereas *Phormidium tenue*

contained 5.24% of amino acids which agreed with the present study.

Cyanobacteria contain a variety of amino acids and hormonal substances along with sugars and are involved in the improvement of soil texture by acting as chelating agents for heavy metals; they may stimulate the growth of heterotrophic bacteria and also act as growth promoting substances for the plants (Misra and Kaushik, 1989).

The biochemical constituents of cyanobacteria depends on the nature of strains, physiological state of the culture and the environment (Vargas et al., 1998; Subhashini et al., 2003; Maslova et al., 2004; Rosales et al., 2005). Subhashini et al. (2003) observed significant variations in protein content among the four isolates of *Anabaena azollae.* Authors also stressed about the biochemical variations that enabled to distinguish between the subspecies in several cyanobacterial genera. Rosales et al. (2005) reported about the physiological competence of *Synechococcus* sp. in hypersaline medium. They observed high cell contents of chlorophyll a, carotenoids, proteins and carbohydrates at 100 ppt and in good nutrient conditions. There are certain factors which also influence the protein synthesis (Borbely et al., 1985; Bhagwat and Apte, 1989). Over 300 nitrogen containing metabolites which are lipopeptide in nature have been reported from marine cyanobacteria (Tan, 2007).

Fatty acids

With regard to the fatty acid composition, all the species showed high levels of saturated fatty acids with the values ranging from 0.25 to 45.3%, whereas the levels of monounsaturated and polyunsaturated fatty acids (PUFA) were generally low in the present study. Similar results were also obtained by Vargas et al. (1998) and Caudales et al. (2000). Caudales et al. (2000) studied the cellular fatty acids in *Dermocarpa, Xenococcus, Dermocarpella, Myxosarcina* and *Pleurocapsa* species which contained high proportion of saturated fatty acids (26 - 41% of the total) and unsaturated straight chains (40 - 67%). In the present study, the contents of lauric acid ($C_{12:0}$) was highest in all the seven species. But some researchers have reported palmitic acid as the most prevalent fatty acid in cyanobacteria (Vargas et al., 1998; Sassaki et al., 2005).

The influence of various environmental factors on the fatty acid composition is also unveiled. For example, Olvera - Ramirez et al. (2000) reported the fatty acid content in *Calothrix* sp., isolated from a rice field in Mexico, was influenced by the nitrate content in the culture medium and its polyunsaturated fatty acid content was more. The effect of light, temperature and salinity on the lipid and fatty acid composition in some species isolated from different habitats have also been reported by many workers (Rezanka et al., 2003; Maslova et al., 2004; Liu et al., 2005; Rosales et al., 2005). Besides, lipids and fatty acids play an important role in the tolerance of cyanobacterial cells to various environmental stresses such as desiccation, salt induced damage, low temperature, high light induced photoinhibition (Singh et al., 2002).

One of the factors affecting the value of cyanobacteria as a food source is its PUFA content; consequently isolated species from different polluted water bodies and sulfur spring should be only of a limited value. Kumar et al. (2003) showed that polyunsaturated fatty acids varied among the strains. Similar results were obtained in the present study. Matsunaga et al. (1995) reported high cis – palmitoleic acid content (54.5 and 54.4% of total fatty acid, respectively) in two marine species of *Phormidium* and *Oscillatoria.* However, in the present study, polyunsaturated fatty acid content was low and in few others, it was absent. One of the species lacking PUFA was *O. limnetica* which could be alternatively grown aerobically or anaerobically with sulfide as electron donor as reported by Oren et al. (1985) who observed that *Oscillatoria limnetica* synthesized monounsaturated fatty acids (MUFA) by desaturation of their saturated counterparts in the presence as well as in the absence of molecular oxygen. But Kumar et al. (2003) and Rezanka et al. (2003) reported higher concentration of PUFA in some strains investigated by them. The fatty acid content of cyanobacteria is also influenced by certain effluents. Manoharan and Subramanian (1993) observed changes in the levels of different fatty acids which were influenced by the effluents. This could be one of the factors that affect the fatty acid composition of cyanobacteria isolated from different habitats which was evidenced in the present study.

Fatty acid composition is used as an effective tool in clarifying the taxonomical problems of cyanobacteria (Kruger et al., 1995; Li and Watanabe, 2004). Kruger et al. (1995) divided *Microcystis* isolates into subgroups which were

characterized by a high content of polyunsaturated fatty acids (27 – 44 %) and low content of palmitoleate. According to them, the toxic strains and nontoxic strains of *Microcystis* should be placed in separate groups.

Mineral content

The cyanobacteria are also rich in minerals as they form an integral part of the cell. In the present study, higher concentration of magnesium was observed in all the species as they formed a key mineral of the chlorophyll molecule. Heavy metals such as copper, ferrous, manganese, nickel, mercury, cadmium, zinc, lead, molybdenum, etc. are the essential micronutrients required for the growth of cyanobacteria and in higher concentration, they may have an inhibitory effect on the growth (Kannan and Subramanian, 1992; Reddy et al., 2002).

Venkataraman and Becker (1985) reported some minerals in different species of *Spirulina*. There is a wide variation in mineral composition between the species of *Spirulina*. Similar trend was also noticed among *Oscillatoria* species in the present study. Subhashini et al. (2003) analysed four micronutrients, namely, copper, manganese, ferrous and zinc in *Anabaena* species. Of the four isolates of *A. azollae*, *A. azollae* – AM recorded maximum copper and manganese content in the concentration of 233 and 1217µgmL^{-1}, respectively. *A. azollae* – AF recorded maximum ferrous and zinc content in the concentration of 2365 and 900 µgmL^{-1}, respectively.

Heavy metals are important environmental pollutants. Cations uptake and their toxicity was extensively studied in cyanobacteria by many investigators (Ahluwalia and Kaur 1989; Khare and Bisen 1991; Pandey et al., 1996). Less toxicity of nickel and copper was due to their use as essential elements for various metabolic processes. Mercury was more toxic to the test organisms than nickel and copper. At acidic pH, heavy metals were more toxic to the growth; EDTA was more effective chelator than citrate and glutamine which played a protective role against the metal toxicity. Moffett et al. (1997) reported that cell densities of cyanobacteria declined drastically in harbours subjected to high anthropogenic copper inputs which indicated the copper toxicity at higher concentration.

The ferrous was the second richest mineral component after magnesium in all the species of the present study. Ferrous acts as a cofactor of many enzymes and even its deficiency leads to the loss of biochemical pathway in cyanobacteria. The ability of cyanobacteria to accumulate heavy metals from the polluted water bodies and transform them into nontoxic form has been successfully utilized in bioremediation processes (Samal et al., 2004; Hernandez and Olguin, 2002). The investigation undertaken by Saxena and Kumar (2004) on the effect of mercuric chloride on testicular phosphatases and to evaluate the modulatory potential of *S. fusiformis* on mercury induced testicular toxicity in Swiss albino mice revealed that the cotreatment of *Spirulina* with mercuric chloride effectively reduced the mercury-induced testicular changes.

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