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Effect of Iron Deficiency and Supplemented Conditions on the Antioxidant System, Membrane Lipid Peroxidation and Some Metal Levels in *Spirulina maxima*

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Effects of iron deficiency (10 μM) and excess (50-100 μM) conditions, causing oxidative stress, on enzymatic and non-enzymatic antioxidants, also lipid peroxidation (LPO) were investigated in blue-green cyanobacterium, *Spirulina maxima* depending on time. Increasing iron concentration in the medium resulted in increases in intracellular iron levels. Uptakes of iron and magnesium indicated positive correlation, whereas Mn levels in deficiency condition showed a similar trend with control and increased in excess conditions. While catalase activity wasn't observed in all conditions, the highest superoxide dismutase and ascorbate-dependent peroxidase activities were determined in 50μM on 13th day. However, chlorophyll contents in deficiency condition were similar to control up to 13th day, they were over control in excess conditions. Then, they tended to increase in all conditions except control. Phycocyanin contents in deficiency condition up to 7 day were slightly lower than controls' though significantly higher than it in excess conditions. Then, phycocyanin levels for control were determined over all conditions, except 17th day, for excess conditions. Ascorbate and total carotenoid contents showed similar, but converse trends, depending on iron concentration except control. Control's LPO levels indicated insignificant change, yet they in other conditions were higher than control's and showed decreasing variations during time.

Key words: *Spirulina maxima;* Phycocyanin; Superoxide dismutase; Ascorbate-dependent peroxidase; Lipid peroxidation; Iron.

Blue-green algae (cyanobacteria) have evolved as the most primitive, oxygenic, plant-type phosynthetic organisms[1]. They were the first produced molecular oxygen as a byproduct of photosynthetic activity. Moreover, since it was the cyanobacteria which themselves produced the free reactive oxygen species (ROS) that can damage cellular components leading to cell death, they necessarily had to find a way to counter act it.

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It is generally believed that the production of ROS in photosynthetic organisms is an unavoidable consequence of the operation of the photosynthetic electron transport chain in an oxygen atmosphere. The chloroplast is a particularly rich source of ROS since it contains the highly energetic reactions of photosynthesis and an abundant oxygen supply[2]. It is also possible that ROS are produced under certain oxidative growth conditions. Apart from photosynthesis, another important source of ROS in cells is respiration[3]. For the efficient removal of the ROS such as superoxide radical, hydroxyl radical, hydrogen peroxide (H_2O_2), the cyanobacteria as well as other living cells attempt to adapt to the oxygen-rich environment through a number of different redox enzymes including superoxide

dismutase (SOD) which catalyzes the dismutation of superoxide radical to hydrogen peroxide and molecular oxygen, and peroxidases which detoxify H_2O_2 as well as non-enzymatic system including ascorbate, phycocyanin, carotenoids[1,4]. However, lipid peroxidation (LPO) is linked to the production of superoxide radical, an indicator of free ROS production and consequent tissue damage.

ROS formation is also increased in the presence of trace amounts of iron or other transition metal ions and metal chelators[5]. On the other hand, the availability of iron is crucial for growth, since this element is present in numerous cellular processes, particularly for photosynthetic organisms[6]. It functions to accept and donate electrons and plays important roles in the electron-transport chains of photosynthesis and respiration. Iron limitation generally causes decreased synthesis of pigments, an even greater relative decrease in cytochrome synthesis and impaired organizational structure of the thylakoid membrane[7]. Iron deficiency also affects cytochrome synthesis since it is required for heme synthesis. However, since iron–stressed cyanobacteria remain viable and grow[8], it hypothesized that these cells must exhibit a potential to adjust metabolically to iron stress conditions. Photosynthetic organisms have developed strategies that compensate for metal limitation. Iron-limited cyanobacteria produce a photosynthetic antenna complex that facilitates excitation transfer to photosystem I (PSI)[9].

In spite of the intensive research over the years, most of the studies on the effects of iron deficiency in cyanobacteria such as *Anabaena variabilis* and *Synechococcus* have been done on cells that clearly experience major alterations in their photosynthetic apparatus. However, research about the relation of phycocyanin production and antioxidant system of cyanobacteria to iron deficiency and supplement conditions is limited. The aim of this study was to research antioxidant response capacity of *Spirulina maxima* SAG 84.79 under iron deficiency and supplemented growth conditions. Therefore, phycocyanin, chlorophyll, total carotenoids, ascorbate levels, and also SOD, ascorbate-dependent peroxidase (AsA- POD) and catalase (CAT) enzyme activities as well as LPO levels were investigated with respect to incubation period and the obtained results were compared with that of control (36 µM).

MATERIAL AND METHODS

Organism and Culture Conditions

The cyanobacterium *S. maxima* SAG 84.79 used in this study was obtained from the algae culture collection of the University of Gottingen, Germany. Inoculums of the *S. maxima* were maintained in Zarrouk's medium[10]. A carbonate-bicarbonate buffer maintained a pH of 9.5 ± 0.2. No adjustments were made throughout the experiments. All reagents were of analytical grade.

S. maxima was cultivated in batch cultures containing 650 ml of medium at 10-100 µM iron concentrations at 28°C. 36µM iron concentration as control condition was chosen because Zarrouk's medium include. Cultures were inoculated to an initial optical density (600 nm) of ca. 0.2. The cultures were mixed and bubbled using filtered air continuously. Illumination at 2500 lux (30 µmol photon m^{-2} s^{-1}) light intensity was provided by white fluorescent lamps continuously. The light intensity was measured by a digital light meter (Luxtron LX-101).

Preparation of Crude Extract

S. maxima cells were harvested periodically by centrifugation (4000 g, 10 min, 4°C), washed with distilled water, following potassium phosphate buffer (20 mM, pH 7.4) and resuspended in the same buffer containing polypropylene glycol 1200 in a volume equal to 1.5 times its weight. A 600 µl cell suspension was ground in 1.5- ml plastic vials with 0.5 g of glass beads (0.5 mm ø) in a mixer-mill for 5 min. Cell debris was removed by centrifugation at 16600 g, 4 °C for 15 min. The obtained supernatant was used to assays.

Analytical Methods

Phycocyanin level was determined by the method of Benett and Bogard[11]. Levels of chlorophyll and total carotenoids were measured as described by Lichtenthaler and Wellburn[12], after extraction with 80% acetone. The protein content was estimated by the method of Bradford using bovine serum albumin as standard[13]. Ascorbic acid analysis of *S.maxima* was performed in HPLC. 0.5 mL of 5% aqueous dithiothreitol and 3.5 mL of 1% aqueous meta-phosphoric acid were added to the

samples of 1 mL [14]. The samples were filtered (0.45 µm) and injected (10 µL) onto a heated (30°C) Supelcogel C610H column (30 cm x7.8 mm) that was protected with a Supelcogel C610H guard column (5cm x 4.6mm) (Supelco, Inc, Bellefonte, PA). The HPLC system had UV detector set at 210 nm (Hewlett-Packard, Palo Alto, CA). Mobile phase was 0.1% phosphoric acid. Flow rate was 0.5 mL/min and run time was 40 min per sample. Quantification was obtained using external L-ascorbic acid standards prepared in 5% dithiothreitol and 1% meta-phosphoric acid.

The concentration of the metal was measured by inductively coupled plasma optical emission spectrophotometer (ICP-OES, Optima 2100DV, Perkin Elmer, USA). 1 g wet weight of *S. maxima* cells was digested in 2 ml nitric acid followed by 2 ml perchloric acid. All glassware washed with 0.1 M nitric acid and ultra-pure water before use.

Assay Methods in Cell Free Extract

SOD activity was determined in cell free extract by the method of Crosti based on the inhibitory effect of SOD on the spontaneous autoxidation of 6-hydroxydopamine (6-OHDA) at 490 nm and 25 °C [15]. One IU is defined as the amount of SOD required to inhibit the initial rate of 6-OHDA autoxidation by 50%.

The activity of AsA-POD was measured according to Nakano and Asada[16], by monitoring the rate of ascorbate oxidation at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). One IU of AsA-POD activity represents the amount of enzyme which oxidizes 1 µmol of ascorbate per minute at 25 °C.

Catalase activity was determined in cell free extract by the method of Aebi. The decomposition of H_2O_2 was followed directly by the decrease in extinction at 240 nm and 25 °C [17].

LPO was estimated based on thiobarbituric acid (TBA) reactivity. Samples were evaluated for MDA production using a spectrophotometric assay for TBA[18]. The coefficient of extinction at 532 nm of 153 000 M^{-1} cm^{-1} for the chromophore was used to calculate the MDA-like TBA produced.

Statistical Analysis

The Tukey test was used for statistical significance analyses. The values were the mean of three separate experiments. Comparisons were also made with Pearson correlation.

RESULTS AND DISCUSSION

Trace metals play an important role in the regulation of elements in the various metabolic processes and affect the uptake of macronutrients[19]. Availability of micronutrients such as iron, manganese, zinc and nickel determine the productivity based on macronutrient[20]. Especially, iron, magnesium and manganese have been regarded as essential elements for cyanobacteria. Therefore, this study has been undertaken to find out the effect of iron concentration, in the range of 10-100 mM, on the antioxidant systems and LPO in *S. maxima*.

Variation of Intracellular Some Metal Levels in *S. maxima* depending on the Iron Concentrations in the Culture Medium

Iron, magnesium and manganese uptakes of *S. maxima* depending on incubation time are presented in Fig. 1. Iron and magnesium uptakes in deficiency condition were lower than that of control, while they in excess conditions were generally stable and higher than that of it. The levels of intracellular iron and magnesium of *S. maxima* indicated positive correlation with iron concentration ($r=0.940$, $r=0.845$, $p<0.01$). However, manganese uptakes of *S. maxima* in deficiency condition up to 13th day showed similar values with control, they in excess conditions were higher than that of it and then reached their maximum by slightly increasing. On the 13th day, levels of intracellular iron, magnesium and manganese in 100 µM supplemented iron concentration when compared to those of controls increased by 3.8-, 2.5- and 3.3- fold, respectively.

All of these metals are required for photosynthesis in cyanobacteria. Magnesium also influences growth, cell division, enzyme activity and stabilizes the membrane bilayer. Manganese has an important role in catalytic reactions as an activator and photosystem II (PSII) as well as its toxicity[21-22].

Effects of Iron Concentration on the SOD, CAT and AsA-POD Activities in *S. maxima*

To determine the type of SOD in *S. maxima*, assays were performed with 5 µM NaCN and 5 µM H_2O_2. The activity was present with cyanide, indicating that it does not represent CuZnSOD, which is inhibited by cyanide. Hydrogen peroxide can be used to distinguish

FeSOD and MnSOD because only FeSOD is inactivated. The SOD activity of *S. maxima* was inactivated with H_2O_2. Therefore, *S. maxima* SOD was detected as FeSOD.

As seen from Fig. 2, FeSOD activities of *S. maxima* depending on iron concentration reached the maximum level on the 13th day, and then decreased with the prolongation of the incubation period. SOD activities in deficiency and excess conditions except 50 mM were lower than that of control on the 13th day. The highest FeSOD activity was determined in presence of 50 mM iron as 22.65 ± 1.5 IU/mg, which is insignificantly different from control. The lowest FeSOD activity in deficiency condition was determined as 8.14 ± 0.9 IU/mg. The increase in the activity of FeSOD of the *S. maxima* may be a consequence of production of superoxide radical at PSI in the cells that has to be scavenged. The highest FeSOD activity of *S. maxima* in the study was 3.6- and 2.1-

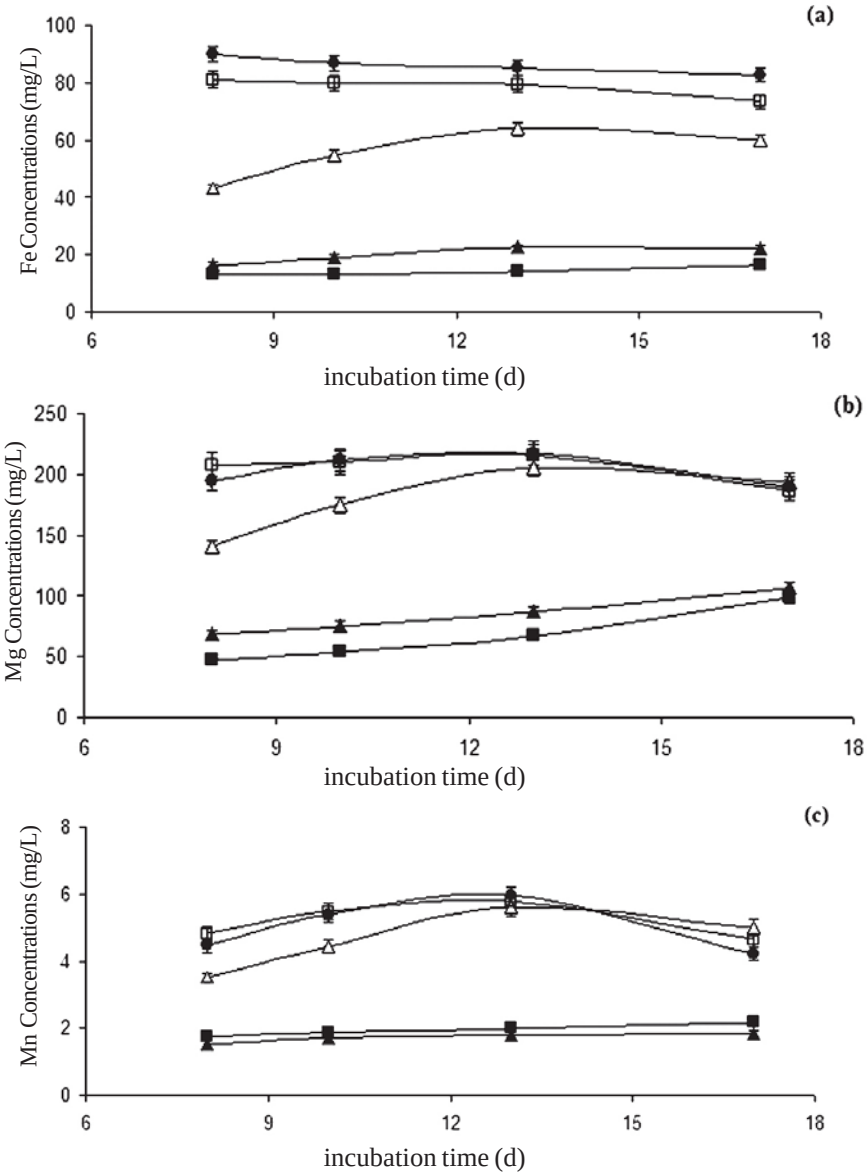

Fig. 1. Variations of Fe (a), Mg (b) and Mn (c) levels in *S. maxima* depending on the incubation period in medium containing: 10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (- □ -); 100 mM (-●-) iron concentrations. The values are the mean ±SEM for experiments of three separate experiments.

fold higher than that of *Spirulina platensis* ARM728 grown in inorganic growth medium[23] and *Anabaena variabilis* grown in BG_{11} medium, respectively[5]. FeSOD activity in deficiency condition including 10 mM iron was determined as minimum value on the 13th day. FeSOD activity was negatively affected in deficiency and also excess iron concentrations except 50 µM. This data indicate that iron concentration is important for *S. maxima* FeSOD activity as a cofactor. The fact that the increase in FeSOD activity is not in parallel to the increase in iron level shows that iron can stimulate the expression of the FeSOD enzyme to some extent. It was reported that the transcript levels of the FeSOD were decreased under iron limiting conditions suggesting a down regulation of the gene expression in response to lack of available iron within the cell[24]. In another study, marine cyanobacterium *Synechococcus* sp. in ASW medium containing no added iron resulted in no alteration in FeSOD enzyme activity[25].

Another antioxidant enzyme, AsA-POD, which is H_2O_2-scavenging enzyme with high specificity for ascorbate as the electron acceptor, removes H_2O_2 enzymatically, as well as non-enzymatically via AsA. AsA-POD activities determined in *S. maxima* depending on iron concentration are illustrated in Fig. 3. Asa-POD activities in control and excess conditions when compared to deficiency condition were higher, but the changes in values close to each other have demonstrated by showing up maximum on the 13th day. AsA-POD activity variations depending on incubation period and all studied iron concentrations except 10 µM showed insignificantly differences ($p>0.05$). The highest activity in comparison with that of deficiency condition was significantly increased by approximately 2-times ($p<0.05$). The highest AsA-POD activity of *S. maxima* is 4.3-fold higher than

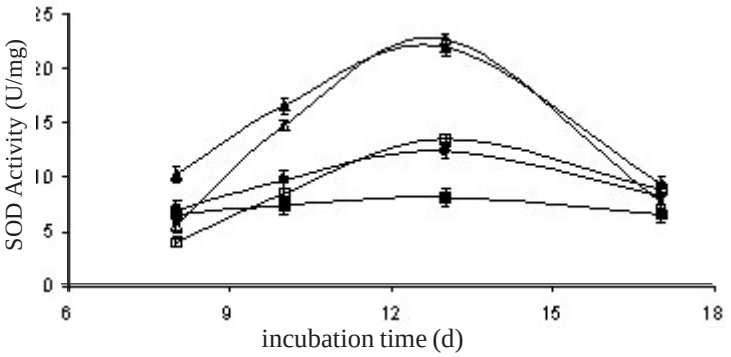

Fig. 2. Variations of SOD activity in *S. maxima* depending on the incubation period in medium containing: 10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (-□-); 100 mM (-●-) iron concentrations. The values are the mean ±SEM for experiments of three separate experiments

that of *S. platensis*[26]. AsA-POD activity in comparison with their controls in excess conditions showed a similar trend with control despite much more intracellular iron levels. This situation may indicate that iron concentration induces the production of hydroxyl radical, which may decrease enzyme activities.

In addition, the other antioxidant enzyme CAT, which contained iron as a cofactor, was not determined in *S. maxima* for investigated growth conditions and also control. This suggested that the CAT in *S. maxima* has no role for H_2O_2 detoxification. Interestingly, AsA-POD activities generally increase along with FeSOD in response to iron suggesting that the components of ROS scavenging systems are co-regulated.

Effects of Iron Concentration on the Phycocyanin, Chlorophyll, Total Carotenoids and Ascorbate Contents of *S. maxima*

Differences in phycocyanin levels between control and deficiency conditions depending on incubation period reached maximum on 8th day and these levels in deficiency condition were lower than that of control (Fig. 4). They in excess conditions up to 6th day were higher than those of controls but they reached the value of deficiency condition on the 8th day. Following incubation period, they showed insignificantly

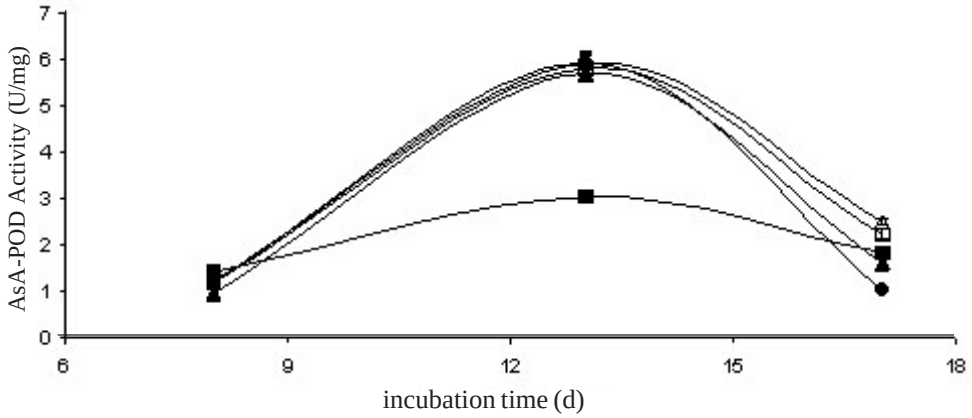

Fig. 3. Variations of AsA-POD activity in *S. maxima* depending on the incubation period in medium containing: 10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (-□-); 100 mM (-●-) iron concentrations. The values are the mean ±SEM for experiments of three separate experiments

changes in deficiency and control conditions and had been increasing trend in excess conditions after 13th day. The obtained levels, which are insignificantly different from each other, exceeded its value obtained in control condition on the 17th day and reached up to 178.2 ± 6.2 mg/g wet weight.

Chlorophyll contents of *S. maxima* depending upon iron concentration are depicted in Fig. 5. Chlorophyll levels up to 13th day in deficiency condition were similar to control, while they in excess conditions showed variations very close to the values but over the control levels. After the 13th day, chlorophyll levels in all conditions except for control tended to increase. According to the results; chlorophyll levels indicated positive correlation with increasing iron concentration, and incubation time (r=0.463, $p<0.05$; r=0.719, $p<0.01$, respectively) and the highest level reached up to 49.5 ± 1.8 mg/g at the 100 µM iron on the 17th day. This chlorophyll level in *S. maxima* was higher than that of *Anabaena variabilis* grown in BG_{11} medium included 100 µM iron concentration[5].

As expected, the lowest phycocyanin and chlorophyll levels compared to the other investigated iron concentrations were determined in iron deficiency condition. Iron is important in photosynthesis because of a key component of chromophore synthesis[27]. It affects the synthesis of the major photosynthetic pigments, chlorophyll and phycocyanin, and its incorporation into ferredoxin and the iron-sulfur proteins on the

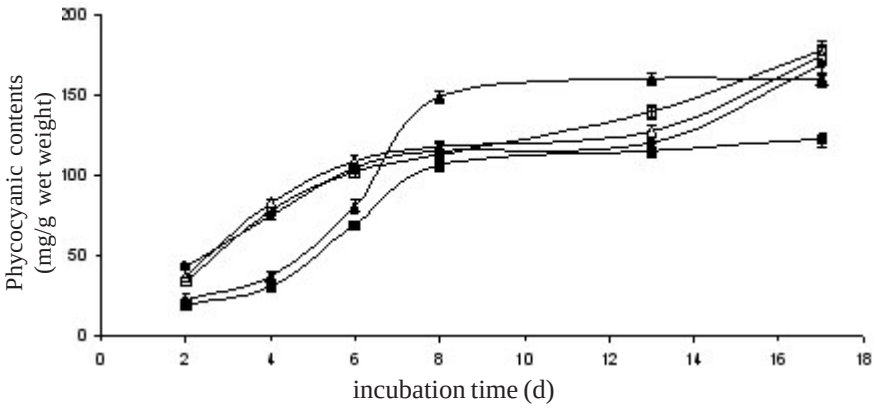

Fig. 4. Variations of phycocyanin level in *S. maxima* depending on the incubation period in medium containing: 10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (-□-); 100 mM (-●-) iron concentrations. The values are the mean ±SEM for experiments of three separate experiments

acceptor side of PSI make it a vital component of photosynthesis[28]. According to the results; it was determined that cells of *S. maxima* grown under iron-deficient conditions, exhibit decreased phycocyanin and chlorophyll levels. These results are consistent with cyanobacterium *Synechococcus* sp., PCC 7942 [7]. It was also reported that the decrease of pigments is paralleled by a decrease in thylakoid membranes and then reduced PSII as well as PSI photochemistry and restricted photosynthetic electron transport between PSII and PSI [29].

The other important pigments, carotenoids in cyanobacteria are found on the cell walls and cytoplasmic membranes as well as photosystems. Response against stress with carotenoid production in deficiency condition further increased until 13th day in compared with

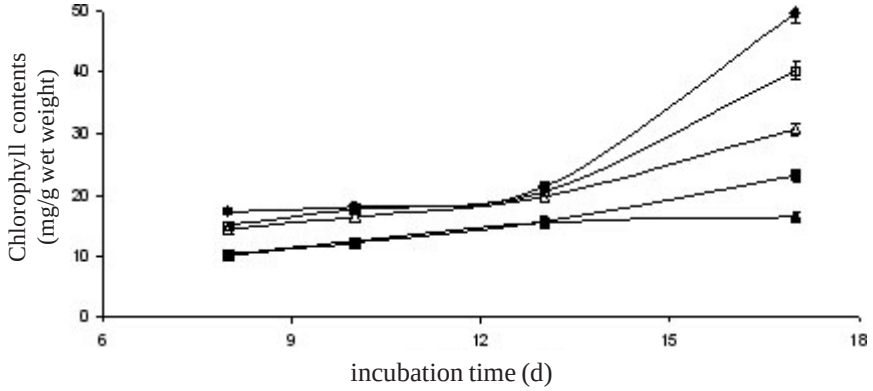

Fig. 5. Variations of chlorophyll level in *S. maxima* depending on the incubation period in medium containing:10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (-□-); 100 mM (-●-) iron concentrations. The values are the mean ±SEM for experiments of three separate experiments

control and excess conditions (Fig. 6). Total carotenoid levels were generally higher than that of control for all incubation period. Carotenoid levels reached their maximum on the 13 day as 8.04 ± 0.6 mg/g for deficiency condition. However, total carotenoid levels in presence of 75 and 100 µM iron concentrations on the 17th day were higher than those of the other iron concentrations ($p< 0.05$). Total carotenoid levels in *S. maxima* showed positive correlation with incubation period ($r=0.712$, $p<0.01$) and reached up 12.05 ± 0.6 mg/g wet weight at the 100 µM iron on this day.

As known, the increase in total carotenoids served as structural components of

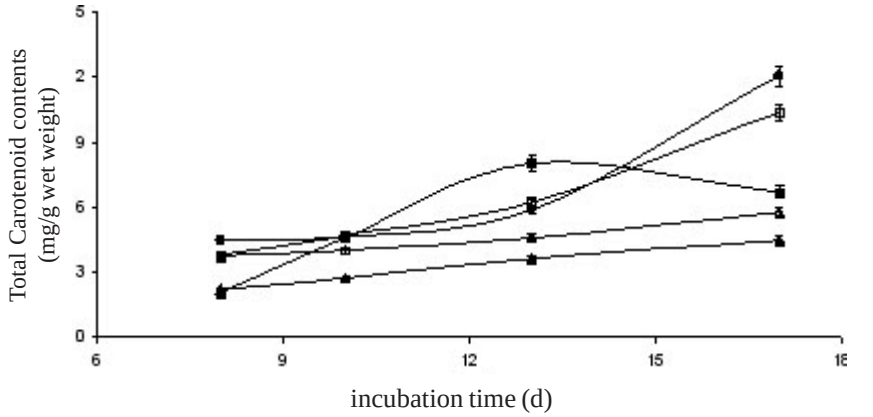

Fig. 6. Variations of total carotenoids level in *S. maxima* depending on the incubation period in medium containing: 10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (-□-); 100 mM (-●-) iron concentrations. The values are the mean ±SEM for experiments of three separate experiments

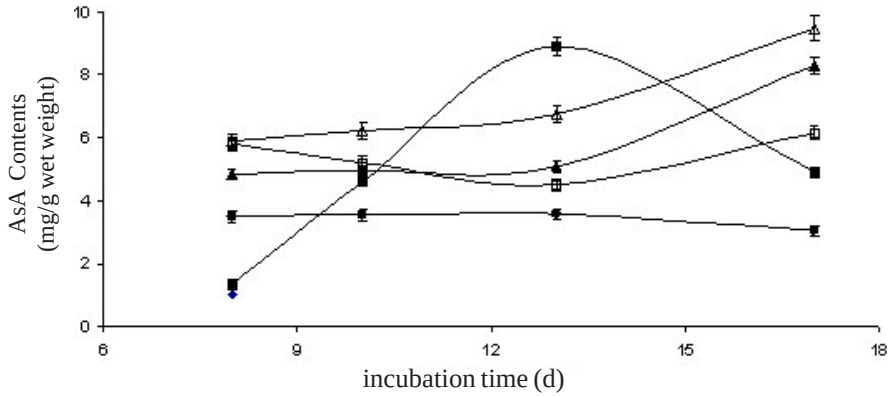

Fig. 7. Variations of AsA level in *S. maxima* depending on the incubation period in medium containing: 10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (-□-); 100 mM (-●-) iron concentrations The values are the mean ±SEM for experiments of three separate experiments

PSI. Carotenoids are involved in photosynthesis in various ways: they act as light-harvesting pigments, contribute to the structure of thylakoid membranes and play an important role in photoprotection[30] and worked synergistically to enhance antioxidant protection. Perhaps the most important functions of carotenoids include the dissipation of excess energy of excited chlorophyll and the elimination of ROS [31].

AsA level in deficiency condition when compared to control condition on day 13 reached up to maximum as 8.9 ± 0.6 mg/g wet weight by 1.8-fold increase (Fig. 7). Unlike total carotenoids, they showed a decrease in increasing iron concentrations except for 50 µM. But, they in excess conditions except 100 µM indicated increases after the13th day. And then, though decreases of AsA-POD enzyme activity, estimation of increases in AsA levels except deficiency condition indicates the contribution of AsA in the antioxidant system of *S. maxima.* In the meantime, AsA functions as a reductant source for many ROS, thereby minimizing the damage caused by oxidative stress. Ascorbate scavenges not only H_2O_2 but also superoxide, hydroxyl radicals, and lipid hydroperoxides without enzyme catalysts and it can indirectly scavenge ROS[32, 33]. In addition, since carotenoids are interacted with AsA existing in the aqueous phase, the produced carotenoid radical cations are efficiently reconverted to the parent carotenoid by ascorbate[34].

Variation of LPO Levels

As an indicative marker of oxidative membrane damage, LPO levels in *S. maxima*

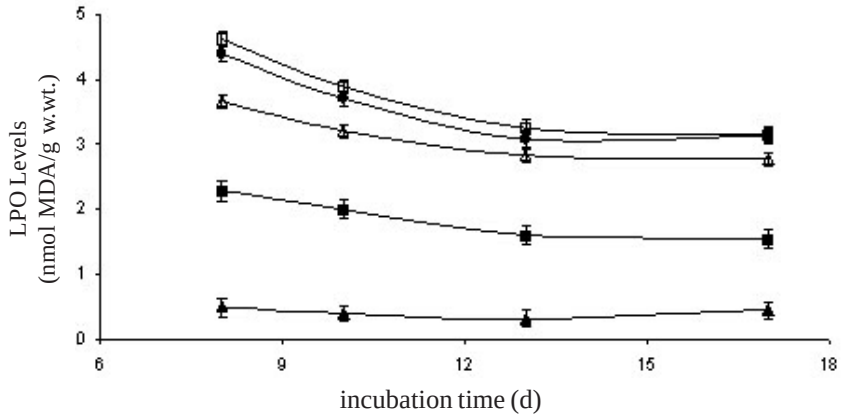

Fig. 8. Variations of LPO level in *S. maxima* depending on the incubation period in medium containing: 10 µM (-■-); 36 µM (-▲-); 50 mM (-Δ-); 75 mM (-□-); 100 mM (-●-) iron concentrations. The values are the mean ±SEM for experiments of three separate experiments

depending on all testing iron concentrations are shown in Fig. 8. The obtained LPO levels in excess conditions were higher than that of deficiency condition. LPO levels in both the deficiency and excess conditions up to 13th day showed significantly decreases depending upon incubation period.

Generally, contents of chlorophyll, total carotenoids, AsA and also phycocyanin up to 6th day were higher than those of control, and activities of FeSOD and AsA-POD were similar, while to have higher LPO levels in comparison to control stem from the increase of intracellular iron concentration. Increased intracellular iron level in the *S. maxima* has led to hydroxyl radical formation by triggering mechanism of Fenton and consequently damage on membrane namely increasing of the level of MDA. After the 13th day, despite the aging process in *S.maxima*, it similar to that in the control to maintain the levels of LPO may be explained by the observed increases in antioxidants such as chlorophyll, total carotenoids, AsA and high level phycocyanin contents. It was similarly showed that an extract of *Spirulina* containing phycocyanin is a potent free radical scavenger and inhibits microsomal lipid peroxidation[35]. Similarly Miki and coworkers reported that carotenoids serve as effective quenchers of ROS and can prevent LPO in marine animals[36]. LPO levels in iron deficiency condition depending on incubation time were lower than those of excess conditions but higher than control's. Although, intracellular iron levels were under the control and very close to it, LPO levels in compared to control showed at least 3-fold increases. This situation may result from insufficient antioxidant defense system of *S. maxima* for this condition. As ROS balancing is a continuous process in living systems, LPO products are always present. In *S. maxima*, capacity of antioxidant defense system shows that iron level in culture medium is important to be kept under control.

In conclusion; biotechnological processes based on cyanobacteria have been attracting more interest due to their potential to produce a diverse range of chemicals and biologically active compounds, such as vitamins, carotenoid pigments, proteins, lipids and polysaccharides. Actually *Spirulina* has gained more attention because of its nutritional and various medicinal properties. In this study, the results indicated that balance between ROS production and antioxidant systems of *S. maxima* impaired in ROS favor by intracellular iron levels in both deficiency and excess conditions.

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