IRON TRANSPORTATION AND ITS IMPACT ON NITRATE ASSIMILATION IN THE CYANOBACTERIUM *ANABAENA SPHAERICA*: A PROMISING BIOACCUMULATOR OF IRON

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Abstract

To investigate the iron transport system in cyanobacteria and its impact on nitrate assimilation, cyanobacterium *Anabaena sphaerica* was treated with increasing concentrations of iron viz. 0 (-FeCl$_3$), 20, 75 and 100 µM FeCl$_3$. An increasing concentration of iron triggered significant production of carbohydrate and carotenoids whereas nonsignificant reduction in pigment and protein content. Nitrate and iron uptake were substrate dependent and maximum uptake rate was recorded at 100 µM FeCl$_3$. Although the intracellular nitrate content was increased at all the tried concentrations of iron but a noticeable increase in nitrate reductase and glutamine synthetase activity was observed only upto 75 µM FeCl$_3$. A minimum toxicity (relative ROS level and membrane injury index) was observed at 75 µM FeCl$_3$ that may be correlated with efficient iron efflux system, minimum residual iron (similar to 20 µM FeCl$_3$ treated cells) during immediate effect but during the longer exposure a substantial level of antioxidants (carotenoids), well developed antioxidative detoxification machineries (SOD, CAT, APX and GPX activity) and nitrate assimilating enzymes (NR and GS) were most effective. Despite the highest accumulation of iron, relative ROS level and membrane injury index, only a slight reduction in growth was observed at 100 µM FeCl$_3$ which may be attributed to the extremely high antioxidative enzyme activities and greater nitrate demand. Based on bioconcentration factor (> 1), *A. sphaerica* may be considered as a potential iron hyperaccumulator to extract iron from iron contaminated site and also be used for biofortification of plants grown in iron deficient soils.

Keywords:Anabaena sphaerica, Iron, Iron transport, Relative ROS level, Bioconcentration factor, Hyperaccumulator.

Introduction

Among the micronutrients, iron plays an important role in the growth and development of living organisms. It is the fourth most abundant element on the earth but due to present oxidant environment forms insoluble complexes at neutral to basic pH and causes poor iron bioavailability. The primary source of iron is soil and its optimum level must be maintained for adequate and balanced supply of iron as a nutrient for the healthy plant growth and better soil fertility to maintain the intense crop productivity and global food production in countries like India (Kaushik *et al*., 2019; Khan *et al*., 2019; Kunui and Singh, 2020). Chhattisgarh state of India is known for natural resources like iron and coal mines. Therefore established iron and steel industries are day by day affecting the microbial, plant and human health due to the successive deposition of iron at different trophic level which increases the level of iron in the soil and water. The contaminated soil and water are used by the people in their daily life for drinking, irrigation and agricultural practices and cause various types of illness. Apart from this, microbial system is not untouched from this impact.

As compared to heterotrophic organisms, photosynthetic and nitrogen fixing organisms require a substantial amount of iron for their iron containing electron carriers, cofactors and enzymes involved in carbon and nitrogen metabolism. Though, worldwide 30% of the soil faces iron limitation but microbes have acquired several strategies to capture iron from the environment in biologically useful forms and reduce iron from the insoluble ferric iron (Fe^{+3}) to the soluble ferrous form (Fe^{+2}). On the other side, 18% of the global soils are heavily loaded with the iron either through mining industries or iron ores but plants and microbes have the ability to regulate the homeostasis of Fe and respond accordingly in the surplus level of Fe through alternative means for their proper growth and survival (Krohling *et al*., 2016). But an excess iron availability (beyond permissible limits) or its starvation (below permissible limits) plays an extremely deleterious effects and catalyzes the formation of reactive oxygen species (ROS) leading to the reduced growth, nutrient imbalance, membrane injury and abnormal physiological and biochemical behaviour and also recognized as important initiators and mediators of cell death in a variety of organisms (Kaushik *et al*., 2019; Kaushik *et al*., 2016; Kunui *et al*., 2017).

Among the microbes, cyanobacteria are known for their omnipresence occurrence, oxygenic photosynthesis and global nitrogen fixation (Singh *et al*., 2014; Singh *et al*., 2016; Minj *et al*., 2017; Singh *et al*., 2017). Cyanobacteria have a tremendous plastic behavior to accommodate with any extreme conditions (Minj *et al*., 2017; Singh *et al*., 2017). Cyanobacteria are fast grower and outrageous stores of protein, carbohydrate, fatty acids, vitamins, growth hormones and secondary metabolites so their contributions as a diet supplements, biofuel, biofertilizer and metal accumulator etc. are highly appreciable. Intense researches have been carried over the years related to every aspects of cyanobacteria against metal stress including iron but most of the studies are confined to iron limitation in cyanobacteria (Kaushik *et al*., 2019; Minj *et al*., 2017; Verma *et al*., 2018). However, very few reports are available concerning the time course of the structural and functional rearrangement of the cellular

constituents, photosynthetic apparatus and nitrogen fixing ability, iron induced generation of reactive oxygen species and detoxification machineries during the acclimation of cyanobacteria towards iron sufficient conditions. So, during the present investigation much attention has been given to the study of the iron transport, accumulation and tolerance in cyanobacterium *A. sphaerica* isolated from the iron contaminated region of Chhattisgarh and simultaneously also dealt with the assessment of the alterations in the physiological and biochemical behavior in iron sufficient and deficient conditions to corroborate the impact of accumulated iron on the enzymes of nitrate metabolism and antioxidant defence system and also the potentiality of *A. sphaerica* as an iron tolerant and hyperaccumulator species which can be further used in iron contaminated soil and water for extensive extraction of iron.

Materials and Methods

Detection of iron level in the soil

The iron concentration of the soil was determined using the Atomic Absorption Spectrophotometer (Schimadzu). Before detecting the concentration of iron the soil samples were digested with the help of aqua regia (HNO_3: HCl in a ratio of 3:1).

Maintenance of Culture

The axenic culture of *A. sphaerica,* isolated from iron contaminated region of Chhattisgarh, was brought in the laboratory and was maintained in $BG-11_0$ nutrient medium (pH 7.2) at standard growth condition i.e. 28 $\pm$ 2° C temperature and 50-55 $\mu E\ m^{-2}\ s^{-1}$ light intensity with photoperiod of 14:10 hr light dark cycle (Singh *et al*., 2014). The cultures were properly maintained and subcultured in fresh medium after every 15 days. The cultures were shaken twice a day. Before experimentation, the exponentially grown cyanobacterium *A. sphaerica* was transferred to iron starved condition for 8 days. Then cyanobacterium was transferred to different regimes of iron i.e. 0 ($-FeCl_3$), 20 (concentration present in BG11 medium also known as control) 75 and 100 $\mu M\ FeCl_3$ condition for 96 hrs. All the experiments were performed in six replicates.

Iron transport (Iron Uptake, efflux and its residual iron)

Short term iron uptake by the cell of cyanobacteria treated with different concentration of iron i.e. 0, 20 i.e. control, 75 and 100 μM) was determined at different time interval (0, 5, 10 and 15 minutes) by using atomic absorption spectroscopy. After incubation at different time interval, cyanobacterial culture was centrifuged and the absorbance of the supernatant was taken which represented the depletion of the iron content from the medium. The depleted amount of iron has been assumed as taken inside the cells of cyanobacterium and it was determined in terms of $\mu mol\ mg^{-1}$ protein whereas rate was in terms of $\mu mol\ /mg\ protein\ /\ min$ using atomic absorption spectroscopy.

Iron efflux by the cells of cyanobacteria was determined using atomic absorption spectroscopy. The cultures were centrifuged at 10,000 rpm and put into the deionized water for 5 min. The iron content in the water was measured as iron efflux. The iron efflux was determined in terms of $\mu mol\ mg^{-1}$ protein min^{-1}. The iron uptake rate was calculated on the basis of the uptake of iron at 5 minutes.

After efflux of iron from the cell the iron remained in the cell was represented as the residual iron. The residual iron was calculated as

Residual iron = Iron uptake rate- Iron efflux rate.

Bioaccumulation of iron in cyanobacterial biomass

The iron accumulated in the cyanobacterium cells was determined by using protocol of Bhat *et al*. (2016). The cells were subjected to grow at iron starved condition for 8 days and then they were exposed to different iron concentration (i.e. 0, 20 i.e. control, 75 and 100 $\mu M\ FeCl_3$) for 4 days. Once the cyanobacterial cell reached to the exponential phase, the cultures were centrifuged and the pellet was collected. The pellets obtained from different iron concentrations were weighed separately and dried in oven at 65° C for 72 hrs. The dried content was powdered and acid digested with 0.1 N HCl in a sterilized mortar and pestle. The prepared samples were analyzed through Atomic Absorbtion Spectrophotometer (AAS).

Efficiency of iron removal

The efficiency of iron removal from the soil by the cyanobacterial cells is considered as iron remediation. The Fe remediation from soil was estimated using bioconcentration factor (BCF) on the basis of long term exposure of iron (in terms of iron accumulation in cyanobacterial biomass). The BCF at different concentrations was estimated by using following formula (Niu *et al*., 2007).

$$BCF = \frac{\text{Iron concentration in cyanobacterial biomass}\,(mg\ kg^{-1})}{\text{Iron concentration in the medium}\,(mg\ kg\text{-}1)}$$

Effect of different iron regime on the nitrate uptake and its accumulation

Nitrate uptake in presence of different concentration of iron was estimated by measuring the depletion of nitrate from the nutrient medium. Initially, cyanobacterium was kept in iron starved condition for 8 days and then transferred to different regimes of iron (i.e. 0, 20, 75 and 100 $\mu M\ FeCl_3$) with supplementation of 5 mM KNO_3 and then the cultures were withdrawn at different time interval for estimating the nitrate (Cawse, 1967). The nitrate accumulation in *A. sphaerica* was estimated in terms of $\mu mol\ /mg$ protein (Cawse, 1967).

Estimation of Nitrate Reductase (NR) Activity (EC 1.7.7.2)

The culture of *A. sphaerica* treated with different concentration of iron with supplementation of 5 mM nitrate for 4 days and Nitrate reductase activity was expressed in terms of $\mu mol\ /\ mg$ protein (Herrero *et al*., 1981; Snell and Snell, 1949).

Glutamine Synthetase (GS) activity (EC 6.3.1.2)

A. sphaerica cultures treated with different iron concentration (0, 20, 75 and 100 $\mu M\ FeCl_3$) and 5 mM nitrate for 4 days were centrifuged at 10,000 rpm for 10 minutes and the pellets obtained were permeabilized using toluene. The permeabilized cyanobacterial cells were used for estimation of GS activity (Shapiro and Stadtman, 1970).

Relative ROS level and Membrane Injury Index

The cyanobacterial cells (5μL) from each treatment were treated with phosphate buffered saline (PBS) (50 mM, pH 7.0) and the relative ROS level was determined using the formula provided by Hong *et al*. (2008).

$$\text{Relative ROS level (\%)} = \frac{\text{Mean DCF fluorescence intensity (iron treated)}}{\text{Mean DCF fluorescence intensity (control)}} \times 100$$

The membrane injury index was determined using following formula (Sullivan, 1972).

$$\% \text{ injury} = [\{1-T1/T2\}/ \{1-C1/C2\}] \times 100$$

Where T1 and T2 are the electrical conductivity of the iron treated cells and C1 and C2 are the respective electrical conductivity of the control before and after boiling. The electrical conductivity was measured using Water and Soil Analysis Kit.

Estimation of percentage change in antioxidative enzymes

All the enzymatic activities i.e. Superoxide dismutase activity (EC 1.15.1.1), Catalase activity (EC 1.11.3.6), Ascorbate Peroxidase activity (EC 1.11.1.11), Guaiacol peroxidase (GPX) activity were detected in *A. sphaerica* as per the protocol described in earlier literature (Stewart and Bewley, 1980; Nakano and Asada, 1981; Tatiana *et al.*, 1999). All the antioxidative enzyme activities at different concentration of iron were compared with their control (20 µM) and represented in percent increase and decrease.

Results and Discussion

The iron concentration detected via Atomic absorption spectrophotometer revealed that the soil consisted of 250 mg kg^{-1} of iron. In spite of this, some other physico-chemical properties of the soil were also measured in terms of pH (7.2), temperature (32.2 ℃), salinity (148.9 ± 12.90 g/ L), total dissolved solids (600.5 ± 52.3 mg/ L), dissolved oxygen (12.3 ± 0.09 mg / kg) and conductivity (128.6 ± 11.64 µ Seimen/ cm).

Stress factors such as availability of nutrient, temperature, light, pH always been a prime reason for devastating the microbial growth (Singh *et al.*, 2017). Among all stress prone environment, metal stress is one of the emerging field of study. Current observation in presence of iron provides a clear cut outline of the substantial involvement of iron concentration in affecting the physiology and biochemistry of the cyanobacterium *A. sphaerica*.

It was evident from the result that the uptake of iron was proportional to the iron concentration provided in the medium. A linear uptake was observed at 5 min of incubation at all the regimes of iron. The linearity of iron uptake started to deviate after 5 min of incubation. Maximum iron uptake was observed by the cyanobacteria when exposed to 100 µM FeCl$_3$ followed by 75 and 20 µM FeCl$_3$ concentration (Fig. 1a). The iron taken by the cell of *A. sphaerica* treated with 75 µM FeCl$_3$ was found to be increased by 32.02 and 34.16 % at 10 and 15 min of incubation. Similarly, at 100 µM FeCl$_3$ the iron uptake was increased by 26.13 and 27.54 % at 10 and 15 min of incubation as compared to control at the same time of incubation.

The iron uptake rate was calculated at 5 min of incubation and it was increased in substrate dependent manner (Fig. 1b). Maximum iron uptake was found in the cyanobacterium subjected with 100 µM FeCl$_3$ (0.0458 ± 0.001 µmol/ mg protein) followed by 75 µM FeCl$_3$ (0.0458 ± 0.001 µmol / mg protein) and 20 µM FeCl$_3$ (0.0288 ± 0.0064 µmol / mg protein).

The iron efflux rate was found to be minimum at 20 µM FeCl$_3$ followed by 100 µM FeCl$_3$. Maximum iron efflux was observed in case of 75 µM FeCl$_3$. The iron resided inside the cells after efflux was considered as residual iron. Maximum iron that resided inside the cell was in case of 100 µM FeCl$_3$ followed by 20 µM FeCl$_3$. Minimum iron resided inside the cell was at 75 µM FeCl$_3$ concentration (Fig. 2).

4 days of iron exposure caused a significant increment in (4.325 and 5.13 times) iron accumulation at 75 µM FeCl$_3$ and 100 µM FeCl$_3$ when compared with the accumulation of iron at 20 µM FeCl$_3$ (Fig. 3a). The bioconcentration factor for iron was recorded to be 2.096, 2.133 and 1.9 at 20, 75 and 100 µM FeCl$_3$ respectively (Fig. 3b).

It is clearly evident from the figure 1a, 1b, 2 and 3a that the cells of *A. sphaerica,* exposed to higher regimes of iron i.e. 100 µM FeCl$_3$, took maximum iron inside the cell. Possibly, maximum exposure of cyanobacteria in iron starved condition and thereafter transference to the higher regimes of iron concentration may be correlated with maximum upregulation of H$^+$-ATPase proteins that help the cyanobacterium *A. sphaerica* to solublize (reduce Fe^{3+} to Fe^{2+}) the surplus amount of iron at 100 µM FeCl$_3$. The activation of H$^+$-ATPase at an elevated level may resulted an extreme acidic condition that supported maximum iron uptake rate at higher concentration of iron (Kunui *et al.*, 2017).

As compared to the higher concentration of iron, less iron uptake was reported in case of 75 and 20 µM FeCl$_3$ concentration. Probably, less availability of iron might have failed to create acidic condition at a greater extent for iron uptake mechanism (Kunui *et al.*, 2017). In spite of this, minimal efflux rate may also cause highest iron accumulation as well as residual iron at 100 µM FeCl$_3$. Thus, at each minute if iron transport has been discussed (Fig. 1a, 1b), residual iron may have accumulated slowly in due course of time and possibly participated in Reactive Oxygen species generation. The ROS generated was the sole component of damaging the membrane integrity and caused membrane injury (Fig. 6a, 6b). Thus, such accumulated iron (after 4 days) takes part in various physiological and metabolic activity of the cell. Our experimental organism showed bioconcentration factor more than 1 i.e. 2.09, 2.133 and 1.9 in 20, 75 and 100 µM FeCl$_3$. This clearly reveals that *A. sphaerica* accumulated iron more than the concentrations present in that medium which proved that this species is one of the efficient hyperaccumulators of iron as per the rules of accumulation (Bhat *et al.*, 2016). *A. sphaerica* at 75 µM FeCl$_3$ showed highest BCF which clearly clarifies that the organism works very efficiently in Fe removal. It also meant that though the organism is found to be good iron excluder in short term iron uptake experiment at 75 µM FeCl$_3$ but even at longer exposure, efflux rate may be slowed and accumulation proceeded. However, low BCF at higher concentration (100 µM FeCl$_3$) may be attributed to slower ratio of iron bioaccumulation rate or Fe removal from the soil as compared to the presence of highest iron level in the medium. Further, higher concentration also caused highest level of toxicity inside the cell which can easily be pointed out in form of enormous amount of relative reactive oxygen species formation and membrane injury index (Fig.6a, b). Some of the hyperaccumulators of metals have also been reported in *Bacillus subtilis* and other green, brown and red algae (Banerjee *et al.*, 2019).

The Fig. 4 A clearly depicted that *A. sphaerica* showed a linear uptake of nitrate at 5 min of incubation at iron regimes (20, 75 and 100 µM FeCl$_3$) after which the linearity deviated. There was an exception observed in case of 0 µM

$FeCl_3$ whose exponential phase was observed at 10 min of incubation. There was an increment of 48.91, 27.94, 10.99 and 14.41% was observed in case of 0, 20, 75 and 100 µM $FeCl_3$ at 10 min time of incubation when compared to that of the 5 min time of incubation. The trend of nitrate uptake at 15 min time of incubation was found to be similar as 10 min time of incubation. Iron starved cells showed minimum nitrate uptake whereas 100 µM $FeCl_3$ treated cells showed maximum nitrate uptake. It is clearly evident that nitrate uptake rate was calculated at 5 min of incubation and minimum was recorded at 0 µM $FeCl_3$. It is also evident that gradual increase in the concentration of iron increased the nitrate uptake rate (Fig. 4b).

Fig. 5a showed nitrate accumulated inside the cells of cyanobacterium *A. sphaerica* after 4 days. Minimum nitrate was accumulated at iron starved condition whereas maximum nitrate was accumulated at 100 µM $FeCl_3$ followed by 75 and 20 µM $FeCl_3$.

There was a positive correlation between the iron and nitrogen uptake and accumulation (Fig. 4, 5). Once the nitrate enters inside the cell it is metabolized into the simplest form i.e. from nitrate to nitrite, nitrite to ammonia and ammonia to glutamine through NR, NiR and GS enzyme activities which is available for the various metabolic activities. Though iron concentrations were very high at 75 (3.75 times of control) and 100 (5 times of control) µM $FeCl_3$ yet growth was not reduced significantly (Kunui and Singh, 2020) because nitrogen available through higher nitrate uptake and accumulation may supported the growth by involving themselves in maintenance of phenotypic and physiological constituents such as proteins and chlorophyll synthesis (Fig. 4, 5) (Kunui and Singh, 2020). More iron toxicity was observed at 100 µM $FeCl_3$ in terms of relative ROS level and membrane injury index justified more requirement of nitrogen in *A. sphaerica*. Further, NR and GS activity was accordance to the demand of nitrogen or its uptake and accumulation at each level of iron but trend was slightly changed and the nitrate accumulation rate was not accordance with NR and GS activity at 100 µM $FeCl_3$ (Fig. 5a, 5b, 5c). Additional requirement of nitrate may suggested the nonspecific function of a nitrate as osmoticum under stress conditions mainly during the shortage of organic compounds due to reduced pigment content and ultimately photosynthesis. Nitrogen is also considered as energy reservoir under stress condition and same may be enhanced in iron stressed *A. sphaerica* for fulfilling the energy demand to cope against the iron stress (Singh *et al*., 2017). Cellular nitrate demand with increased iron concentration has also been suggested in some phytoplankton and different microorganisms including *Anabaena* sp. during metal stress (Kunui *et al*., 2017).

The maximum nitrate reductase activity was observed in case of the cells of *A. sphaerica* treated with 75 µM $FeCl_3$ and the percent increased by 8.93 % as compared to that of the control. The reduction of NR activity was 28.44 % and 55.30 % in the cells of *A. sphaerica* treated at 100 µM $FeCl_3$ and 0 µM $FeCl_3$ respectively (Fig. 5b).

The cells of *A. sphaerica* were managed to perform less glutamine synthetase activity at iron deficient condition with respect to control (20 µM $FeCl_3$). The activity of GS was enhanced by 41.81 % at 75 µM $FeCl_3$ concentrations respectively with reference to the GS activity of 20 µM $FeCl_3$

treated cells of *A. sphaerica*. The GS activity of 100 µM $FeCl_3$ treated cells was also found to be enhanced by 6.3 % in contrast to 20 µM $FeCl_3$ treated cells of *A. sphaerica*. Moreover, the GS activity was drastically reduced at - $FeCl_3$ condition (Fig. 5c).

Enhancement of nitrate reductase activity upto 75 µM $FeCl_3$ proved the enzyme is a substrate inducible and also suggested the increased nitrogen demand upto these concentrations (75 µM $FeCl_3$) (Fig. 5b). It was earlier proved that the presence of nitrate induced the protein synthesis and sometimes plays critical role in osmotic stability (Kunui *et al*., 2017). At the highest concentration of iron (100 µM $FeCl_3$), NR and GS activity was reduced which might be associated with limited supply of ATP during iron toxicity. It was evident from the published data that the chlorophyll a synthesis was lost upto some extent at 100 µM $FeCl_3$ (Kunui and Singh, 2020). Hence, the ultimate product of photosynthesis in form of ATP has reduced and the supply of energy has also been reduced for NR and GS activity (Perej *et al*., 2012).

Enhanced NR and GS activity at 75 µM $FeCl_3$ was also due to the uptake and accumulation of more nitrates into the cells which might have induced the synthesis of NR protein. Moreover, lower iron accumulation upto 75 µM $FeCl_3$ may lead to less energy consumption in protection from iron toxicity. This result was strengthened with the minimum relative ROS level and minimum membrane injury index. On the other hand, iron deficient condition would not be able to fulfill the cellular iron requirement for procuring normal NR and GS activity in *A. sphaerica* (Fig. 5b, 5c). Since iron is required during synthesis of ferredoxin which contributes important role in NR activity. Some of the similar reports are also available from the *Anabaena* sp. BI42 and *Calothrix* sp. BI22 under different stress condition (Perej *et al*., 2012; Ekinci *et al*., 2020).

Another, plausible cause of reduction in NR and GS activity was the enormous production of ROS at 100 µM $FeCl_3$ because the cells of *A. sphaerica* might have shifted surplus energy in performing various antioxidative defence mechanism (synthesis of antioxidants and antioxidative enzymes) at elevated level that probably resulted into reduced enzyme activities of nitrogen metabolism (Fig. 5b, 5c). A reduction in the GS activity in presence of iron deficient condition might be due to improper supplement of nutrient to the cell to influence proper ATPase activity as reported in case of Ca^{2+} and Pi starved condition (Singh and Singh, 2000).

The highest ROS generation was visualized in cyanobacterium *A. sphaerica* treated with 100 µM $FeCl_3$ (78.89 % increase) whereas there was 30 % relative ROS level was increased at iron deficient condition (-$FeCl_3$). 16.7 % of the relative ROS level was observed in the cells of *A. sphaerica* at 75 µM $FeCl_3$ as compared to that of control (Fig. 6a). Membrane injury index was maximally depicted at 100 µM $FeCl_3$ treated cells of *A. sphaerica* and was reported to be 84.4% followed by 0 µM $FeCl_3$ (75.1%) and at 75 µM $FeCl_3$, only 35.4% of membrane injury was noticed as compared to control (Fig. 6b). Excessive availability of iron at 100 µM $FeCl_3$ concentration might have actively participated in Fenton's as well as Haber-weiss reaction to produce $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$ which caused highest membrane injury (Kaushik *et al*., 2016; Kunui *et al*., 2017).

The antioxidative enzyme activity was measured in terms of % increase or decrease with respect to the activities at control (20 µM FeCl$_3$). Iron deprived condition showed a decrement of 23 % and 37.5 % of reduction at SOD and CAT activity whereas 77.03 % and 68.7% of enhancement was recorded in APX and GPX activity. At 75 µM FeCl$_3$, a sequential increment of 18 %, 73.4 %, 20 % and 38.24 % was recorded for each of the antioxidative enzyme status. Similar to 75 µM FeCl$_3$, 100 µM FeCl$_3$ also showed sequential increment of 18.3 %, 81.12 % and 63.87 % in case of SOD, APX and GPX activity (Fig. 6c) but the catalase activity was decreased by 45 %. It was evident from the data that though maximum iron accumulation caused almost 80% relative reactive oxygen species and membrane injury index at 100 µM FeCl$_3$ condition but cyanobacterium *A. sphaerica* somehow managed only 20 % reduction in the growth due to a pronounced SOD, APX and GPX activity. Likewise, APX and GPX managed to combat the ROS production in the cell at 0 µM FeCl$_3$ condition. Contrastingly, though there were considerable accumulation of iron but growth was reduced only by 5 % at 75 µM FeCl$_3$ condition (Fig. 6c). Possibly, enhanced level of efficient antioxidative machineries i.e. SOD, APX, CAT and GPX were equally responsible to neutralize the toxic effect of ROS at 75 µM FeCl$_3$ condition (Fig. 6c). An iron is also one of the structural components of superoxide dismutase enzyme in form of Fe-SOD. An ample supply of iron at 75 and 100 µM FeCl$_3$ condition also justifies the active participation in structural management of the enzyme. APX is known to work more efficiently in ROS scavenging procedure to detoxify H$_2$O$_2$ production which can be easily correlated at each iron condition as compared to 20 µM FeCl$_3$ condition in our findings (Fig.6c). GPX comprises of an aromatic group that acts as an electron donor. This electron donor group attracts and scavenges the ROS generated within the cell. GPX actually converts hydrogen peroxide radicals to water and reduced glutathione. Similar findings were reported in various *Nostoc muscorum*, *Nostoc sp.*, *Spirulina* sp. etc. at different stress regimes (Kannaujiya and Sinha, 2017; Kumar *et al.*, 2018).

From the above observations, it can be concluded that the cyanobacterium *Anabaena sphaerica* exhibits elevated iron tolerance upto 100 µM with only 27% reduction in growth and is an outstanding bioaccumulator of iron. Interestingly, the organism efficiently sustains its ability to acquire, reduce and assimilate nitrate even at the highest concentration of iron used in this study. These peculiar intrinsic features of *A. sphaerica* are supposed to be the cumulative outcome of efficient antioxidative defense machineries. Therefore, the impeccable capacity to store high amount of iron inside the cells with moderately reduced growth and metabolism make this organism a suitable candidate for bioremediation application.
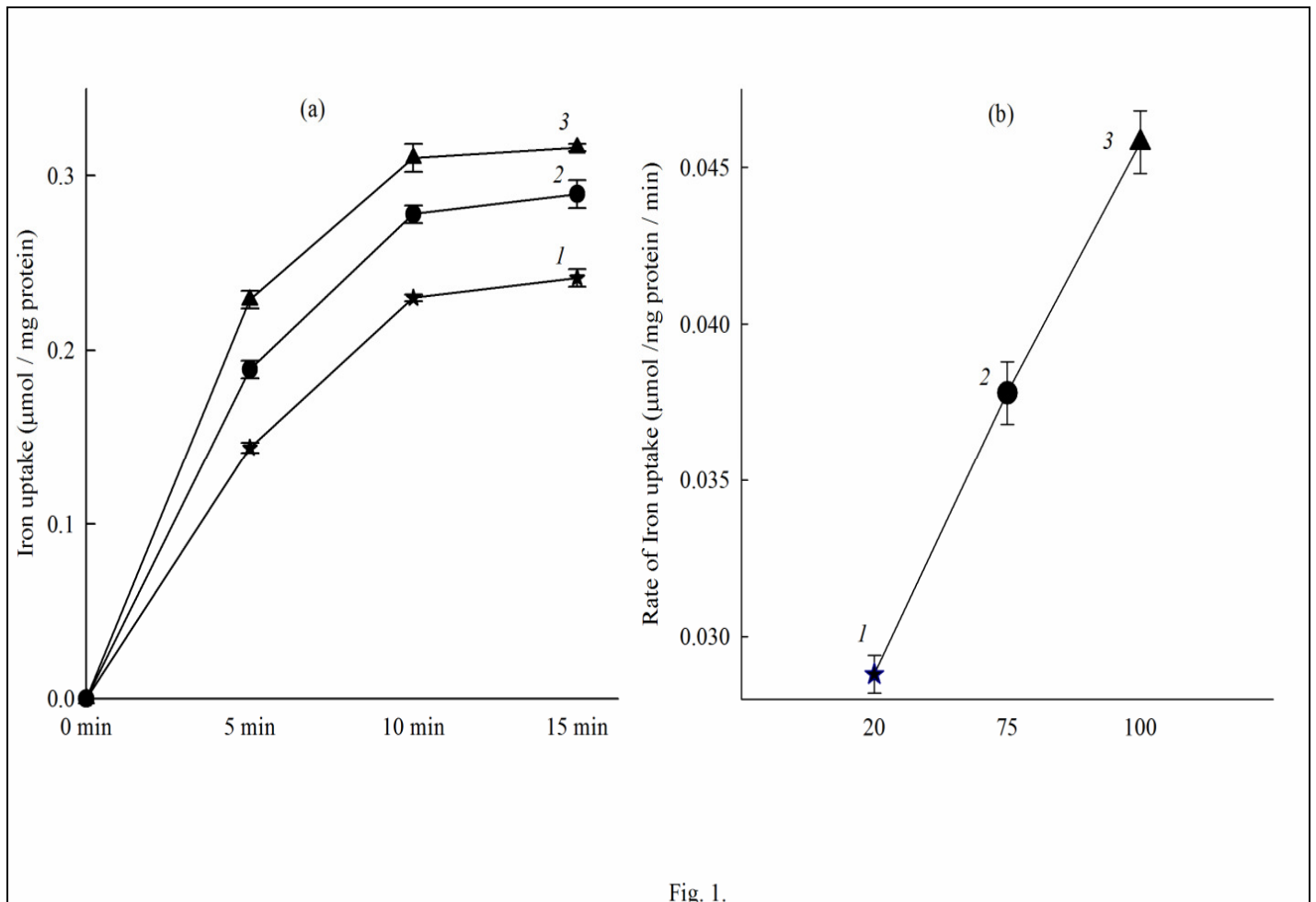

Fig. 1.

Fig. 1. Representation of the iron uptake (a) at different time interval (0, 5.10 and 15 minutes) and rate of iron uptake (b) in *A. sphaerica* at different iron regimes 20 (*1*), 75 (*2*), 100 (*3*) µM FeCl$_3$ and different time intervals. Data represents mean ± SD (n=6).
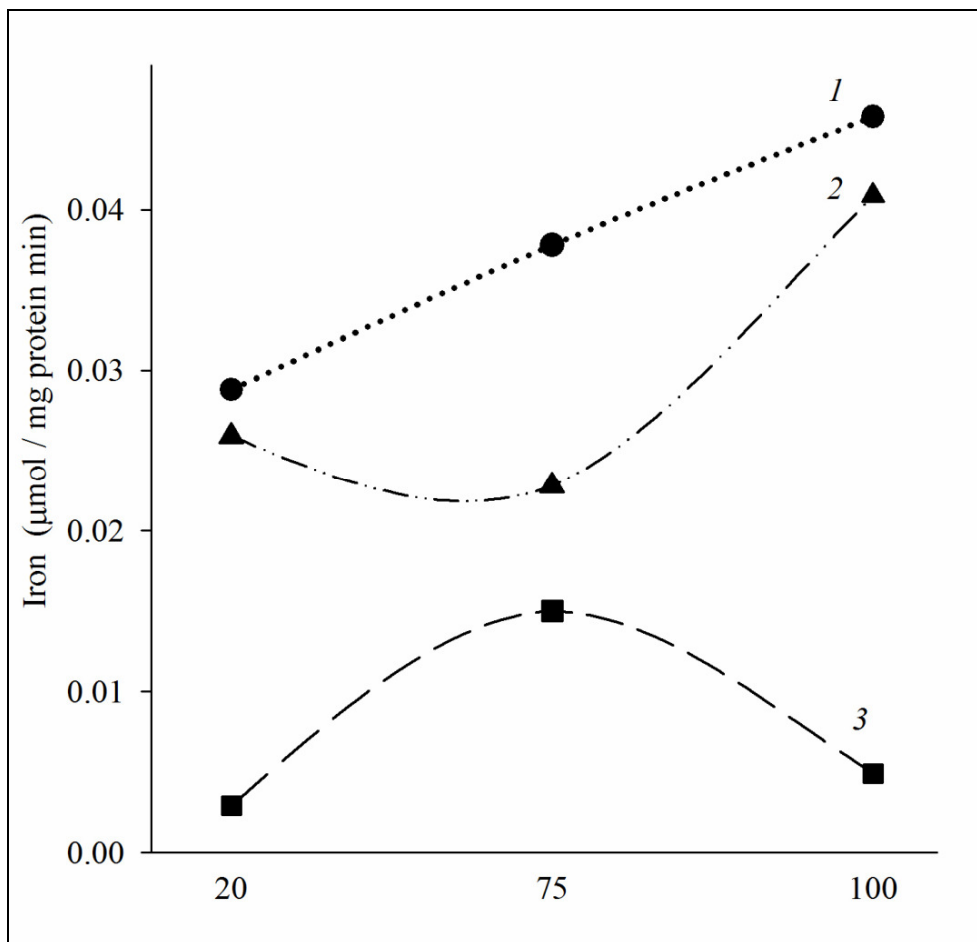
Fig. 2 : Representation of relationship among iron uptake rate (*1*), iron efflux rate (*2*) and the residual iron (*3*) in the cells of *A. sphaerica* incubated at different concentrations of iron (FeCl$_3$) i.e. 20, 75 and 100 µM. Data represents mean ± SD (n=6).
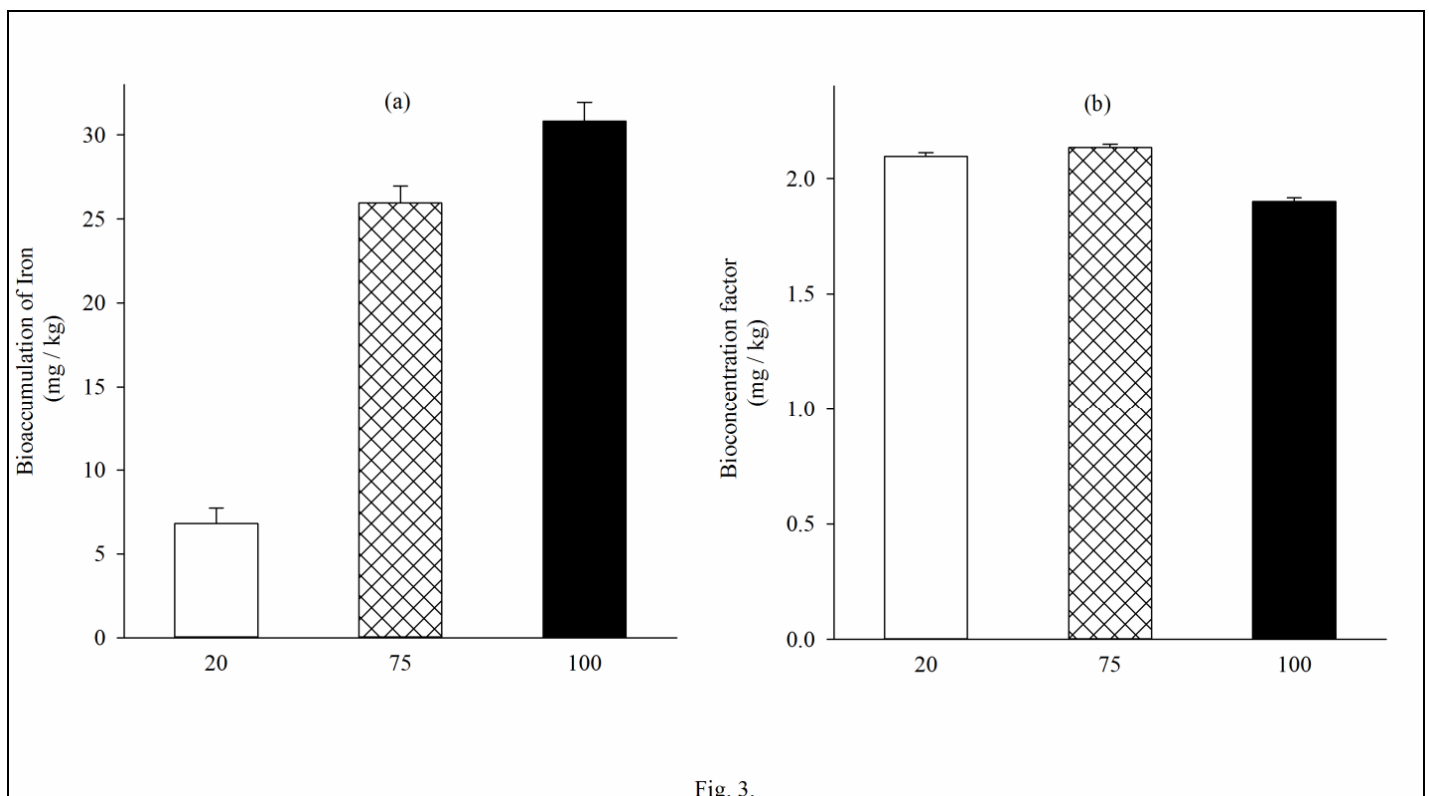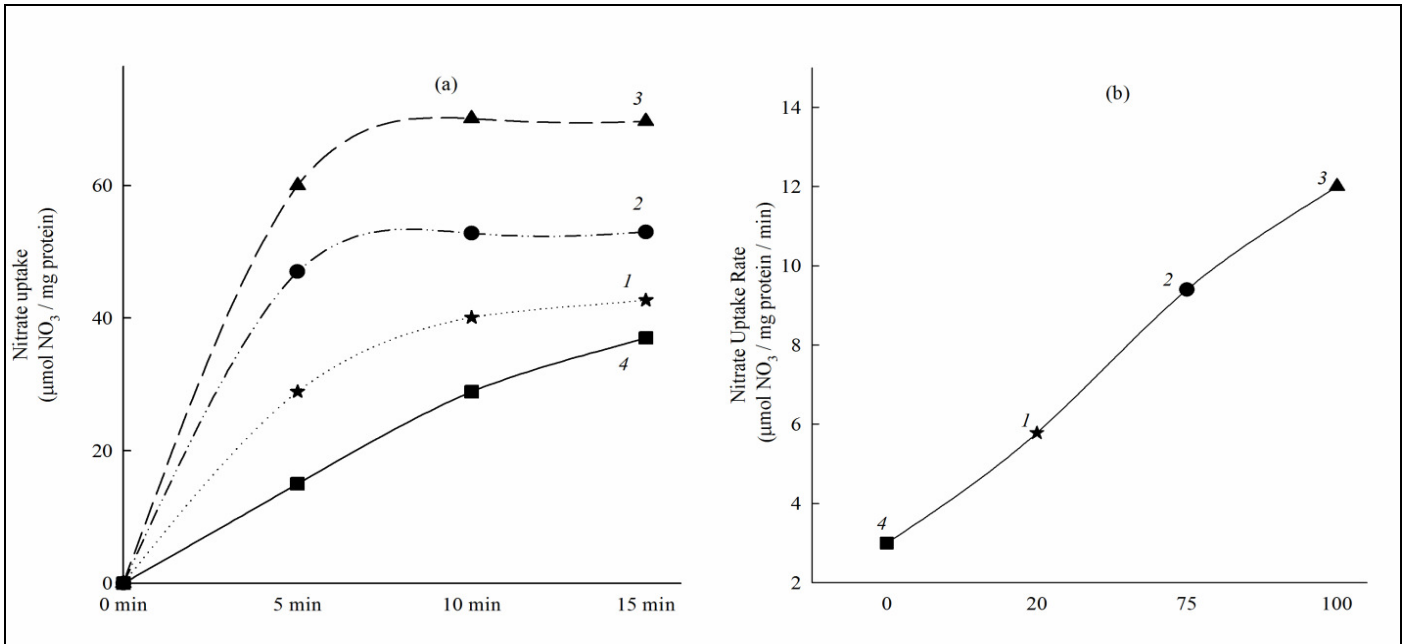

Fig. 3.

Fig. 3 : Representation of bioaccumulation of iron (a) and its bioconcentration factor (b) in the cells of *A. sphaerica* under different concentrations of FeCl$_3$ (µM) i.e. 20, 75 and 100. Data represents mean ± SD (n=6).

Fig. 4 : Representation of the Nitrate uptake (a) at different time interval (o, 5, 10 and 15 minutes) and nitrate uptake rate (b) in *A. sphaerica* incubated at different concentrations of FeCl$_3$ (0 (*4*), 20 (*1*), 75 (*2*) and 100 (*3*) µM FeCl$_3$). KNO$_3$ (5 mM) was used for nitrate uptake. Data represents mean ± SD (n=6).
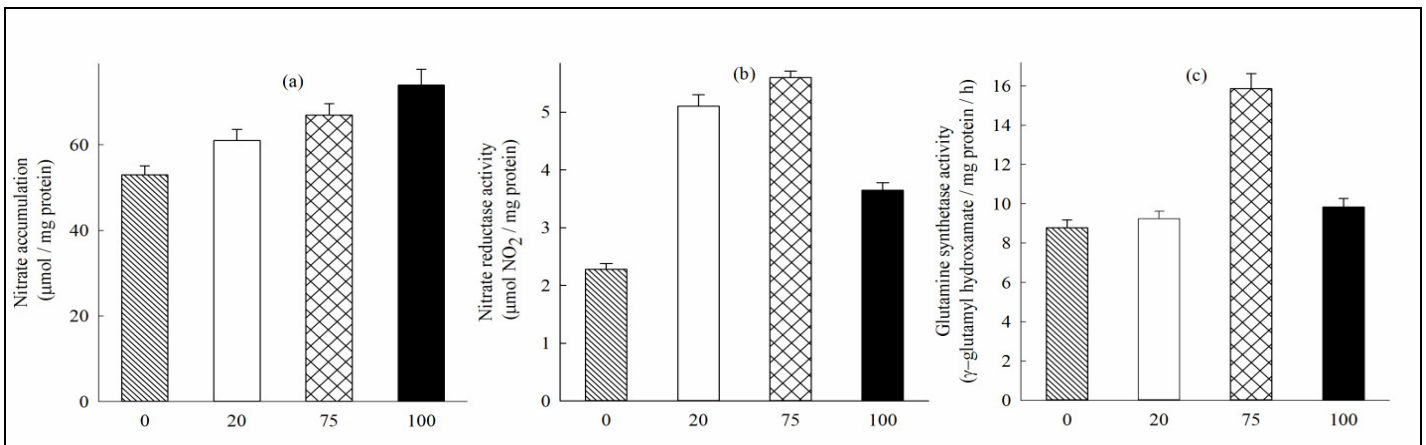

Fig. 5 : Effect of iron on Nitrate accumulation (a), Nitrate reductase activity (b) and Glutamine synthetase activity (c) in *A. sphaerica* incubated at different concentrations of FeCl$_3$ (0, 20, 50, 75 and 100 µM) supplemented with 5 mM KNO$_3$. Data represents mean ± SD (n=6).
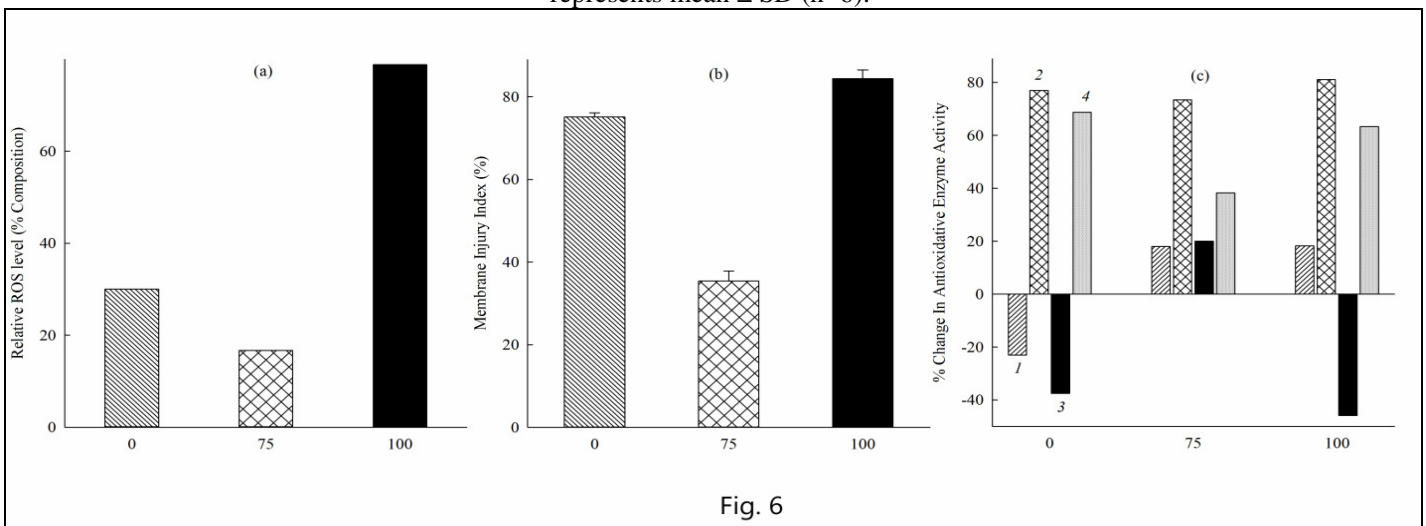

Fig. 6

Fig. 6 : Comparative study of relative ROS level (a), Membrane Injury Index (b) and % change in antioxidative enzyme activity (c) *viz.* SOD (*1*), APX (*2*), CAT (*3*) and GPX (*4*) at different regimes of iron (0, 75 and 100 µM FeCl$_3$) against control (20 µM FeCl$_3$ concentration) in *A. sphaerica* exposed to the different concentrations of iron (0, 75 and 100 µM FeCl$_3$). Data represents mean ± SD (n=6).

Acknowledgements

Conflict of interest: The authors declare that they have no conflict of interest.

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