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Differential Responses of Superoxide Dismutase in *Anabaena variabilis* Kutz to Metal Chelators

V. Padmapriya* and N. Anand

Centre for Advanced Studies in Botany, University of Madras,
Guindy Campus, Chennai - 600 025, India.

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Cyanobacteria have an evolved antioxidant system that catalyzes the harmful oxy radicals produced during photosynthesis. The differential responses of superoxide dismutase (SOD), nitrogenase, growth and physiological processes in the presence of five different chelators at varying concentrations with different chelating properties were investigated in the heterocystous cyanobacterium, *Anabaena variabilis*. Growth and enzyme activities were found to be influenced by the chelator properties. Ethylene diamine tetra acetic acid (EDTA) was the most potent chelator while 2, 2, bipyridyl (2,2-BP) stimulated growth. Nitrogenase and SOD activities decreased in the presence of EDTA, diamino tetraethyl penta acetic acid (DTPA) and 2,2-BP. The absence of SOD activity at higher concentrations of EDTA and DTPA was also detected in the native gel electrophoresis assays. Of the five chelators, EDTA, DTPA and 1, 10-phenanthroline (Phen) degraded cells at high concentrations. Increased nitrogenase activity with a decreased SOD activity at low chelator concentration and decreased nitrogenase activity with an increased antioxidant activity was observed at high concentrations of 8-hydroxyquinoline (HQ). These results suggest that for cyanobacteria, even tightly bound iron is biologically available but iron bound to some extremely strong chelators may become biologically unavailable.

Key words: Superoxide dismutase, Cyanobacteria, *Anabaena variabilis*, Nitrogen-fixing enzymes, Metal chelators.

Cyanobacteria have been subjected to various unfavorable environmental changes and their existence till date is a proof of an effective stress combat system prevalent in these organisms. The organisms cope with this pressure by the help of a cascade of antioxidants, where the SOD act initially and the catalase and peroxidase enzymes function in the subsequent step. It was in 1969 that Mc Cord and Fridovich[1] discovered the catalytic activity of the metalloenzyme, SOD, and named it as such. SOD is actively involved in the defense of the organism against oxy radicals (O_2). Cyanobacteria are oxygen evolving photosynthetic prokaryotes and produce reactive oxygen species that can damage cellular components leading to cell death. Therefore, the co-evolution of an antioxidant system occurred with the ubiquitous metalloenzymes, SODs, catalyzing the disproportion of superoxide radicals to peroxide and molecular oxygen through alternate oxidation

* To whom all correspondence should be addressed.
Tel.: +91-044-26210089; Mob.: +91-9884247317;
E-mail: priyajc1@gmail.com

and reduction of their metal ions. SOD was first commissioned in 1985 to be researched as an enzyme drug in the USA for defence of donor organs against oxidative stress during periods of ischemia and reperfusion[2]. SODs are highly valued as therapeutic 'enzyme drugs'[3,4]. O_2^- radicals are unable to cross the cytoplasmic membranes and it becomes imperative that they are removed from the source and this may have contributed to the compartmentalization of SOD enzymes[5,6]. Thus, a clear demarcation of the territorial boundaries of the SOD isozymes seems to have been chalked out. In the presence of metal chelators, a significant increase in catalase and SOD activities was observed[7,8,9]. Reactive oxygen species (ROS) formed in the biological system during the normal course of metabolism are potentially harmful agents, since they attack membranes, proteins and even DNA molecules. ROS formation is also increased in the presence of trace amounts of iron or other transition metal ions. To counteract this effect, chelators have been used as protective agents in *in-vivo* studies. Cyanobacteria produce siderophores under iron-limiting conditions for efficient iron acquisition. Chelators are known to affect the iron acquisition from the environment. In the present study, the response of *A. variabilis* grown in the presence of the chelators, ethylene diamine tetra acetic acid (EDTA), diamino tetraethyl penta acetic acid (DTPA), 1,10-phenanthroline (Phen), 8 Hydroxyquinoline (HQ) and 2,2-bipyridyl (2,2-BP) was investigated.

MATERIAL AND METHODS

The *A. variabilis* cultures were obtained from the Culture Collection at the Center for Advanced Studies in Botany, University of Madras, Chennai, India. All chemicals and reagents were obtained from Sisco Research Laboratories, Mumbai, India. Cultures were grown and maintained in BG_{11} medium[10]. Nitrate free BG_{11} medium was prepared by omitting sodium nitrate (BG_{11N0}). The cultures were maintained at 27°C±1°C under fluorescent illumination of 30 to 40 $\mu Em^{-2}s^{-1}$ provided by fluorescent tubes (Philips Trulite, Col 82). Cultures were exposed to a 12 h light / 12 h dark photoperiod and swirled manually for five minutes, thrice daily. Growth was determined every fourth day up to 20 days by measuring the chlorophyll *a* content (11). Protein estimation was carried out using bovine serum albumin as standard and the results expressed in µg/mL (12). Photosynthetic oxygen evolution and respiratory oxygen consumption in the intact filaments were measured at 27°C using Clark type Oxygen Electrode (YSI model 53) at a light intensity of 100 $\mu Em^{-2}s^{-1}$ (13). Hydrogen peroxide estimation was carried out in the extracellular medium and the extinction coefficient of the quinoneimine dye (product) at 505nm was taken as $6.4 \times 10^3 M^{-1} cm^{-1}$ (14). Nitrogenase activity was measured in whole cells by the Acetylene Reduction Assay (ARA)[15] and the activity was expressed as nM of C_2H_4 h^{-1} mg Chl a^{-1}. Glutamine synthetase (GS) in cell free extract was measured by the biosynthetic assay and 1U of the enzyme corresponded to 1ìg Pi formed min^{-1} [16]. The GS activity was expressed as U mg $protein^{-1}$. The SOD activity was expressed in units (U) where 1 unit of the enzyme corresponded to the amount causing half the maximum inhibition of nitroblue tetrazolium (NBT) to blue formazan. The activity was calculated using the formula- U/mL = [(V_0/ v) – 1] (dilution factor); $V_{0:}$ A_{560} of control ; v: A_{560} of sample. The results were expressed as U mg $protein^{-1}$ [17]. The polyacrylamide gel electrophoresis (PAGE) for native gel-assay was carried out under a constant current of 10 mA under non-denaturing conditions. SOD bands appear colorless against a blue background and isozymes were detected by the method of Beyer and Fridovich[18]. The gels were cut and soaked in 2 mM of KCN or 2 mM of H_2O_2 for 10 mins prior to soaking in NBT. FeSOD is inactivated by H_2O_2, Cu-ZnSOD is inactivated by KCN while MnSOD remains unaffected by these compounds. All the experiments were conducted in triplicates. Standard deviation and One-Way ANOVA were used for the statistical analysis of the data.

RESULTS AND DISCUSSION

Table 1 gives the growth, oxygen rates and peroxide content in the external medium of the chelators-treated cells. A significant decrease in the chlorophyll *a* levels (by 64% at 1 mM concentration) and protein content (by 37% at

0.75 mM) of the cells grown in EDTA amended medium was observed when compared with control. A similar effect was seen in the oxygen rates also. A marked decrease (nearly 55% at 0.25 mM) in the photosynthetic rates was noted in the amended cells when compared with control. Photosynthesis rates were totally absent and

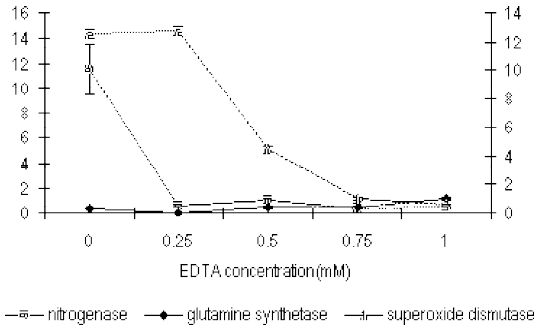

Fig. 1. Enzyme activities in the presence of EDTA

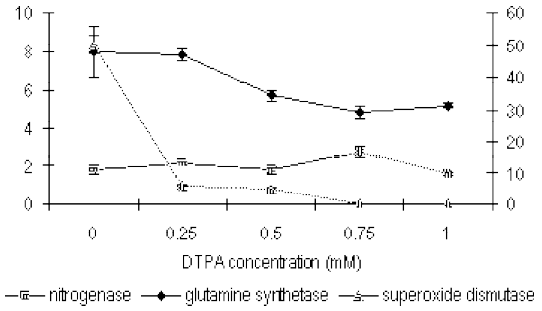

Fig. 2. Enzyme activities in the presence of DTPA

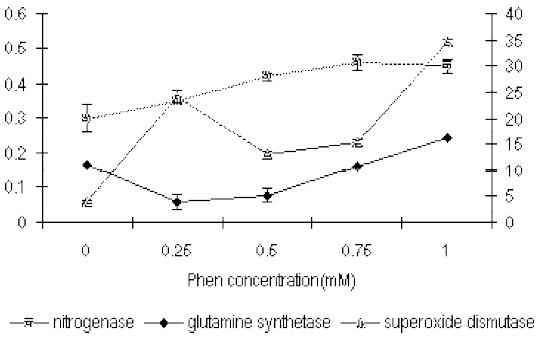

Fig. 3. Enzyme activities in the presence of HQ

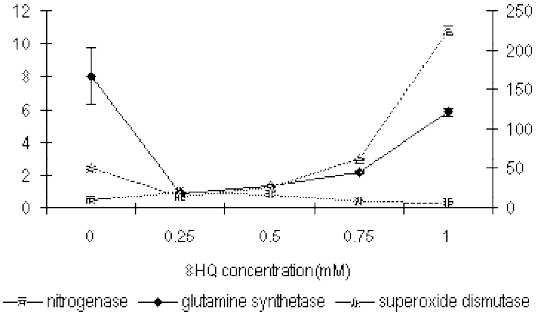

Fig. 4. Enzyme activities in the presence of Phen

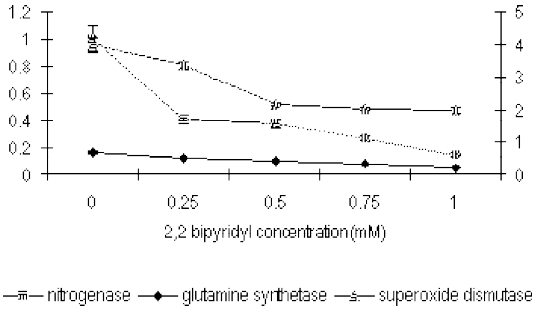

Fig. 5. Enzyme activities in the presence of 2,2-BP

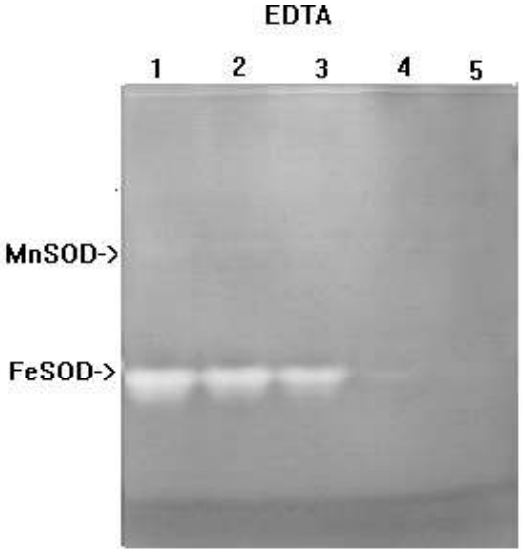

Fig. 6. Native PAGE showing the isozymes of SOD in *A. variabilis* treated with EDTA. Lane 1: Control, Lane 2: 0.25mM, Lane 3: 0.5mM, Lane 4: 0.75mM, Lane 5: 1.0mM

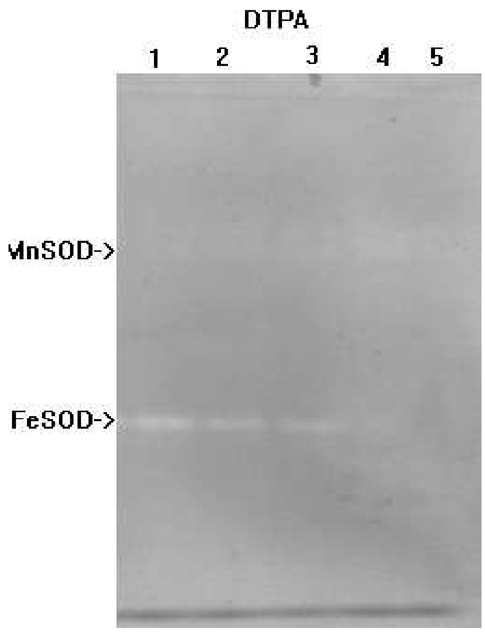

Fig. 7. Native PAGE showing the isozymes of SOD in *A. variabilis* treated with DTPA. Lane 1: Control, Lane 2: 0.25mM, Lane 3: 0.5mM, Lane 4: 0.75mM, Lane 5: 1.0mM

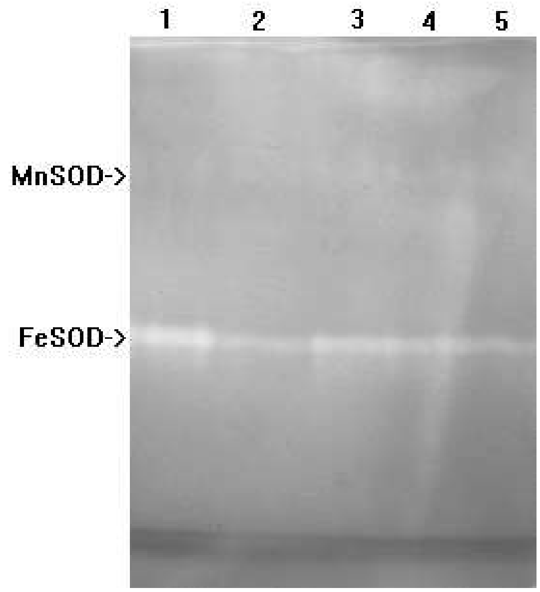

Fig. 8: Native PAGE showing the isozymes of SOD in *A. variabilis* treated with Phen. Lane 1: Control, Lane 2: 0.25mM, Lane 3: 0.5mM, Lane 4: 0.75mM, Lane 5: 1.0mM

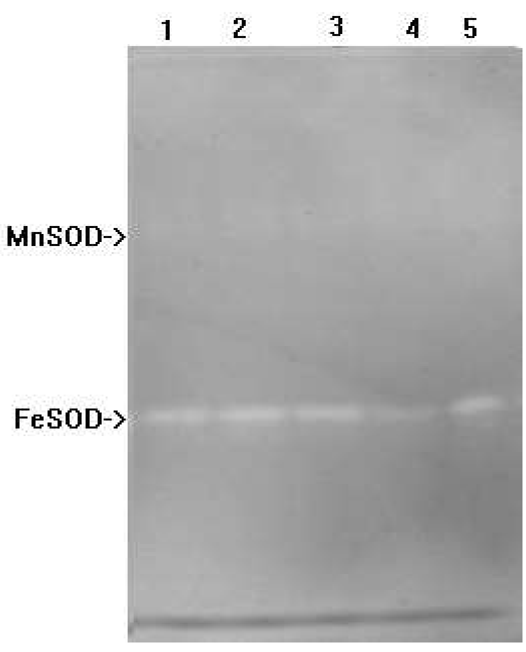

Fig. 9. Native PAGE showing the isozymes of SOD in *A. variabilis* treated with HQ. Lane 1: Control, Lane 2: 0.25mM, Lane 3: 0.5mM, Lane 4: 0.75mM, Lane 5: 1.0mM

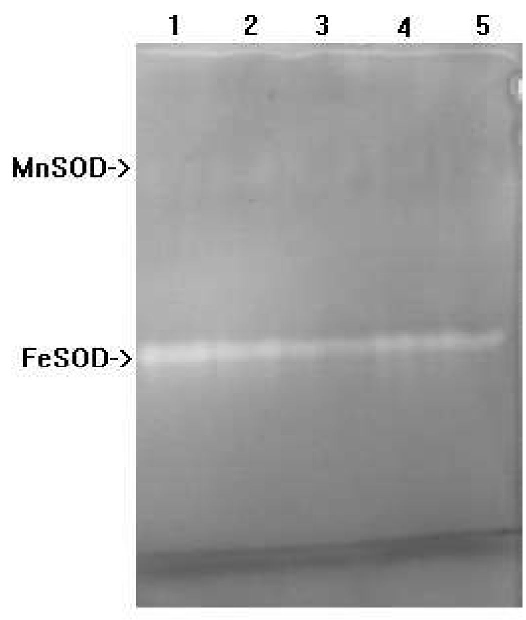

Fig. 10. Native PAGE showing the isozymes of SOD in *A. variabilis* treated with 2,2-BP. Lane 1: Control, Lane 2: 0.25mM, Lane 3: 0.5mM, Lane 4: 0.75mM, Lane 5: 1.0mM

respiratory rates were significantly decreased at high concentrations of EDTA. At EDTA 1 mM, the extracellular peroxide content was nearly 9 ìM/mL. Significant decreases in the antioxidant and nitrogenase activities were seen in the EDTA-treated cells (almost 96% inhibition of both the enzymes at EDTA 1 mM). The GS activity was higher at EDTA 1mM compared with nitrogenase and SOD activities (Fig 1). The chelator seems to adversely affect the metal containing enzymes. Figs 6-10 show the activity of the SOD isozymes in polyacrylamide gel electrophoresis assays. The FeSOD is barely detectable at higher chelator concentrations of 0.75 mM and 1.0 mM (Fig 6). As seen in the EDTA amendments, DTPA also inhibited the growth of the organisms in terms of protein and chlorophyll *a* content. Significant decrease in growth was seen as the DTPA concentration increased, resulting in nearly 78% reduction in protein content of cells grown in DTPA 1 mM and 36% reduction in chlorophyll *a* levels. At DTPA 0.25 mM, photosynthetic rates were reduced by 25% compared with control and at higher concentrations, photosynthesis was completely absent. Respiratory rates at DTPA 0.5 mM and 1 mM were significantly reduced by 53% and 67%, respectively. Peroxide levels increased significantly and at DTPA 1 mM, it was nearly 17±1 µM/mL in the medium. A highly significant decrease in the antioxidant activities was observed in DTPA exposed cultures compared with control. At DTPA 0.75 mM and 1 mM, there was a total absence of the enzyme activity (Fig 2). GS activity was the lowest at DTPA 0.75 mM concentration

Table 1. Protein, Chlorophyll *a*, oxygen rates and external medium peroxide levels in *A. variabilis*

	Chelator concentration (mM)	Protein	Chlorophyll	Photosynthesis	Respiration	Peroxide content
EDTA	0	35.9±7.0	3.65 ±0.1	570.82±21.8	304.64±87.6	5.7±1.7
	0.25	26.9±5.7	2.16 ±0.3	242.01±0.1	135.83±43.4	9.78±0.4
	0.5	27.0±1.5	2.01 ±0.3	0	211.55±15	4.58±0.2
	0.75	22.6±1.3	1.8 ±0.3	0	124.31±49.8	8.05±0.12
	1.0	25.7±4.1	1.31 ±0.08	0	63.74±19	9.29±0.7
DTPA	0	81.5 ±11	0.73±0.02	407.5±10.5	140.06±3.6	2.35±0.8
	0.25	72.9±8.5	0.57±0.05	294.8±26.6	179.14±27.2	1.78±1.6
	0.5	35.7±13	0.58±0.06	0	64.41±6.3	3.98±1.4
	0.75	23.8±5.2	0.41±0.02	0	99.41±3.6	10.9±2
	1.0	18.3±1.5	0.47±0.02	0	55.24±2	16.84±0.9
Phen	0	22.8±3.4	2.2±0.02	190.67±14.6	92.08±14.7	7.73±0.7
	0.25	21.5±0.9	1.83±0.18	0	42.4±2.8	8.6±0.7
	0.5	22.7±0.3	1.54±0.03	0	14.81±2.2	7.24±0.6
	0.75	23.4±0.8	1.4±0.05	0	23.35±0.7	11.77±0.6
	1.0	18.6±0.3	1.73±0.02	0	7.09±0.9	9.75±0.6
HQ	0	35.5±0.9	2.03±0.1	128.91±20.9	72.77±13.4	2.95±0.3
	0.25	22.6±4.5	1.54±0.05	0	106.51±17.4	2.7±0.1
	0.5	19.5±1.7	1.46±0.02	0	60.15±22.6	2.64±0.1
	0.75	12.3±2.3	1.51±0.06	0	46.15±27.4	1.73±0.5
	1.0	16.3±0.9	1.55±0.07	0	39.84±18.5	3.96±0.1
2,2-BP	0	41.6±0.3	2.63±0.3	158.45±18.5	54.71±9.8	11.9±3.0
	0.25	36.8±0.4	1.62±0.4	0	62.17±6.2	22.3±2.0
	0.5	39.3±0.2	1.55±0.3	0	59.47±2.5	16.4±2.6
	0.75	42.9±0.1	1.8±0.6	0	39.42±10.6	14.4±2.3
	1.0	45.4±0.1	3.17±0.1	0	17.74±0.1	16.9±2.0

with a decrease of nearly 32% compared with control. The nitrogenase activity was significantly high at DTPA 0.75 mM (around 59% more than control) (Fig 2). Fig 7 shows the presence of the SOD isoforms detected in native-PAGE of DTPA amended cells. FeSOD was not observed in cell extracts of organisms grown at DTPA 0.75 mM and 1.0 mM. In the Phen-treated cells, chlorophyll *a* content was reduced significantly (28% in 1 mM treated cultures) compared with control. Protein content though was not affected with only 17% reduction seen at Phen 1 mM. Total absence of photosynthesis was observed in the Phen-treated cells. Significant decrease in respiration was noted, similar to the previous experiments. There was a significant increase in peroxide levels at Phen 0.75 mM and 1.0 mM concentrations, nearly 22% and 48%, respectively. The enzymes do not seem to be adversely affected by the presence of the chelator (Fig 3). At Phen 0.25 mM, the SOD activity was nearly increased by 9-folds compared with control. All the enzymes showed significantly higher activities at Phen 1 mM concentration (SOD: ~8.7 fold, GS: ~1.5 fold and Nitrogenase: ~15 folds). Fig 8 shows the isozymes of SOD when *A. variabilis* was treated with varying concentrations of Phen. HQ also shows similar effect of growth inhibition as the above chelators. Significant decrease in the chlorophyll *a* and protein content was observed. Chlorophyll *a* content though did not decrease as chelator concentration increased and was present at 1.5±0.04 µg/mL throughout the amendment series. Total absence of photosynthesis was noted in the amended cultures. Respiratory activity at HQ 1mM was decreased by approximately 47%. Peroxide concentration was approximately 3.8±0.2 mM/mL at HQ 1mM. Both SOD and GS activities were significantly low at HQ 0.25 mM 80% and 87.5% decrease, respectively (Fig 4). At HQ 1 mM, high activities of SOD were observed (Fig 4). Nitrogenase activity was significantly decreased at HQ 1 mM compared with control. In-gel assays of the SOD isozymes are shown in Fig 9. In the presence of 2,2-BP, the protein content decreased only at low chelator concentrations of 0.25 mM and 0.5 mM but increased at 0.75 mM and 1 mM when compared with control. There was complete absence of photosynthesis and the respiratory rates were decreased in the chelator-amended cells compared with control. Peroxide concentration increased from 25-100% in the extracellular medium of the 2,2-BP amended cells. Both the antioxidant and nitrogenase enzymes were adversely affected by the presence of chelator (Fig 5). The nitrogenase enzyme showed inhibitions of approximately 60% (0.25 mM) to 84% (1 mM). SOD activity was inhibited by approximately 13% (0.25 mM) to 50% (1 mM). GS activity increased by nearly 2 folds at 2,2-BP 1 mM compared with control. Fig. 10 shows the presence of the SOD isoforms resolved on 10% gel when 2,2-BP was amended to the medium. The presence of MnSOD was detected as very faint bands while FeSOD bands were distinct. FeSOD bands in control lanes are prominent than those of the treated cells. Metal chelators are supplied in the medium to ensure that the essential elemental nutrition is available to the organisms. Chelators are widely used in the protection from pro-oxidant conditions, in the prevention of *in vitro* iron-mediated $OH^{\bullet}$ formation and also as tools to study the role of iron oxidative stress process[19-21]. Schiavone and Hassan[22] carried out experiments with the metal chelator 2,2-BP to study the effect on SOD in some prokaryotes (bacteria). They found that in some forms the chelator induced MnSOD, in others there was a reduction in FeSOD activity while in still others, there was no induction or inhibition of the enzyme activity. Studies on the iron chelator, Phen, showed that the chelator was not able to fully block the Fenton reaction and thus damage to the 2-deoxyribose oxidative degradation and plasmid DNA was observed (23). It was also noted that though Phen reduces $OH^{\bullet}$ formation from Fe (II) plus H_2O_2, the interaction between Fe $(Phen)_3^{2+}$ (and / or Fe $(Phen)_2^{+2}$) and H_2O_2 still produces a highly active oxidizing agent[23]. FeSOD activity in *Plectonema* was partially inhibited when treated for 24 hours with Phen. Among the several chelators tested, dithizon and toluene-3, 4 dithid inhibited the FeSOD even after the removal of the chelator[24]. In studies using the metal chelators-EDTA, DTPA and HQ, a biphasic response of all the chelators along with the increased in SOD content in cells due to the synergistic action with metal ions and paraquat was reported[25]. In another study, it was observed that *A. flos-aquae* cells exposed to various

chelators of greatly varying affinity for Fe^{3+} (HEDTA, EDDHA, desferrioxamine mesylate, HBED, HQ) did not show any iron uptake in the presence of HBED, the strongest of the tested chelators[26]. Cyanobacteria are able to avail even tightly bound iron but in the presence of very strong chelators, the iron may become biologically unavailable resulting in an increase in the stress leading to an increase in the antioxidant enzymes, as observed in the current study.

CONCLUSION

In the present study, of the five chelators chosen to study their effect on the SOD enzyme in *A. variabilis*, 2,2-BP stimulated growth and EDTA was the most potent chelator. EDTA, DTPA and Phen degraded cells at higher concentrations. Both nitrogenase and SOD enzymes were decreased in the presence of EDTA, DTPA and 2,2-BP while DTPA was the most potent inhibitor of enzyme activity. Increased nitrogenase activity with a decreased SOD activity at lower concentration and vice-versa at higher concentration was observed in the presence of HQ treatments. The absence of SOD enzyme at higher concentrations of EDTA and DTPA was observed in the native PAGE.

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REFERENCES

1. Mc Cord, J. M., Fridovich, I. Superoxide dismutase: An enzymic function for erythrocuprein (hemocuprein). *J. Biol. Chem.* 1969; **22**: 6049-6055.
2. Vellard, M. The enzyme as drug: Application of enzymes as pharmaceuticals. *Curr. Opinion in Biotech*. 2003; **14**: 1-7.
3. Bannister, J.V., Bannister, W.H., Rotilio, G. Aspects of the structure, function and applications of superoxide dismutase. *CRC Critical Rev. Biochem*. 1987; **22**: 111-180.
4. Proctor, P.H., Reynolds, E.S. Free radicals and disease in man. *Physiol. Chem. Phy. Med. NMR*, 1984; **16**: 175-195.
5. Takahashi, M.A., Asada, K. Superoxide anion permeability of phospholipid membranes and chloroplast thylakoids. *Arch. Biochem. Biophys*. 1983; **226**: 558-566.
6. Hassan, H.M., Fridovich, I. Paraquat and *Escherichia coli*. Mechanism of production of extracellular superoxide radical. *J. Biol. Chem*, 1979; **254**: 10846- 10852.
7. Asada, K., Kanematsu, S., Uchida, K. Superoxide dismutases in photosynthetic organisms: absence of the cuprozinc enzyme in eukaryotic algae. *Arch. Biochem. Biophys*, 1977; **179**: 243-256.
8. Pugh, S.Y.R., Fridovich, I.Induction of superoxide dismutase in *Escherichia Coli* by metal chelators. *J. Bacteriol*, 1985; **162**: 196-202.
9. Schiavone, J.R., Hassan, H.M. Biosynthesis of superoxide dismutase in eight prokaryotes: effects of oxygen, paraquat and an iron chelator. *FEMS Microbiol. Lett*, 1987; **42**: 33-38.
10. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J. Gen. Microbiol*, 1979; **111**: 1-61.
11. Mc Kinney, G. Absorption of light by chlorophyll solution. *J. Biol.Chem*, 1941; **140**: 315-322.
12. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*, 1976; 72: 248-254.
13. Allen, J. F., Holmes, N.G.The Liquid Phase Oxygen Electrode. *In*: Photosynthesis energy transdution, a practical approach. (Eds.) M.F. Hipkins and N.R. Baker. IRL press, 1986; 108-116.
14. Frew, J. E., Peter, J., Scholes, G. Spectrophotometric determination of hydrogen peroxide and organic hydroperoxides at low concentrations in aqueous solution. *Anal. Chem. Acta*, 1983; **155**:139-150.
15. Stewart, W.D.P., Fitzerald, G.P. Burries, H. *In situ* studies on nitrogen fixation using the acetylene redution technique. *Proc. Natl. Acad. Sci,* USA, 1967; **58**: 207: 2078.
16. Shapiro, B.M., Stadtman, E.R.Glutamine synthetase (*E. coli*). *Methods in Enzymology*, 1970; **17**A: 910-913.
17. Beauchamp, C., Fridovich, I. Improved assays and assay applicable to acrylamide gels. *Anal.*

Biochem, 1971; **44**: 276-287.

18. Beyer Jr, W. F., Fridovich, I.. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Analytical Biochemistry*, 1987; **161**: 559-566.
19. Halliwell, B., Gutteridge, J. M. C. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. Arch. *Biochem. Biophys,* 1986; **246**: 501-514.
20. Halliwell, B., Gutteridge, J. M. C. Role of free radicals and catalytic metal ions in human disease: an overview**.** *Methods Enzymol*, 1990; **186**: 1-85.
21. Halliwell, B., Gutteridge, J. M. C. Antioxidant defences. *In:* Free Radical in Biology and Medicine (Eds.) Halliwell, B., Gutteridge, J.M.C. 3rd Edn. Oxford Univ. Press, Oxford, UK, 1999; 105-245.
22. De Avellar, I.G.J., Magalhães, M.M.M., Silva, A.B., Souza, L.L., Leitão, A.C., Hermes-Lima, M. Reevaluating the role of 1, 10-phenanthroline in oxidative reaction involving ferrous ions and DNA damage. *Biochimica et Biophysica Acta*, 2004; **1675**: 46-53.
23. Schiavone, J. R,. Hassan, H. M. Biosynthesis of superoxide dismutase in eight prokaryotes: effects of oxygen, paraquat and an iron chelator. *FEMS Microbiol. Lett*, 1987; **42**: 33-38.
24. Asada, K., Yoshikawa, K., Takahashi, M., Maeda, Y., Enmanji, K. Superoxide dismutase from a blue-green alga, *Plectonema boryanum*. *J. Biol. Chem*, 1975; 250: 2801-2807.
25. Pugh, S. Y. R., Fridovich, I. Induction of superoxide dismutase in *Escherichia coli* by metal chelators. *J. Bacteriol*, 1985; **162**: 196-202.
26. Gress, C. D., Treble, R. G., Matz, C. J., Weger, H. G. Biological availability of iron to the freshwater cyanobacterium *Anabaena Flos-Aquae. J. Phycol.*, 2004; **40**(5): 805-807.