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ANTIOXIDATIVE ACTIVITY OF SUPEROXIDE DISMUTASE IN BLOOM FORMING CYANOBACTERIA

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ABSTRACT

Cyanobacteria are autotrophic prokaryotes with excellent antioxidative system that scavenges reactive oxygen species produced during photosynthesis. Superoxide dismutase (SOD) represent the primary defence against oxygen damage. The antioxidative activity of SOD enzymes was determined in cultures of the bloom forming cyanobacteria *Anabaena variabilis* and *Synechococcus elongates*. Experimental results depict higher antioxidative activity of SOD enzyme in *S. elongates* than *A. variabilis.* The isozymes of SOD in *A. variabilis* and *S. elongates* cultures were assayed by negative staining. Two bands were visualised in *S. elongates,* while one band was seen in *A. variabilis*. The activity of these bands was insensitive to 2 mM KCN but disappeared when 30% H_2O_2 was added to the activity staining solutions. Because Fe-SOD is insensitive to KCN but sensitive to H_2O_2, the bands present were of Fe-SOD. Growth analysis and Pigment profile of cultures was also studied. Screening cyanobacteria for SOD activity can help us in exploring the immense potential of this enzyme as therapeutic enzyme drug.

Keywords: **Antioxidative enzymes, Cyanobacteria, Superoxide dismutase (SOD).**

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INTRODUCTION

Cyanobacteria are unique photosynthetic prokaryotes. These are the first oxygen evolving organisms to convert the early reducing atmosphere into an oxidizing one. They can occupy various ecotopes by virtue of having high ecological adaptivity. Accumulation of nutrients results in the excessive growth of cyanobacteria in eutrophic water, which is commonly known as bloom. The bloom forming cyanobacteria found in fresh and marine waters whose blooms can produce toxins that are fatal to animals and human health. Cyanobacteria utilize the energy of sunlight to drive photosynthesis, a process where the energy of light is used to split water molecules into oxygen, protons, and electrons. The reactive oxygen species (ROS) generated during redox processes can cause a major damage to the DNA, proteins and other biological molecules. These ROS can be removed by both enzymatic as well as non-enzymatic methods. The superoxide anions, generated by Mehler –type reaction, are scavenged by superoxide dismutase and hence prevent the formation of other reactive oxygen species (i.e. H_2O_2 and $OH^{\cdot}$). Other ROS are destroyed by the phycobiliproteins and enzymes like catalase and peroxidase. Phycobiliproteins are the most accessory light-harvesting pigments in cyanobacteria (Glazer, 1988). It is reported that phycobiliproteins are used in the measurement of peroxy radical damage (DeLange *et al*., 1989). In *Spirulina* the phycocyanobilin have antioxidizing activity and therefore may act as an effective antioxidant in humans (Hirata *et al*., 2000). Superoxide Dismutase (SOD) catalyzes the dismutation of 0_2- to H_2O_2, and O_2, protecting cells from the effects of oxygen that are likely to be largely due to the primary production of 0_2-. SOD activity in free living cyanobacteria has been reported in *Anabaena cylindrica* (Mackey and Smith, 1983), while Fe-SOD and Mn-SOD were detected in *Plectonema boryanum*, *Anacystis nidulans*, and *Anabaena variabilis* (Okada *et al*., 1979). The objective of the present study was to determine the antioxidative activity of SOD enzyme and to establish the presence of its different isozymes in bloom forming cyanobacteria- *A. variabilis* and *S. elongates.* Growth analysis and pigment profile was also studied.

MATERIALS AND METHODS

Cyanobacterial Strains

Cyanobacterial strains *Synechococcus elongates* and *Anabaena variabilis* were isolated from natural blooms of northern India. The strains were purified and enriched in the laboratory in BG-11 medium (Stanier *et al*., 1972).

Growth Conditions

A. variabilis was grown in nitrate free BG_{11} medium. The cells were grown photoautotrophically in 500 ml Erlenmeyer flasks containing liquid BG_{11} medium at 25 ± 2°C under fluorescent illumination provided by fluorescent tubes exposed to a 12 h light / 12 h dark photoperiod and swirled manually for five minutes, thrice daily. Growth of two strains in the culture media was determined (every fifth day up to 35 days) by measuring the optical density (OD) of culture media at 660 nm.

Pigments

Chlorophyll-a was quantified in acetone extracts by taking absorbance at 630 nm, 645 nm, 665 nm according to equations derived by Richard and Thompson (1952) and modified by Parson and Strickland (1965), while allophycocyanin, phycocyanin and phycoerythrin were quantified in phosphate buffer extracts by taking absorbance at 562 nm, 615 nm and 652 nm using the equations given by Bennett and Bogorad (1971).

Preparation of cellular extracts

The fresh grown cultures were harvested by centrifugation for 10 min at 5,000 rpm. The pellets were washed. The cells were broken by following the procedure of osmotic shock treatment (Tao *et al*., 2002). The pellets were resuspended in buffer A (20mM Tris HCl, pH-

8.0, 10mM NaCl) containing 0.5 M sucrose. The temperature of pellets was raised to 35°C. To the cell suspension, lysozyme was added to a final concentration of 0.5 mg ml^{-1} and incubated at 30°C for 30 min (occasional shaking) before it was centrifuged at 5,000 × *g* for 5 min. The cell pellets were gently resuspended in an equal volume of buffer A without sucrose and centrifuged at 13,000 rpm for 30 min. The supernatant was used for electrophoresis, protein determination (Bradford method, 1976) and SOD enzyme assay (Beauchamp *et al*., 1971)

Quantitative analysis

The spectrophotometric method used for measuring the SOD activity in this study, was a slight modification of indirect inhibition assay developed by Beauchamp and Fridovich (1971).

Electrophoresis

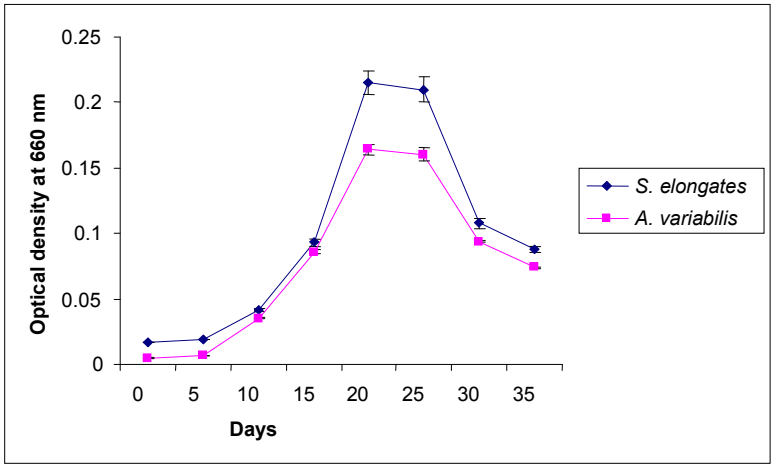
Electrophoresis was carried out at 4°C according to a modified procedure (Sambrook *et al*., 2001) with 10% polyacrylamide mini-slab gel in standard tris-glycine buffer (pH 8.3). Isoforms of SOD in solution were determined according to their sensitivity to 2mM KCN and 30% H_2O_2.

RESULTS AND DISCUSSION

Growth Measurements

From the beginning of inoculation to the end, cyanobacterial culture passed through several stages or phases (Graph 1). After 6th day to 20th day, the cyanobacterial cells showed exponential growth. But from 20th day to 25th day, the cyanobacterial cells entered into the stationary phase in which some cells died and some grew. After 25th day onwards the number of cells in the culture media gradually decreased due to decrease in the nutrients, accumulation of the toxic metabolites and cell-death. However, some of these cells were still viable and were used to initiate new cultures.

Graphs and Figures

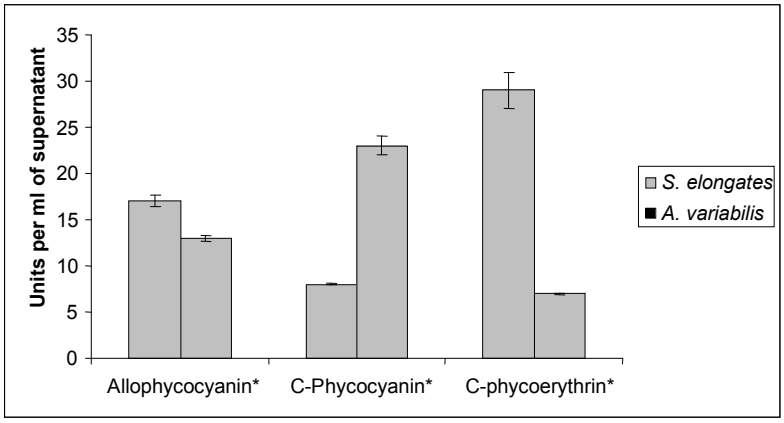


Graph 1
Growth curve of A. variabilis and S. elongates.

Pigment analysis

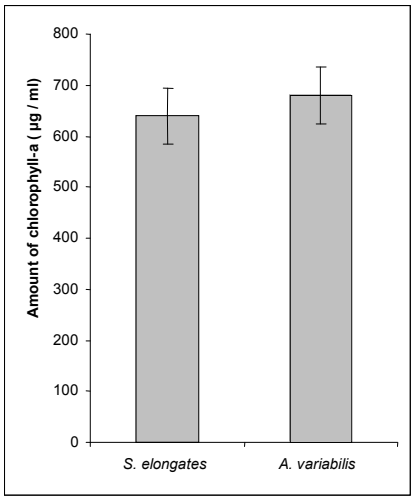
The amount of Chlorophyll-a and Phycobiliproteins(C-phycocyanin, C-phycoerythrin and allophycocyanin) was determined.Among the two species tested (Table1), the amount of chlorophyll-a and C-Phycocyanin was higher in *A. variabilis* than in *S. elongates* (Graph-3). While the amount of allophycocyanin and C-phycoerythrin was higher in *S. elongates* than in *A. variabilis* (Graph-2). Differences among species in phycobiliprotein content may be related to the

light environment in which the algae grow. Cyanobacteria that grow in shallow freshwater environments generally contain red-light-absorbing phycocyanin as the primary accessory pigment, whereas cyanobacteria from the open ocean also possess phycoerythrin, which absorbs the blue-green and green light that penetrates to greater depths. Cyanobacteria contain abundant phycobiliproteins. Major producers of phycobiliproteins are the cyanobacteria *Arthrospira, Spirulina* and many other strains. These pigments can be employed in various purposes like markers and in cosmetics and can prove to be very useful. Many cyanobacteria have been explored for their suitability for commercial potential. *Spirulina* sp. has been used successfully to produce high concentrations of valuable compounds such as lipids, proteins and pigments (Abd El-Baky *et al*., 2002).



Graph 2

Cyanobacterial pigments in A. variabilis and S. elongates. Mean values ± standard errors of data obtained from two different cyanobacterial extracts. Three replicate determinations were carried out with each extract.



Graph 3

Amount of Chlorophyll-a in A. variabilis and S. elongates. Mean values ± standard errors of data obtained from two different cyanobacterial extracts. Three replicate determinations were carried out with each extract.

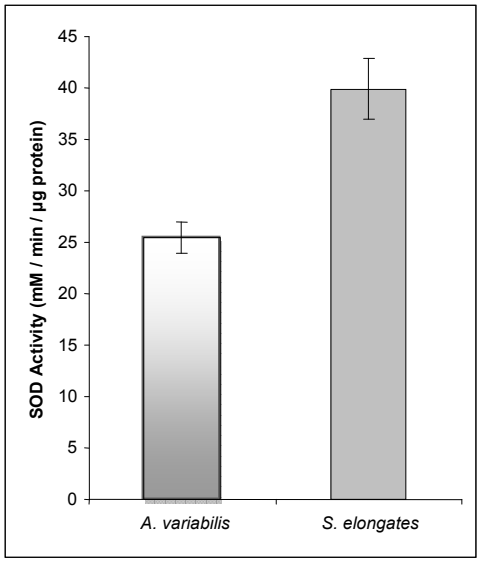
Table 1
Different amounts of cyanobacterial pigments; allophycocyanin, C-phycocyanin, C-phycoerythrin and chlorophyll-a (in µg/ml) in A. variabilis and S. elongates.

Species	Allophycocyanin*	C-Phycocyanin*	C-phycoerythrin*	Chlorophyll-a*
	(in µg / ml)	(in µg / ml)	(in µg / ml)	(in µg / ml)
S. elongates	17.0 ± 0.6	8.0 ± 0.08	29.0 ± 1.9	640.0 ± 54.0
A. variabilis	13.0 ± 0.3	23.0 ± 1.0	7.0 ±0.06	680.0 ± 57.0

Mean values ± standard errors of data obtained from two different cyanobacterial extracts. Three replicate determinations were carried out with each extract.

Enzymatic assay of Superoxide Dismutase

There was a significant difference in SOD activity (Table 2) among *S. elongates* and *A. variabilis* species. *S. elongates* had higher SOD activity than *A. variabilis* (Graph- 4). The spectrophotometric method used for measuring the SOD activity in this study, was a slight modification of indirect inhibition assay developed by Beauchamp and Fridovich (1971). In this method, riboflavin was utilized to generate a superoxide flux. Illumination of a riboflavin solution in the presence of EDTA causes a reduction of the flavin. It then reoxidizes and simultaneously reduces oxygen to O_2^- which reacts with a detector molecule such as nitro-blue tetrazloium (NBT).



Graph 4
Superoxide dismutase (SOD) activity (U/ml) in two different cyanobacterial extracts of A. variabilis and S. elongates. SOD activity was determined in two different extracts. Three replicate determinations were carried out with each extract. Mean values ± standard errors are presented.

Table 2
Different amounts of soluble protein content in A. variabilis and S. elongates.

Species	SOD activity* (mM / min / µg protein)
A. variabilis	25.41 ± 1.5
S. elongates	39.92 ± 2.9

Mean values ± standard errors of data obtained from two different cyanobacterial extracts of A. variabilis and S. elongates. Three replicate determinations were carried out with each extract.

The absorbance obtained from NBT reduction to blue formazan by superoxide was determined spectrophotometrically at 560 nm. The SOD in the sample competes for superoxide, inhibiting the reaction rate of superoxide with NBT. In the NBT negative staining system, after the gels have been soaked with NBT then riboflavin, exposing them to light causes the riboflavin to generate a superoxide radical flux in the presence of oxygen and TEMED. NBT and SOD in the gels compete for the superoxide radical at the same time. At the locations where SOD exists, the gel remains transparent, in contrast to those areas without SOD where the gel becomes purple-blue due to reduced NBT. The isozymes of the SOD family were separated by electrophoresis. Two bands were present in *S. elongates,* Fig. 1(lane 1), while one band was present in *A. variabilis,* Fig. 1(lane 2). The activity of these bands was insensitive to 2 mM KCN but disappeared when 30% H_2O_2 was added to the activity staining solutions, Fig. 1(lane 3 & 4). Because Fe-SOD is insensitive to KCN but sensitive to H_2O_2, thus it was assumed that the bands present were of Fe-SOD. No other hybrid-SOD (Hy-SOD) or Mn-SOD bands were detected. Earlier, presence of only two forms, Fe-SOD and Mn-SOD, was demonstrated in *Plectonema boryanum, Anacystis nidulans, A. variabilis* and *A. cylindrical* (Obinger *et al*., 1998). Canini *et al.,* (1991) reported presence of an additional type of SOD, i.e. hybrid-SOD (Hy-SOD or Fe-Mn-SOD) in *Anabaena azollae*, a symbiont to *Azolla filiculoides*. However, in the growing mats of *Lyngbya arboricola,* five different isoforms of SOD were observed (Tripathi *et al*., 2001). SOD assays reveal great information about the wonderful enzyme. SOD plays a significant role in protecting the organisms. The main function of SODs is to scavenge superoxide radicals generated in various physiological processes, thus preventing the oxidation of biological molecules, either by the radicals themselves, or by their derivatives.

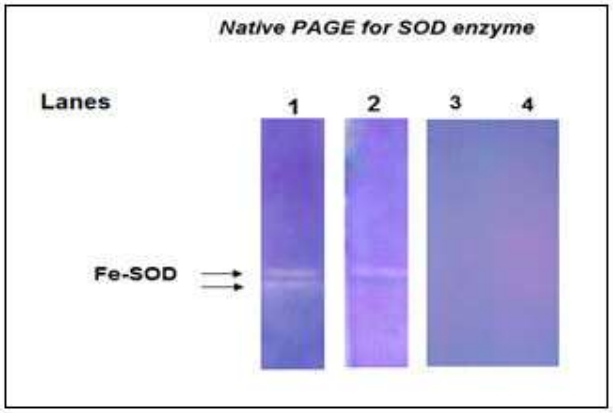


Figure 1
Electrophoresis analysis. Activity staining of SODs after native-PAGE. Lanes 1. S. elongates, 2. A. variabilis. Lanes 3 & 4 treated with 30% H_2O_2 before negative staining of gel to determine the isoforms of SODs. (Arrows indicate isoforms).

Superoxide dismutase is an enzyme found in all living cells. Superoxide dismutase is taken by mouth for removing wrinkles, rebuilding tissue, and extending the length of life. However, there is no evidence that superoxide dismutase products that are taken by mouth are absorbed by the body. As a shot, superoxide dismutase is used for treating pain and swelling (inflammation) caused by osteoarthritis, sports injuries, and rheumatoid arthritis; a kidney condition called interstitial cystitis; gout; poisoning caused by a weed-killer called paraquat; cancer; and lung problems in newborns. Superoxide dismutase is also given as a shot for improving tolerance to radiation therapy, improving rejection rates in kidney transplantation, and minimizing heart damage caused by heart attacks (Noor *et al*., 2002). SOD has powerful antinflammatory activity. Treatment with SOD decreases reactive oxygen species generation and oxidative stress and, thus, inhibits endothelial activation.. Therefore, such antioxidants may be important new therapies for the treatment of inflammatory bowel disease (Seguí *et. al*., 2004). A sterile solution containing superoxide dismutase is sometimes applied directly to the eyes for treating ulcers on the cornea. Superoxide dismutase is an enzyme that helps break down potentially harmful oxygen molecules in cells, which might prevent damage to tissues. It is being researched to see if it can help conditions where oxygen molecules are believed to play a role in disease. The superoxide dismutase that is used as medicine is sometimes taken from cows. Exploring cyanobacteria as source of SOD can open new horizons in antioxidative therapy.

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