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Effect of short-term hydrogen peroxide stress in the marine cyanobacterium *Synechococcus aeruginosus*

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Abstract
Effect of 1mM H_2O_2 stress in the marine cyanobacterium *Synechococcus aeruginosus* showed a marked increase in chlorophyll, ascorbic acid, glutathione and polyphenolics contents at 15 min while phycobiliproteins at 30 min. Astaxanthin content increased during all time periods of H_2O_2 stress. Gels stained for antioxidant enzyme activities in native PAGE showed the preservation of superoxide dismutase, glutathione peroxidase, glutathione S- transferse and esterase activities against H_2O_2 stress.

Keywords: Cyanobacterium *Synechococcus aeruginosus*

Introduction
Marine ecosystem is subjected to different kinds environmental pollution viz. municipal and domestic wastes, dredged spill dumpings, oil spills and leakages, chlorinated and petroleum hydrocarbons, pesticides and herbicides, heavy metals and photo reactive dissolved organic matter, etc. (Torres *et al*. 2008; Verlecar *et al*. 2006) [4]. These toxic pollutants generate large quantities of reactive oxygen species (ROS) namely singlet oxygen, superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, peroxyl radicals, etc in polluted seawater and impart oxidative stress in marine organisms, particularly on microalgae. Amongst various kinds of reactive oxygen species produced in oceanic water, H_2O_2 has the longest lifetime in seawater. The steady-state concentrations (10^{-7}M) of H_2O_2 is high, hence it can rapidly pass through biological membranes, interact with cellular components (Asada 1994) and cause considerable damage in the cell (Lesser 2006).
At the same time, microalgae fight oxidative stress by their innate cellular antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione S-transferase (GST) (Valavanidis *et al*. 2006) [5], besides the non-enzymatic antioxidants namely reduced glutathione, beta carotene, ascorbic acid, alpha tocopherol, polyphenolics, etc. (Halliwell and Gutteridge, 1999, Torres *et al*. 2008) [4].
The antioxidant enzyme, SOD catalyzes the disproportionation of $O_2^{\bullet-}$ radicals to H_2O_2 and molecular oxygen, GPX reduces H_2O_2 to water and alcohols using GSH as electron donor and GST conjugates xenobiotics with GSH for excretion (Regoli and Giuliani 2014).
In the present investigation, H_2O_2 stress was imparted in the marine cyanobacterium *Synechococcus aeruginosus* for a short-term period, under controlled laboratory conditions, to find out its antioxidative potentials.

Materials and methods
Organism
Synechococcus aeruginosus, a fast-growing unicellular marine cyanobacterium, was obtained from the culture collection of Jamal Mohamed College, Tiruchirappalli, India and checked for their axenic nature. The marine cyanobacterium was grown and maintained in sterilized natural seawater at 25±2°C, 14:10h light-dark cycle, and 27 µmol photons m^{-2} s^{-1}.

Hydrogen peroxide stress
Cultures of Synechococcus aeruginosus, 500 mg fresh weight in 250 mL Erlenmeyer flasks containing 100 mL sterilized seawater (control) or seawater containing added H_2O_2 to reach a final concentration of 1mM, were incubated under the above mentioned growth conditions for 15, 30, 45 and 60 min and their biochemical contents and activities of antioxidative enzymes were determined.

Estimation of biochemical components

Chlorophyll *a* was extracted in methanol (90%) overnight at 40 °C in dark and the optical density of the supernatant was read at 663 nm (Jasco V-550 spectrophotometer, Japan) and the quantity estimated (Mackinney, 1941) [17]; total carotenoids were extracted overnight in 85% acetone at 4°C in the dark, centrifuged the contents, read the optical density of the supernatant at 450nm and the amount was estimated by employing the extinction coefficient, A1% of 2500, of Jensen (1978) [18]; phycobilipigments were extracted in cold phosphate buffer (0.05 M, pH 6.8) by repeated freeze thawing until all the phycobilins were extracted, read the absorbance of the extract in a UV-visible spectrophotometer at 562, 615 and 652 nm and the amount of phycocyanin, allophycocyanin and Phycoerythrin were determined using the equation of Siegelman and Kycia (1978) [19]. Standard protocols were followed for the estimation of Astaxanthin (Ma and Chen 2001) [8], glutathione (Moron *et al.* 1979), ascorbic acid (Omaye *et al.* 1979) and total phenolics (Folin-Ciocalteu method as reported by Harbone 1973). Results are average of triplicates. Data were statistically analyzed and results were expressed as means (±SE).

Enzyme preparation

Cell-free enzyme extract was prepared by homogenizing the cyanobacterial biomass in the presence of glass powder (~ 0.5 mm) using pre-chilled mortar and pestle adding ice-cold extraction buffer (0.0625 mM Tris. Cl, pH 6.8, containing 2 mM EDTA, 1% polyvinyl pyrollidone (w/v) and 1 mM PMSF). The homogenate was centrifuged at 15,000 ×g for 30 min in a cooling centrifuge (Remi C20; India). The cell-free supernatant was used as enzyme source. Protein content of the enzyme extracts was determined using bovine serum albumin as standard (Lowry *et al*. 1951) [20].

Native polyacrylamide gel electrophoresis (PAGE)

Antioxidative enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione S- transferase (GST) and esterases were localized on cell-free enzyme extracts by discontinuous PAGE under non-denaturing condition (Laemmli, 1970). The enzymes were resolved based on equal amount of protein (300 µg) in 12% uniform concentration gel at 20 ± 2 °C with a constant power supply of 100V. The gels were stained for respective enzyme activities after electrophoresis.

SOD activity staining

Activity staining of SOD isoenzymes was done by submerging the gel in 50 mL of Tris-HCl (50 mM, pH 8) containing 2 mg riboflavin, 1 mg EDTA (Sigma, USA) and 10 mg NBT (Hi-Media, India), for 30 min, and then illuminated on a light box for 10-15 min at room temperature (25 ± 2 °C). Zones of SOD activity were observed as achromatic regions on dark blue background (Wendel and Weeden, 1989).

GPX activity staining

Activities of GPX isoenzymes were visualized as achromatic bands against a dark background on incubating the gel with 50 ml of Tris-HCl buffer (50 mM, pH 8.0) containing 200 mg of reduced glutathione and 8 µL of 30% H_2O_2 on a shaker at 50 rpm for 15 min. The gel was stained with 50 mL of Tris-HCl buffer containing 25 mg each of NBT and phenazine methosulphate following Lin *et al*. (2002).

GST activity staining

The gel for GST activity was equilibrated in phosphate buffer (0.1M, pH 6.5) for 10 min and then transferred to a reaction mixture containing GSH (4.5mM), 1-chloro-2,4-dinitrobenzene (1 mM) and nitrobluetetrazolium (1 mM) in potassium phosphate buffer (0.1 M, pH 6.5) and incubated at 37 °C for 10 min. The gel was further submerged in Tris buffer (0.1 M, pH 9.6) containing phenazine methosulphate (3 mM) and the appearance of achromatic bands of GST activity was visualized against a dark blue background (Ricci *et al*. **(**1984**)**.

Esterase activity staining

Activity staining for esterase (EST) was performed by placing the gel in 100ml of phosphate buffer (100mM, pH 6.2) containing 50mg α-napthyl acetate (Hi-media, India), 50mg β-napthyl acetate (Hi-media, India) and 100mg fast blue R Rsalt (Sigma, USA). The gel was incubated at 25°C for 1 h to develop black, red or magenta colour bands (Wendel and Weeden, 1989). Both the substrates (α-napthyl acetate and β-napthyl acetate) were dissolved quickly in 1ml of acetone and mixed with buffer solution just before transferring of gel.

Results

The short-term H_2O_2 stress in *Synechococcus aeruginosus* showed an increase in chlorophyll content over the control at 15 min of H_2O_2 exposure. The chlorophyll content then started declining from 30min H_2O_2 treatment and the trend continued up to 60 min. A two-fold decrease in chlorophyll content was recorded at 45 and 60 min of H_2O_2 stress, compared to the control (Table 1). The values of carotenoids were more compared to the control at 15 and 30 min of H_2O_2 stress. A three-fold increase in carotenoid content over the control was observed at 30 min and it declined thereafter at 45 and 60 min exposure. A similar pattern of change was recorded with phycobilipigments such as c-phycocyanin, Allophycocyanin and c-phycoerythrin. However, astaxanthin content in *Synechococcus aeruginosus* started increasing with increase in period of time of H_2O_2 stress. At the end of 60 min of exposing to H_2O_2, *Synechococcus aeruginosus*, exhibited a three-fold enhancement in astaxanthin content (Table 1). In contrast, glutathione and total phenolics contents revealed a different response to H_2O_2 stress showing a small increase in their content over the control at 15 min while, gluthathione and polyphenolics contents declined at 30 min and the trend prevailed up to 60 min exposure (Table 1). The above findings indicated that brief exposure to H_2O_2 promoted the biochemical content and long duration exposure as detrimental. The beneficial and hamful roles of H_2O_2 on living organisms have been reported (Di Meo *et al*. 2016; Wang *et al*. 2011; Pizzino *et al*. 2017) [1, 3, 9].

Synechococcus aeruginosus exhibited a single acromatic isoform of SOD (relative mobility-Rm 1.31) in the native PAGE and the activity of this enzyme was detected during all periods of exposure time of H_2O_2 (Fig. 1).

Glutathione peroxidase showed as many as five isoforms (Rm 1.20, 1.14, 1.11, 1.10 and 1.07) in the control cells of *Synechococcus aeruginosus* and the activities of all the isoforms were preserved at all time periods of H_2O_2 stress (Fig. 2).

Glutathione S- transferase illustrated five isoforms (Rm 3.04, 2.59, 1.50, 1.34 and 1.20) in native PAGE in the control cells of *Synechococcus aeruginosus* (Fig. 3, lane 1). The same level of expression observed in the control cells were noticed at 15 min (Fig. 3 lane 2) while, H_2O_2 stress enhanced the intensity

of GST isoforms with Rm 1.34 and Rm 1.2 at 30 min exposure (Fig. 3, lane 3). Activities of only two isoforms (Rm 1.5 and 1.34) were detected at 45 and 60 min of H_2O_2 exposure (Fig. 3, lane 4 and 5). The activity of GST as observed from the band intensity at 45 min (Fig. 3, lane 4) remained the same as in the control while, H_2O_2enhanced the intensity of isoforms at 60 min (Fig. 3, lane 5) compared to control.

Activity of esterases in native PAGE showed seven isoforms (Rm 2.3, 2.0, 1.7, 1.56, 1.45, 1.31 and 1.14) in the control and the activities of all the isoforms were preserved during H_2O_2 stress in *Synechococcus aeruginosus* (Fig. 4). Antioxidant enzymes are known to protect the organisms against oxidative stress. In the present investigation, *Synechococcus aeruginosus*, combated H_2O_2 stress by preserving SOD, GPX and GST activities as supported other reports (Asada 1994; Torres *et al*. 2008; Valavanidis *et al*. 2006) [5, 4].

Conclusion

The marine cyanobacterium *Synechococcus aeruginosus* tolerated H_2O_2stress by preserving the activities of all the antioxidant enzymes studied and biochemical parameters estimated.

Table 1: Mean values and standard error of biochemical contents in the marine cyanobacterium *Synechococcus aeruginosus* after exposure to H_2O_2 (1mM) for different time duration.

Biochemical parameters	Time duration (min.)				
	0	15	30	45	60
Chlorophyll *a* (µg)	5.432±9.04	6.740±8.43	4.251±3.34	2.796±2.08	2.140±10.35
Total carotenoids (µg)	0.634±3.61	0.739±2.51	1.910±0.072	1.631±1.02	1.43±0.21
Phycocyanin (µg)	0.019±0.931	0.071±0.073	2.070±0.45	0.939±0.93	0.219±0.39
Allophycocyanin (µg)	0.0501±0.041	0.081±0.003	3.97±7.20	0.140±0.59	0.218±0.93
Phycoerythrin (µg)	0.010±0.0813	0.031±0.031	8.40±6.53	3.12±1.46	2.640±0.96
Astaxanthin (µg)	0.313±1.937	0.471±1.640	0.50±1.79	0.740±1.413	0.910±0.43
Ascorbic acid (µg)	0.101±0.490	0.202±0.317	0.302±0.29	0.30±0.491	0.719±0.02
Glutathione (µg)	0.024±0.260	0.032±0.421	0.03±0.071	0.21±0.925	0.102±0.52
Total phenolics (µg)	0.512±6.910	0.791±3.91	0.39±0.20	0.39±0.691	0.311±0.63

Fig 1: Superoxide dismutase (SOD) activity as visualized on native PAGE in *Synechococcus aeruginosus* after exposure to H_2O_2 (1 mM) for various time duration. lane 1: 0 min., lane 2: 15 min., lane 3: 30 min., lane 4: 45 min., and lane 5: 60 min.

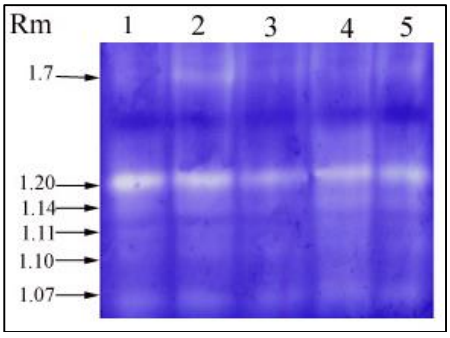

Fig 2: Glutathione peroxidase (GPX) activity as visualized on native PAGE in *Synechococcus aeruginosus* after exposure to H_2O_2 (1 mM) for various time duration. lane 1: 0 min., lane 2: 15 min., lane 3: 30 min., lane 4: 45 min., and lane 5: 60 min.

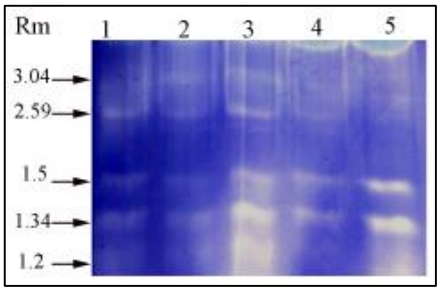

Fig 3: Glutathione S-transferase (GST) activity as visualized on native PAGE in *Synechococcus aeruginosus* after exposure to H_2O_2 (1 mM) for various time duration. Lane 1: 0 min., lane 2: 15 min, lane 3: 30 min., lane 4: 45 min., and lane 5: 60 min.

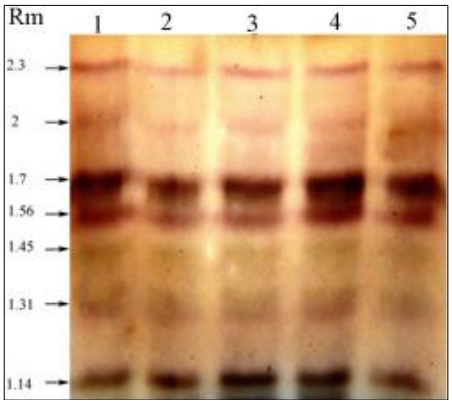

Fig 4: Esterase (EST) activity as visualized on native PAGE in *Synechococcus aeruginosus* after exposure to H_2O_2 (1 mM) for various time duration. Lane 1: 0 min., lane 2: 15 min., lane 3: 30 min., lane 4: 45 min., and lane 5: 60 min.

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