Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(2): 97-104
Received: 10-01-2019
Accepted: 12-02-2019

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Biochemical response of microalga *Synechococcus aeruginosus* to hydrogen peroxide stress

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Abstract
Growth and biochemical response of varying biomass of marine microalga *Synechococcus aeruginosus* exposed to ascending concentrations of H_2O_2were investigated. The growth response of *Synechococcus aeruginosus* to H_2O_2 depended on both H_2O_2 concentrations and biomass concentrations. The effects of H_2O_2 on growth responses were apparent in low biomass concentrations and the effects reduced with increase of biomass concentrations. At low concentrations of H_2O_2, the changes in contents of c-phycocyanin, allophycocyanin, c-phycoerythrin, crotenoids, astaxanthin, beta carotene, glutathione, ascorbic acid and polyphenolics, in *Synechococcus aeruginosus*, were small, while high concentrations of H_2O_2 led to more changes in these biochemicals. These results suggested that the effect of H_2O_2 in *Synechococcus aeruginosus* depended on both H_2O_2 concentration and biomass.

Keywords: Microalga *Synechococcus aeruginosus*

Introduction
Marine microalgae, distributed mostly on upper sunlit layer of seawater (Barra *et al*. 2014) [25], are subjected to a variety of environmental stressors such as municipal and domestic wastes, dredged spill dumping, oil spills and leakages, chlorinated and petroleum hydrocarbons, pesticides and herbicides, heavy metals, solar radiation, dissolved organic matter, etc. (Torres *et al*. 2008; Verlecar *et al*. 2006) [23, 24], is an area of active research. These toxic environmental pollutants release exceedingly large quantities of reactive oxygen species (ROS) such as singlet oxygen, superoxide anion radicals, hydrogen peroxide, hydroxyl radicals, peroxyl radicals, etc in polluted oceanic water, cause oxidative stress in coastal marine environment and adversely affect the precious microalgal biodiversity without any early warning signal. Of all the ROS, H_2O_2 has the longest lifetime in seawater and the highest steady-state concentrations (10^{-7}M) and can quickly diffuse through biological membranes, rapidly react with cellular components and other cellular materials (Asada 1994). H_2O_2 imparts significant damage in the cell because it is not limited to its site of production (Lesser 2006). Therefore, hydrogen peroxide stress was implicated on the marine cynobacterium *Synechococcus aeruginosus* under controlled laboratory conditions to understand the growth and biochemical changes.

Materials and methods
Experimental organism and culture conditions
The fast growing marine cyanobacterium *Synechcoccus aeruginosus* was obtained from the germplasm collections of Department of Botany, Jamal Mohamed College, Tiruchirappalli, India. The axenic nature of the chosen organism was checked and it was grown and maintained in 250mL capacity Erlenmeyer flasks containing 100mL of sterilized natural habitat seawater at 25±2 °C, 14:10h light-dark cycle, and 27 µmol photons m^{-2} s^{-1}.

Hydrogen peroxide (H_2O_2) stress
Marine cyanobacterial culture in its active phase of growth, 50 – 1000 mg fresh weight, were incubated under the above mentioned culture conditions for 7 days in 250 mL capacity Erlenmeyer flasks containing 100 mL of filtered and sterilized seawater (control) or seawater containing daily added H_2O_2 to a final concentration of 1, 5, 10 and 25mM. Growth of microalgae was estimated in the control and microalgal biomass exposed to H_2O_2 by measuring the changes in chlorophyll *a* content (Mac-Kinney 1941) [17]. Biochemicals such as

pigments and non enzymatic antioxidants were determined by measuring the changes of their content both in control and H_2O_2 exposed biomass.

Determination of pigments

Phycobilipigments such as c-phycocyanin, allophycoyanin and c-phycoerythrin were extracted by freez-thawing the microalgal biomass in phosphate buffer (0. 05M, pH 7.0).The absorbencies of the supernatant were measured at 562, 615 and 652 nm. The phycobiliprotein content was calculated from the formula proposed by Siegelman and Kycia (1978) [22].

$$\text{C-Phycocyanin (PC)} = \frac{A_{615} - 0.474(A_{652})}{5.34} \text{ mg}$$

$$\text{Allophycocyanin (APC)} = \frac{A_{652} - 0.208\ (A_{615})}{5.09} \text{ mg}$$

$$\text{C-Phycoerythrin (PE)} = \frac{A_{562} - 2.41\ (PC) - 0.849\ (APC)}{9.62} \text{ mg}$$

Total carotenoid was determined in the 80% methanolic extract of microalgal biomass at 450 nm. The concentration of total carotenoid was calculated using an absorption coefficient, A1% of 2500 following the method of Jensen (1978) [14].

Beta carotene was extracted by homogenizing the microalgal biomass in 1.0 mL of saponification mixer, refluxed for 20 min at 60 ºC in dark and re-extracted with 10mL of petroleum ether at 60 ºC. The extract was dried at 60 ºC and the residue was dissolved in chloroform. An aliquot of the residue was dissolved in chloroform and mixed rapidly with 2 mL of trichloroaceticacid. The absorbance was read at 620 nm immediately in a spectrophotometer. Standards (vitamin A palmitate) of concentrations ranging from 0 - 7.5 µg were treated in the same way (Bayfield and Cole 1980).

Determination of non-enzymatic antioxidants

Non-enzymatic antioxidants such as reduced glutathione, ascorbic acid, and polyphenolics were estimated in the control and microalgal biomass exposed to H_2O_2. Reduced glutathione (GSH) was estimated at 412 nm in a spectrophotometer (Moron *et al*. 1979) [19] by measuring the formation of GS-TNB complex developed from the mixture of deproteinizedmicroalgal extract and DTNB [5, 5' dithiobis (2-nitrobenzoic acid)]. The GSH content was determined from the standard curve.

Ascorbic acid concentration was determined by extracting a known quantity of microalgal biomass with 1.5mL of 5% trichloroaceticacid by centrifugation. An aliquot of supernatant was mixed with 0.5mL of dinitrophenylhydrazine (DNPH) reagent, containing thiourea (0.4%), copper sulphate (0.05%) and 2, 4-dinitrophenylhydrazine (3%) in 9 N sulphuricacid, and incubated at room temperature for 3 h. Then 2.5 mL of ice-cold 65% sulphuric acid was added, mixed well and the absorbance was measured at 520 nm. Standard ascorbic acid in the range of 10 – 50 µg was processed in similarly fashion (Omaye *et al.* 1979) [20].

Total phenolic content of microalgae was determined following the method of Folin-Ciocalteu as reported by Harbone (1973) [13]. Microalgae biomass was homogenized with 50 times the fresh weight with 80% methanol. The sample was vortexed for 1h and then centrifuged at 5000×g for 15 minutes. The methanolic extract was used to determine the phenolics. The reaction mixture containing methanolic extract (0.5mL), 10% Folin-Ciocalteu's reagent (2.5mL) and 7.5% NaHCO3 (2.5mL) was prepared along with a reagent blank. The reaction mixture was incubated at 45ºC for 30 min. The absorbance was measured at 765 nm. The standard gallic acid was processed in the same manner. The total phenolic content of the extract was represented as milligrams of gallic acid equivalent (GAE).

Results

Chlorophyll content as a measure of growth was estimated in the marine microalgal biomass of *Synechococcus aeruginosus* under H_2O_2 stress (Fig. 1). *Synechococcus aeruginosus* successfully tolerated 1 and 2mM H_2O_2 showing 25-20% growth at 50mg biomass concentration and 99-90% growth at 1000mg biomass respectively. However, higher concentrations of H_2O_2 suppressed the growth of microalgae in less biomass concentrations. In the present investigation, 5mM H_2O_2 halted the microalgal growth in 50 and 100mg biomass concentration while, 34%, 50% and 70% growth was manifested respectively in 250, 500 and 1000mg biomass concentrations. In the case of 10mM H_2O_2, microalgal growth was not detected up to 250mg biomass concentration and thereafter about 38% and 57% growth was observed respectively in 500 and 1000mg biomass. At 25mM H_2O_2 treatment, except 1000mg biomass showing 25% chlorophyll content, growth was not observed in any of the biomass concentrations experimented (Fig. 1).

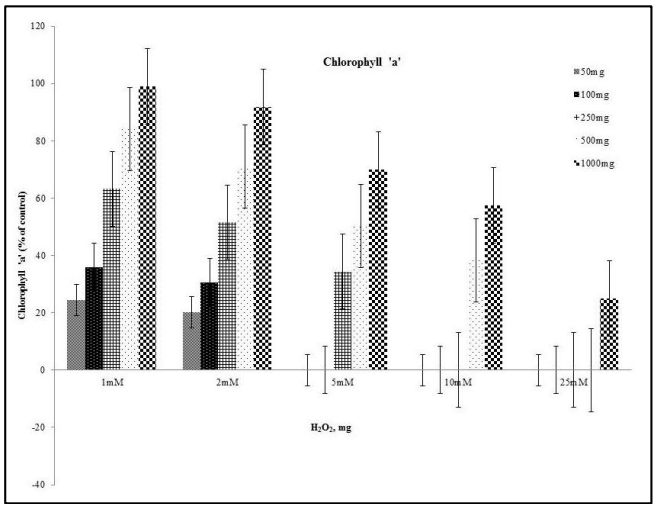


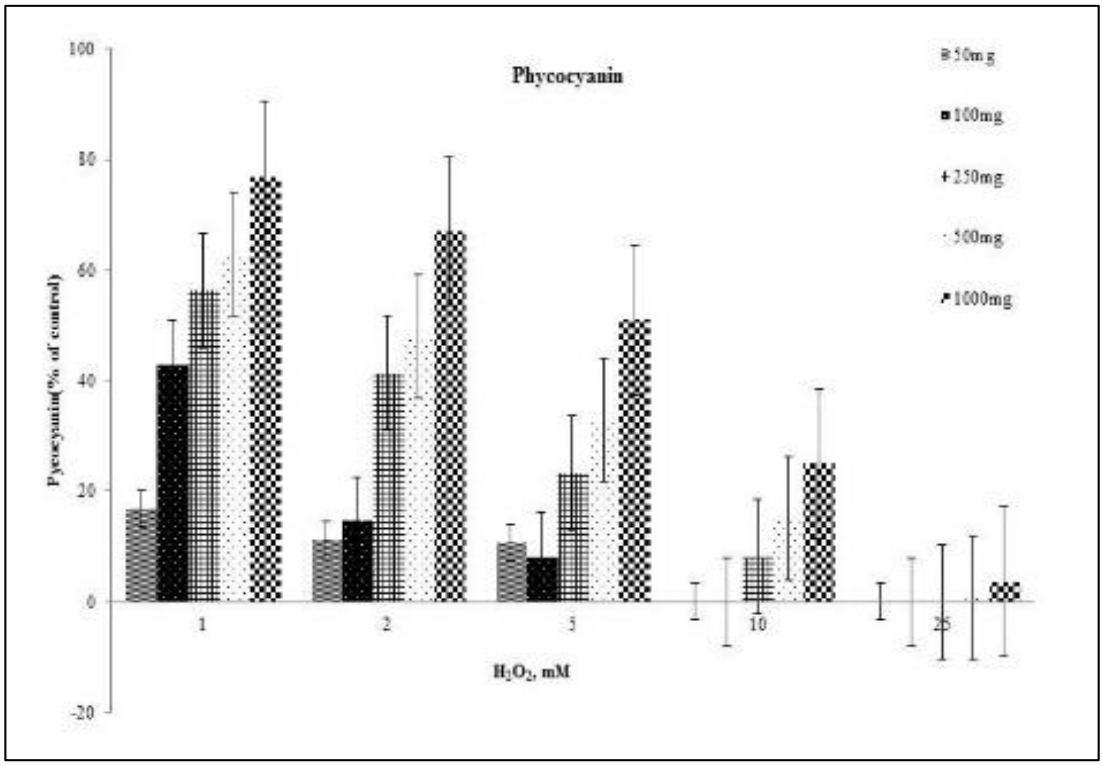
Fig 1: Growth in terms of chlorophyll *a* mean values ± S.E. (% of control) in varying biomass of the marine microalga *Synechococcus aeruginosus* after 7 days of exposure to increasing H_2O_2 concentrations.

The response of phycocyanin, allophycocyanin, phycoerythrin, total carotenoids, astaxanthin and beta carotene content to increasing H_2O_2 concentrations was determined in the marine microalga *Synechococcus aeruginosus* after 7 days of exposure (Fig. 2). H_2O_2, irrespective of its concentrations tested on *Synechococcus aeruginosus*, suppressed the c-phycocyanin content to various levels. The impact of H_2O_2 on phycocyanin content was more when biomass concentration used was less but less when biomass concentration used was more (Fig. 2A). At 1 mM H_2O_2, only 16% phycocyanin content was recorded in 50mg biomass concentration while, 77% in 1000mg biomass concentration. A similar trend prevailed as well in 2 and 5mM H_2O_2 treatment. However, at 10mM H_2O_2 treatment, c-phycocyanin was not detected in 50 and 100mg biomass concentration but at 25mM treatment, c-phycocyanin was not

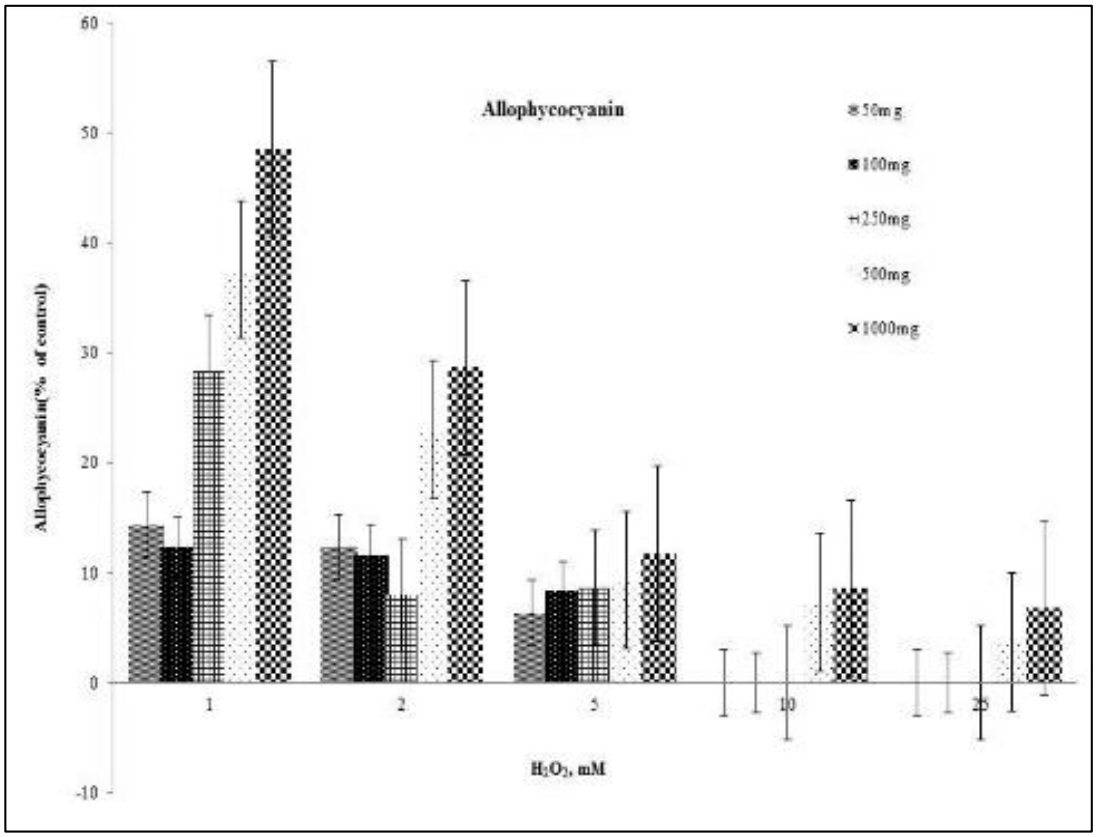
detected up to 500mg biomass. A negligible amount of c-phycocyanin was detected in other biomass concentrations at 10 and 25mM H_2O_2 (Fig. 2A).

Allophycocyanin tolerated all concentrations of H_2O_2 except 10 and 25mM concentrations tested at 50, 100 and 250mg biomass concentrations, wherein allophycocyanin was not detectable (Fig. 2B). Lower concentrations of H_2O_2 viz. 1, 5 and 10mM showed less effect on allophycocyanin content compared to higher concentrations used. At the same time, allophycocyanin content estimated was comparable to biomass concentration (Fig. 2B).

Similarly, c-phcoerythrin (Fig. 2C), total carotenoids (Fig. 2D) astaxanthin (Fig. 2E) and beta carotene (Fig. 2F) too tolerated all concentrations of H_2O_2 except 10 and 25mM concentrations tested at 50 and 100mg biomass, wherein the said biochemicals were was not detectable.



(A)



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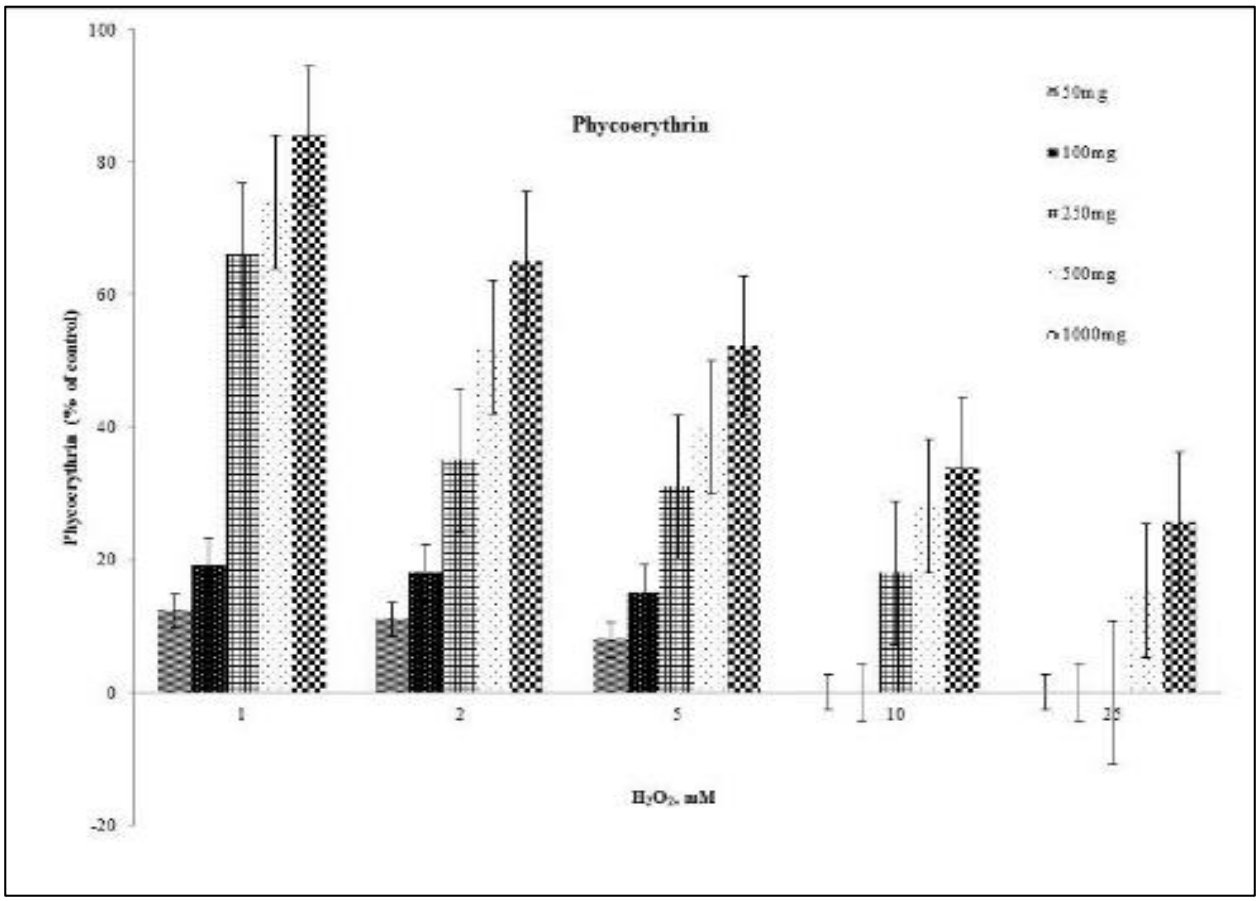
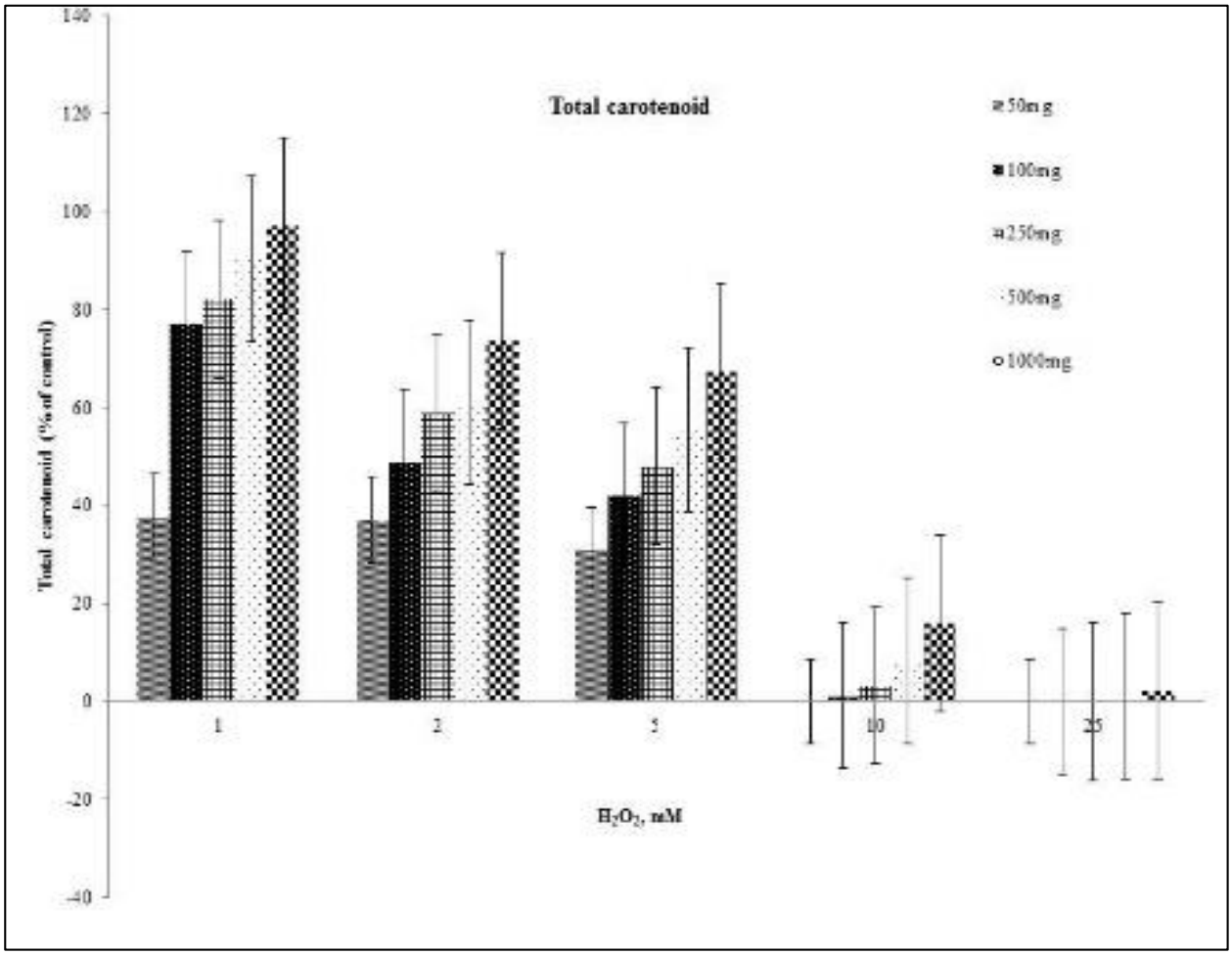
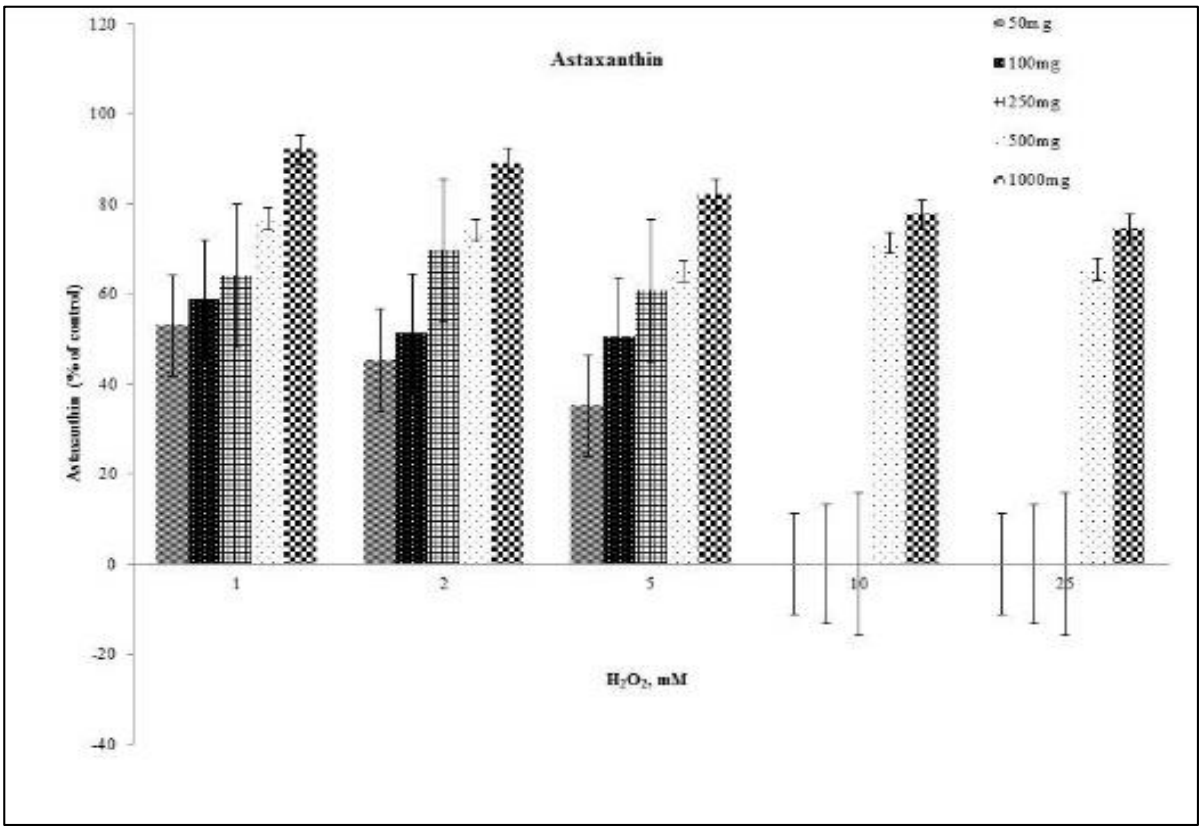

Phycoerythrin
50mg
100mg
250mg
500mg
1000mg
Phycoerythrin (% of control)
100
80
60
40
20
0
-20
1
2
5
10
25
H2O2, mM

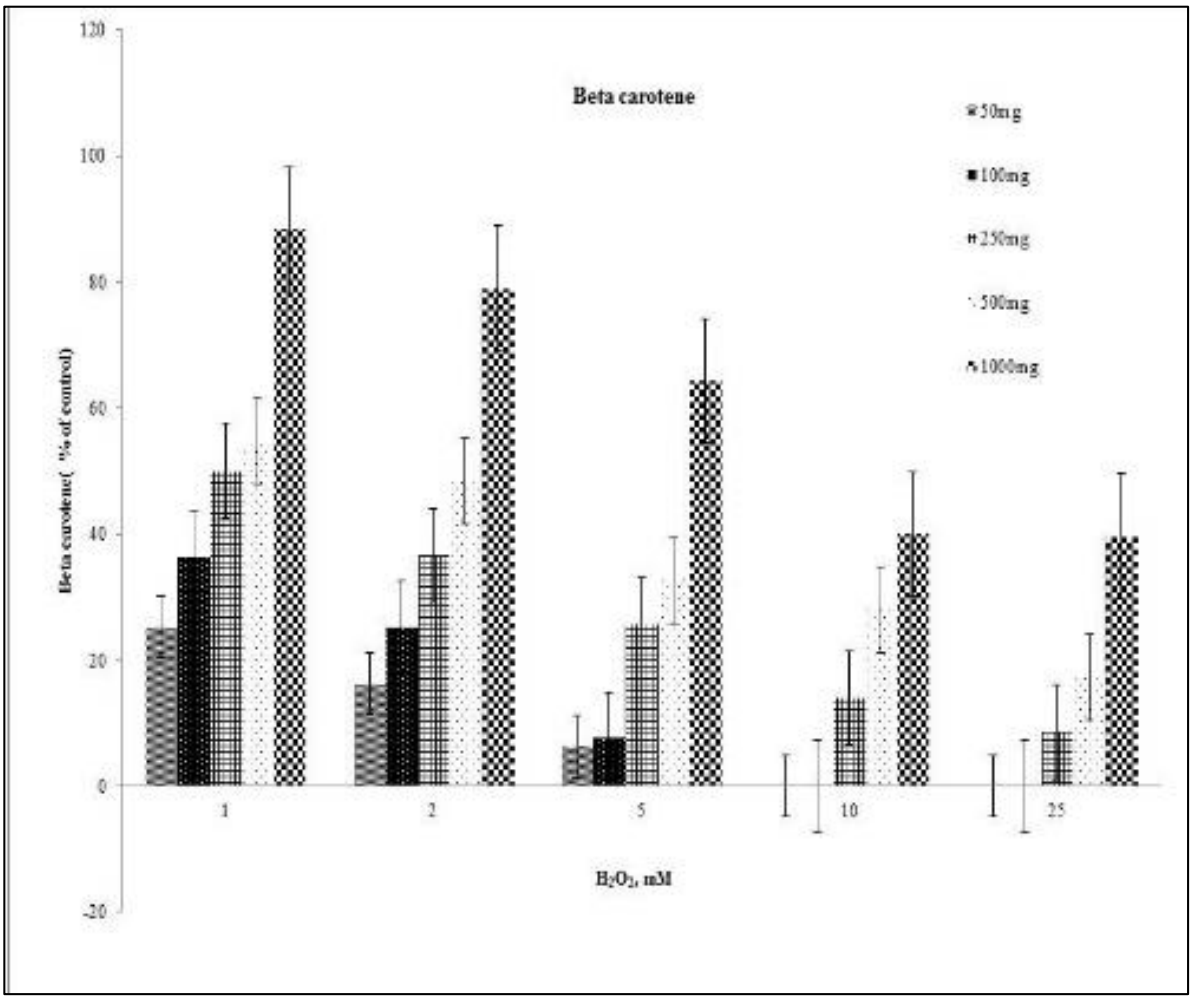
(C)


Total carotenoid
50mg
100mg
250mg
500mg
1000mg
Total carotenoid (% of control)
140
120
100
80
60
40
20
0
-20
-40
1
2
5
10
25
H2O2, mM

(D)



(E)

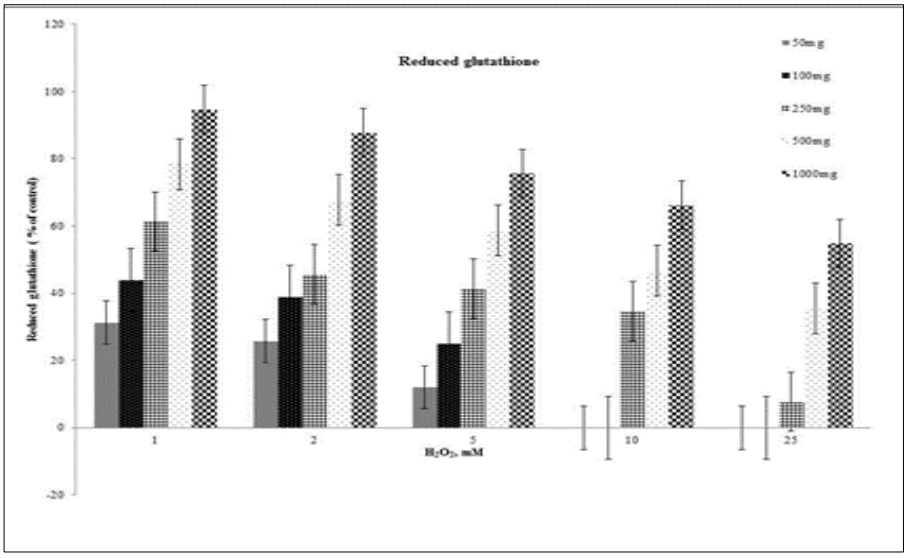


(F)

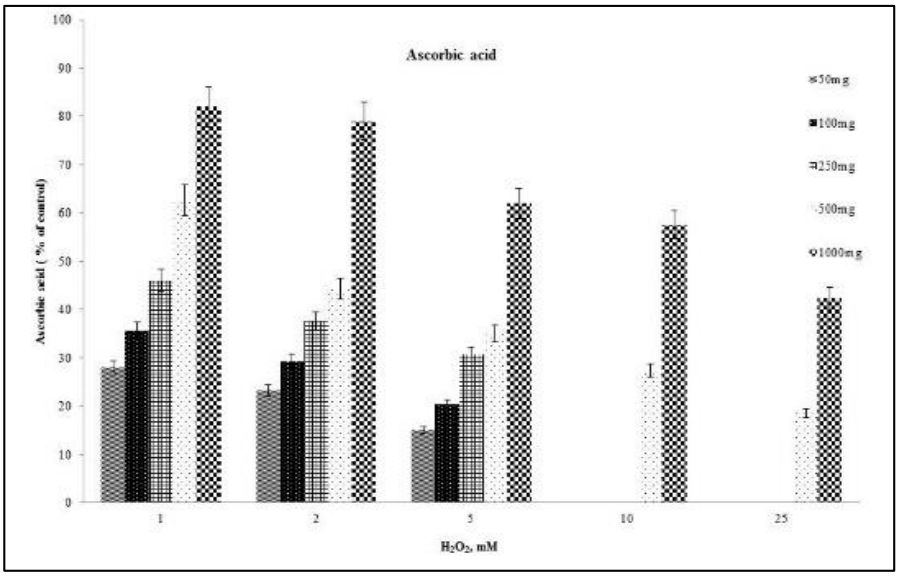
Fig 2: Mean values ± S.E. (% of control) of phycocyanin, allophycocyanin, phycoerythrin, total carotenoids, astaxanthin and beta carotene in varying biomass of the marine microalgae *Synechococcus aeruginosus* after 7 days of exposure to increasing H_2O_2 concentrations.

Glutathione content was preserved to a larger extent in *Synechococcus aeruginosus* exposed to lower concentrations of H_2O_2, for example 1, 2 and 5mM, but to a lesser extent at higher concentrations of H_2O_2 (Fig. 3A). The percentage of glutathione estimated was comparable to the biomass concentration exposed. At 1, 2 and 5mM H_2O_2 treatment, glutathione exhibited a range of 31-95%, 25-87% and 12-75% content respectively. At 10 and 25mM H_2O_2, glutathione was not detectable in 50 and 100mg biomass concentrations while, in 250, 500 and 1000mg biomass concentrations, glutathione content was saved fairly well (Fig. 3A). Ascorbic acid tolerated lower concentrations of H_2O_2. At 1mM H_2O_2 stress, the percentage of ascorbic acid retained in 50mg biomass of *Synechococcus aeruginosus* was 28% (Fig. 3B). The ascorbic acid content continued to show more levels on increasing the biomass concentration. For instance, about 35, 46, 62 and 82% of ascorbic acid was measured respectively in 100, 250, 500 and 1000mg biomass. A similar pattern of change in ascorbic acid content was observed at 2 and 5mM H_2O_2 treatment (Fig. 3B). At 2mM H_2O_2, all biomass concentrations displayed lesser ascorbic acid content compared to 1mM H_2O_2. At 5mM H_2O_2 treatment, still lesser percentage of ascorbic acid was estimated. However, at higher concentrations of H_2O_2 tested, for instance 10 and 25mM, ascorbic acid was not detectable in 50, 100 and 250mg biomass concentrations while in 500 and 1000mg biomass a faily good percentage of ascorbic acid was restored (Fig. 3B).

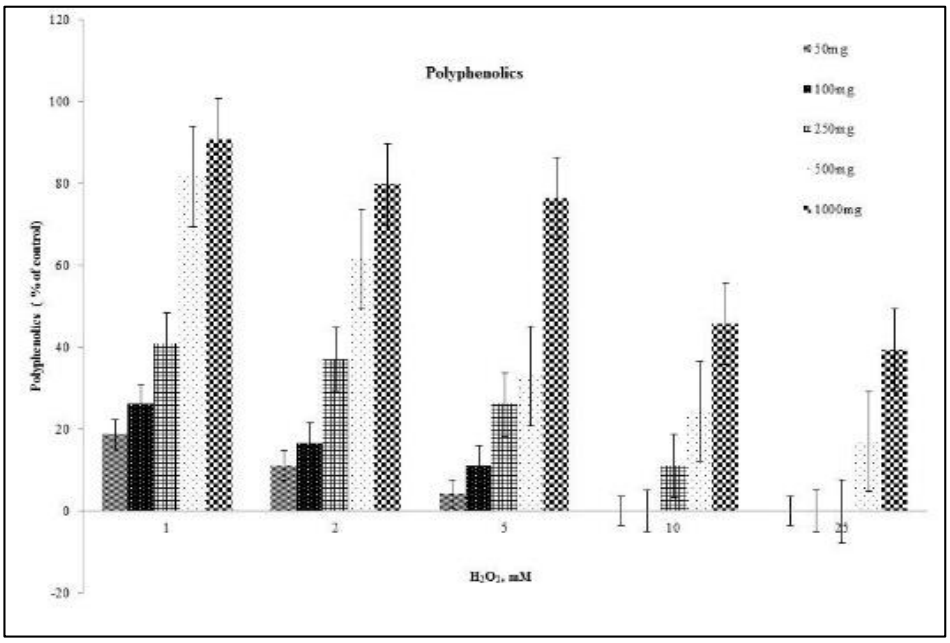
Polyphenolics content of *Synechococcus aeruginosus* was saved against H_2O_2 stress. At 1mM H_2O_2 concentration, about 18% of polyphenolics was restored in 50mg biomass concentration (Fig. 3C). Restoration of ppolyphenolics continued to further levels on increasing the biomass concentration. For example, about 26, 40, 81 and 90% of polyphenolics were determined respectively in 100, 250, 500 and 1000mg biomass. A similar pattern of change in polyphenolics content was witnessed at 2 and 5mM H_2O_2 treatment (Fig. 3C). At 2mM H_2O_2, all biomass concentrations exhibited lesser content of polyphenolics compared to 1mM H_2O_2. At 5mM H_2O_2 stress, still lesser content was recorded. However, at higher concentrations of H_2O_2, for instance 10 and 25mM, ascorbic acid was not detectable in 50 and 100mg biomass while in 500 and 1000mg biomass a considerable percentage of polyphenolics was preserved (Fig. 3C).



(A)



(B)



(C)

Fig 3: Mean values ± S.E. (% of control) of glutathione, ascorbic acid and polyphenolics contents in varying biomass of the marine microalga *Synechococcus aeruginosus* after 7 days of exposure to increasing H_2O_2 concentrations.

Discussion

The present study provides knowledge on response of growth and of biochemicals such antioxidant pigments and non-enzymatic antioxidants in the marine microalga *Synechococcus aeruginosus* under artificial H_2O_2 stress. Chlorophyll content was used as an indicator of growth against H_2O_2 stress. All concentrations of H_2O_2 tested on *Synechococcus aeruginosus*, exhibited a negative response on chlorophyll content. The effect of H_2O_2 on growth was more pronounced when the biomass concentration. This is due to the damage caused to photosynthetic pigment chlorophyll by H_2O_2. Retention of more growth in enhanced biomass indicated that $H_2O_2$was detoxified. The tolerance level of H_2O_2.The modification of growth in *Synechococcus aeruginosus* following H_2O_2 exposure indicated that the photosynthetic pigment chlophyll *a* had experienced damage from H_2O_2 stress. The changes in growth percentage of *Synechococcus aeruginosus* demonstrated that enhanced level of biomass contributed to the detoxification of H_2O_2. In the present investigation, growth did not show any recovery at higher concentrations of H_2O_2, indicated that H_2O_2 detoxification failed due to lack of sufficient biomass.

Pigments such as phycocyanin, allophycocyanin, phycoerythrin, carotenoids, astaxanthin and beta carotene are reported to possess antioxidant property (Wells *et al*, 2017) [1] and also involved in detoxification process of H_2O_2. Levels of antioxidant pigments estimated in the present study correspond to a larger extent with level of growth at repespective H_2O_2 concentrations and the antioxidant property of the pigments could be attributed probably for this.

Glutathione, ascorbic acid and polyphenolics are the important biochemical components of the antioxidant defence systems in cells. These are involved in detoxification process in microalgae (Gao *et al*. 2017) [2]. In the present study, growth at different concentrations of $H_2O_2$was supported by different leves of Glutathione, ascorbic acid and polyphenolics.

Conclusion

The present study demonstrated that growth and biochemical components not only varied among different biomass concentrations, the response of *Synechococcus aeruginosus* to H_2O_2 also depended on H_2O_2 concentrations.

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