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Research Article
Antioxidant System Response in Green Microalga *Chlorococcopsis minuta* Against Nutrient Stress in Growth Media

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Abstract

Background and Objective: Reactive oxygen species (ROS) are produced due to imbalance between cellular ROS production and elimination leading to oxidative stress. Antioxidants are known to involve in protecting cells from oxidative stress induced by various stresses in micro-algae. This study was carried out with the objective of determining the antioxidant system responses in micro-algae grown under nutrient stress conditions. **Materials and Methods:** *Chlorococcopsis minuta* was cultivated in nitrogen and phosphorous reprieve and deprived conditions and enzymatic (superoxide dismutase, catalase, ascorbate peroxidase) and non-enzymatic (Hydrogen peroxide, proline, polyphenol content and lipid per-oxidation level) antioxidants were analyzed. **Results:** Activities of antioxidant enzymes in *C. minuta* during nutrient stress showed that the activities of SOD, CAT and APX were increased in nutrient stress cultures when compared to nutrient fulfilled conditions. There was a 33% increase in lipid per-oxidation N-P- media than N-P+ media. Proline content increased with nutrient stress and reached 8 folds higher in nitrogen and phosphorous deprived media than the control. Total phenolic content was greater in N-P- indicating the nutrient stress triggered antioxidant system in the micro-algae. **Conclusion:** Nitrogen deprivation with phosphorous depletion condition demonstrated the oxidative stress and enhanced activation of enzymatic and non-enzymatic antioxidant systems at various levels.

Key words: Reactive oxygen species, antioxidant enzymes, N starvation, P starvation, lipid productivity, microalgae, *Chlorococcopsis minuta*, nitrogen deprivation, phosphorous depletion

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Molecular oxygen is a terminal oxidant in respiration of aerobic organisms. However, the presence of oxygen in the cellular environment is an oxidative threat to cellular structures and processes. Reactive oxygen species (ROS), such as superoxide (O_2^-), hydroxyl radical ($OH^\cdot$) and hydrogen peroxide (H_2O_2) are formed by the partial reduction of oxygen. All these forms can react with certain biomolecules, altering or inactivating the biochemical activities. Algae minimize free radical damage by enhanced antioxidant defence[1]. Enzymatic antioxidants are able to control the cellular concentrations of superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), thereby preventing the formation of reactive radicals[2,3]. However, under stress conditions, the balance between cellular ROS production and elimination can be disturbed, leading to a locally increased concentration of ROS that leads to oxidative stress. Both non-enzymatic (e.g., ascorbate, glutathione, tocopherols, carotenoids) and enzymatic (e.g., superoxide dismutase, catalase, ascorbate peroxidase) antioxidants are known to involve in protecting cells from oxidative stress induced by various stresses[4,5]. These markers of the oxidative stress are frequently detected and linked with the lipid accumulation by microalgae cultivated under stress conditions[6-10].

Cultivation conditions can stimulate metabolic changes in microalgae[11-13] and one such example is lipid accumulation under varying growth conditions. Nitrogen starvation is an efficient proven stress used to increase lipid accumulation in microalgae[14]. This is attributed to the fact that when there is insufficient N for protein synthesis required for growth, excess carbon from photosynthesis is channelled into storage molecules such as triglyceride or starch[15]. The role of nitrogen depletion in the production of antioxidant enzymes is known but no comparison of antioxidant responses under nitrogen (N) and phosphorous (P) replete and depleted conditions has been made yet. Therefore the aim of the present study was to compare the effects of nutrient depletion in *Chlorococcopsis minuta* and the strategies applied by the alga for its survival. For this purpose, the main activities of the enzymatic and non-enzymatic antioxidants were analyzed in micro algae grown under N, P reprieve and deprived conditions.

MATERIALS AND METHODS

This study was carried out at Department of Biotechnology, Indian Academy Degree College- Autonomous, Bangalore between February-October, 2018.

Organism and growth conditions: Monoalgal, axenic culture of *Chlorococcopsis minuta* was used in this study. The algae were grown in Bristol's media as nutrient fulfilled condition with continuous illumination (white fluorescent light, 132 mmol photons m^{-2} sec^{-1}). In the nutrient depleted experiments, different types of cultivation strategies such as:

- Nitrogen exclusion and phosphorous inclusion (N-, P+)
- Nitrogen inclusion and phosphorous exclusion (N+, P-)
- Exclusion of both nitrogen and phosphorous (N-, P-)
- Control inclusion of both nitrogen and phosphorous (N+, P+) were used

In both the cases, exponential cultures were used for enzymatic and non-enzymatic antioxidant activities.

Superoxide dismutase (SOD) assay: Microalgal cells were homogenized in 0.5 M phosphate buffer (pH 7.5) and centrifuged at 13,000 rpm for 10 min at 4°C. To the supernatant, added 1.5 mL Na_2CO_3 (1 M), 200 mL methionine (200 mM), 100 mL nitroblue tetrazolium (NBT) (2.25 mM), 100 mL EDTA (3 mM), 100 mL riboflavin (60 µM) and 1.5 mL phosphate buffer (pH 7.8, 0.1 M) to determine the inhibition of NBT. The absorbance was recorded at 560 nm and one unit of SOD per milligram of protein was defined as the amount causing 50% inhibition of photochemical reduction of NBT[16].

Catalase (CAT) assay: For catalase assay, 50 mg biomass was homogenized in 2 mL phosphate buffer (0.5 M, pH 7.5), centrifuged at 12,000 rpm at 4°C for 30 min and the supernatant was collected. A reaction mixture containing 1.6 mL phosphate buffer (pH 7.3), 100 µL EDTA (3 mM), 200 µL H_2O_2 (0.3%) and 100 µL supernatant was taken in a cuvette and CAT activity in supernatant was determined by monitoring the disappearance of H_2O_2, by measuring a decrease in absorbance at 240 nm against a blank of same reaction mixture without H_2O_2[17].

Ascorbate peroxidase (APX) assay: For analysis of ascorbate peroxidase activity, 100 µL of microalgal supernatant was added with 1 mL phosphate buffer (pH 7.3), 100 µL EDTA (3 mM), 1 mL ascorbate (5 mM) and 200 µL H_2O_2 (0.3%). The reaction was followed for 3 min at a wavelength of 290 nm against a blank of same reaction mixture without H_2O_2[18].

Non enzymatic antioxidant scavengers

Hydrogen peroxide assay: Hydrogen peroxide levels were determined as described by Alexieva *et al.*[19]. To algal pellets,

5 mL of 0.1% (w/v) ice cold trichloro acetic acid was added followed by sonication for 30 sec. The homogenized mixture was then centrifuged at 12,000 rpm for 15 min. To the 0.5 mL of supernatant, 0.5 mL of 100 mM potassium phosphate buffer (pH 7.4) and 2 mL of 1 M potassium iodide was added. The reaction mixture was incubated at room temperature for 1 h in dark and their absorbance was recorded at 390 nm.

Proline assay: Proline assay was performed by homogenizing the dried micro-algal biomass with 5 mL of sulphosalicylic acid (3%) followed by filtration. To 1 mL of filtrate, equal volumes of glacial acetic acid and acid ninhydrin solution was added and incubated in a boiling water bath for 1 h. The reaction was stopped by placing the filtrate mixture on ice. In the next step, 4 mL of toluene was added, vortexed and the absorbance of the upper pink layer was read at 520 nm. The concentration of proline was calculated from a calibration curve using L-proline as a standard[20].

Polyphenol content: Total polyphenols in the micro-algae were determined according to the Folin-Ciocalteu method[21]. The algal cells were homogenized with 5 mL of 80% ethanol and centrifuged at 10000 rpm for 20 min. To the supernatant, 1 mL of Folin-Ciocalteu's reagent and 5 mL of distilled water were mixed together followed by incubation at room temperature in the darkness for 5 min. After this, 2 mL of sodium carbonate (20%) was added and the contents were incubated at room temperature for 1 h in the darkness. The absorbance of the solution was measured at 650 nm against a blank. Gallic acid was used as a standard and the total phenolic content of algal samples was expressed in mg g^{-1} of gallic acid equivalent (GAE).

Level of lipid peroxidation: Lipid peroxidation was determined in terms of malondialdehyde (MDA) content in the cells[22]. Microalgal cells were harvested by centrifugation, homogenized in 2 mL of 80:20 (v:v) ethanol:water followed by centrifugation at 20000 rpm for 10 min. About 1 mL of the supernatant was mixed with equal volume of thiobarbituric acid (TBA) solution (20.0% (w/v) trichloroacetic acid (TCA), 0.01% butylated hydroxytoluene and 0.65% TBA). The mixture was boiled for 20 min and the contents were centrifuged at 20000 rpm for 10 min. The absorbances of the supernatant were recorded at 450, 532 and 600 nm. The MDA content was calculated using the following formula and expressed on a fresh weight (FW) basis:

$$\text{MDA } (\mu\text{mol g}^{-1}\text{ FW}) = \frac{[6.45\times(A_{532}-A_{600})]-[0.56\times A_{450}]}{\text{FW}}$$

Statistical analysis: The experiment was carried out in triplicates and the data presented are mean values ± standard deviation of three independent replicates. All data were further analyzed by one-way analysis of variance (ANOVA). The mean values were compared with the LSD test and a significant difference was considered at $p<0.05$.

RESULTS

Enzymatic antioxidant assays: Activities of antioxidant enzymes in *C. minuta* during nutrient stress showed that the activities of SOD, CAT and APX were increased in nutrient stress cultures when compared to nutrient fulfilled conditions. The SOD activity had increased to 9.8 U mg^{-1} protein in cells cultivated in N-P- media whereas it was 1.6 U mg^{-1} protein N+P+ media (Fig. 1). Catalase activity was higher in cells of N-P- media than other nutrient conditions. The activity of ascorbate peroxidase did not change considerably among the nutrient combinations studied (Fig. 2).

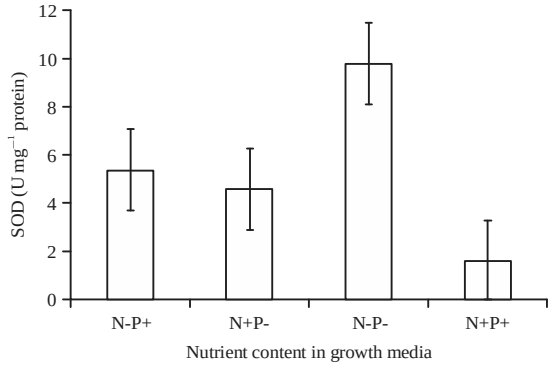

Fig. 1: Superoxide dismutase (SOD) assay of *C. minuta* during nutrient stress

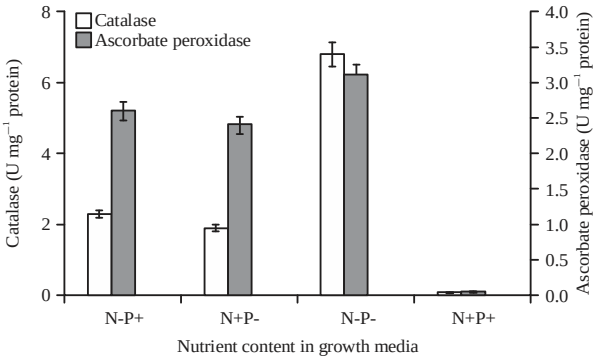

Fig. 2: Catalase (CAT) and Ascorbate peroxidase (APX) assay of *C. minuta* during nutrient stress

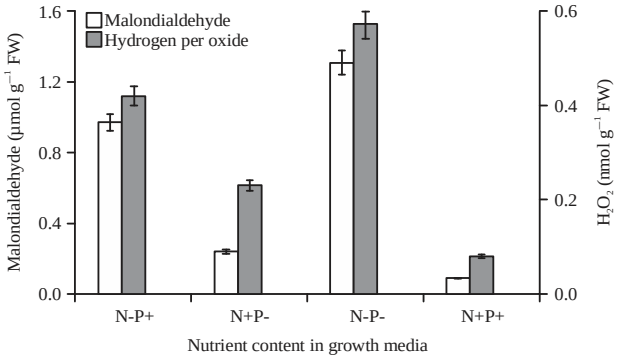

Fig. 3: Malondialdehyde (MDA) and H_2O_2 assay of *C. minuta* during nutrient stress

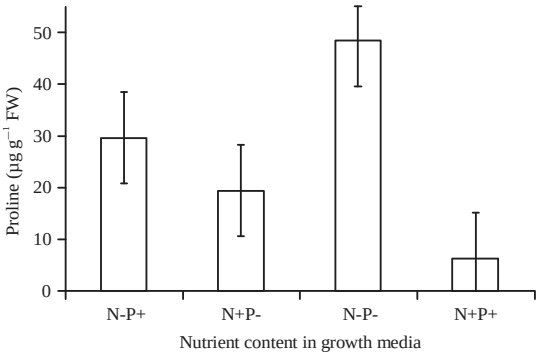

Fig. 4: Proline assay of *C. minuta* during nutrient stress

Non-enzymatic antioxidant scavengers: An increasing trend was observed in malondialdehyde (MDA) assay and 33% increase in lipid per-oxidation was observed in N-P- media than N-P+ media. The MDA is an end product of lipid per-oxidation usually released as an indicator in micro-algae under stress condition. Hydroxyl radicals have sufficient reactivity to initiate membrane per-oxidation by liberation of MDA as an end product. Hydrogen per oxide levels were increased in cells under nutrient stress which recorded 0.42, 0.23 and 0.57 nmol g^{-1} fresh weight in N-P+, N+P- and N-P- media, respectively (Fig. 3). Proline accumulation is a common response to stress in micro-algae in order to maintain chlorophyll level and cell turgor to protect photosynthetic activity under stress. In this study proline content increased with nutrient stress and reached 8 folds higher in nitrogen and phosphorous deprived media than the control (Fig. 4). Besides acting as an excellent osmolyte, proline plays three major roles during stress such as a metal chelator, an antioxidative defense molecule and a signaling molecule. The increase in proline content under nutrient stress indicated the defense mechanism of micro-algae for its survival under nutrient deprived conditions. Total phenolic content was

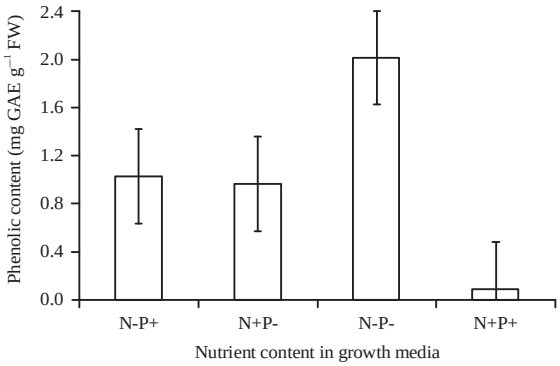

Fig. 5: Total phenolic content of *C. minuta* during nutrient stress

greater in N-P- indicating the nutrient stress triggered antioxidant system in the micro-algae (Fig. 5).

DISCUSSION

High levels of ROS are responsible for abnormal physiological reactions, leading to a condition of oxidative stress. Stress conditions in oxygenic photosynthetic organisms is described as oxidative stress[23] and the scavenging enzymes including super oxide dismutase, catalase, peroxidise will be induced or activated in cells[24-27]. In this study, the activities of enzymatic and non-enzymatic antioxidants were measured in micro algae under nutrient stress conditions.

Superoxide dismutase consists of a group of metallo isoenzymes that neutralize the very reactive super oxide radicals (O_2^-) into oxygen (O_2) and hydrogen peroxide (H_2O_2). Catalase degrades H_2O_2 without consuming cellular reducing equivalents. Hence, catalase provides the cell with a very energy-efficient mechanism to remove hydrogen peroxide. Therefore, when cells are stressed for energy and are rapidly generating H_2O_2 through catabolic processes, H_2O_2 is degraded by catalase in an energy-efficient manner. Catalases are absent in chloroplasts, so degradation of H_2O_2 in chloroplast is achieved by the activity of ascorbate peroxidase. Glutathione is a major water-soluble antioxidant in plant cells. It directly reduces most active oxygen species. Proline acts as an antioxidant by being a scavenger of OH· and O_2. The production of ROS has been indirectly indicated by changes in ROS scavenging compounds/enzymes[1]. The lipid peroxidation, a commonly accepted indicator of oxidative stress was assayed by measuring the level of MDA. Lipid peroxidation in terms of TBA was markedly increased when algal cells were grown under nutrient stress in this study.

To mitigate and repair the damage caused by ROS, enzymes such as SOD, POD and CAT usually accumulate in

cells[28]. Intracellular ROS may in fact be mediators of lipid accumulation in oleaginous micro-organisms[8,29]. Since stress-based strategies are widely used as an environmentally friendly approach to microbial lipid overproduction, understanding the relationship between various stress factors and lipid accumulation is of industrial and biotechnological importance[30]. A number of studies showed that stress-induced lipid accumulation always goes along with increasing antioxidant defenses or increasing intracellular ROS levels[31]. A coupling between neutral lipid accumulation and oxidative stress during N starvation in *Chlorella sorokiniana* was reported by Zhang *et al.*[9]. Similar results were reported in *Acutodesmus dimorphus* by Chokshi *et al.*[29]. Association of increased reactive oxygen species levels and cellular lipid accumulation under different environmental stress conditions was studied in *Scenedesmus* sp.[10]. Mandal and Mallick[32] cultivated the microalgae *Scenedesmus obliquus* under phosphorus starvation and witnessed a lipid content increased from 10.0-29.5%.

Nitrogen starvation triggers many metabolic responses in microalgae like degradation of nitrogenous compounds like protein, chlorophyll, DNA etc., and accumulation of energy rich compounds like lipids and carbohydrates. Nitrogen starvation condition has significantly decreased the photosynthetic activity and crude protein content in *Scenedesmus* sp.[33]. In other reports, nitrogen limitation led to decrease in growth rate, protein synthesis, photosynthesis and cell size in *Nannochloropsis gaditana* and *Miractinium pusillum* and at the same time an increase in lipid and carbohydrate content was observed[34,35]. Similar types of results have also been observed in other microalgal species like *Chlorella* sp.[36] and *Botryococcus braunii*[37], In *Oscillatoria willei*, reduced synthesis of pigments, lipids and fatty acids were observed but the Antioxidative enzymes helped in survival of microalga under nitrogen stress[38]. Changes in the relative total activity of antioxidant enzymes were recorded in micro algae grown under nitrogen depletion and nitrogen resupply in the medium[39]. The results of this study indicate that nitrogen and phosphorous deprivations in the growth media lead to enhanced antioxidant system in micro-algae. However the production of antioxidants with health benefits by the studied micro-algae needs to be explored. At the same time, exploiting the micro-algae for biofuel production is feasible through nutrient stress.

CONCLUSION

This study concluded that nitrogen deprivation accompanied by phosphorous depletion condition in the growth media has enhanced the activation of enzymatic and non-enzymatic antioxidant systems at various levels in *C. minuta.*

SIGNIFICANCE STATEMENT

The present investigation establishes antioxidant response in *Chlorococcopsis minuta* under nutrient limited conditions. Further studies involving correlation between the reactive oxygen species mediated lipid production would provide the strategy thereby to improve the biofuel potential in *C. minuta*.

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