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CHARACTERISATION OF POSSIBLE ENZYMATIC AND NON-ENZYMATIC ADAPTATIONS AGAINST UV-B STRESS IN THE CYANOBACTERIUM, SPIRULINA PLATENSIS.

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Abstract:

Depletion in the ozone layer leads to the entry of ultraviolet-B (UV) rays on to the earth surface which causes the damage to the cyanobacterial systems. The aim of the present study is to observe the characterisation of possible enzymatic and non-enzymatic antioxidant adaptations against UV-B radiation in the cyanobacterium Spirulina platensis. The enzymatic antioxidants like superoxide dismutase (SOD) and catalase (CAT) were measured in control and treated results in increase in the concentration of the enzymatic antioxidants results in the protection. Preliminary phytochemical screening of the enzymatic antioxidants like carotenoids, phenols, flavonoids and anthocyanins are measured which results in increase in the concentrations upon treatment with UV-B radiation. Methanolic extracts of the Spirulina platensis were screened for the presence of mycosporine like aminoacids (MAAs). Increase in the concentration of MAAs results in the protection of the cyanobacterium against UV-B.

Keywords: *Anthocyanins, Catalase, Carotenoids, Flavonoids, MAAs, Phenols.*

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INTRODUCTION:

Cyanobacteria are ubiquitous in nature that they exist in different kinds of environmental conditions. These cyanobacteria are a primitive group of gram negative, oxygenic photoautotrophic prokaryotes. Since from the last decade depletion of the ozone layer due to the environmental pollution leads to the entry of the harmful Ultraviolet radiation (UVR) on to the earth surface which results in the damage of the physiological process of the cyanobacteria and the terrestrial plants. The changes in these composition results in various primary UV-B mediated events such as loss of permeability membrane changes, pigment destruction, direct photosynthetic damage, protein and enzyme inactivation, reduction in protein and DNA synthesis, reduced uptake of nutrients, inactivation of hormones, effect in signal transduction [1,2].Cyanobacteria due to the adverse effects of harmful UV-rays have adopted many protection mechanisms. The mechanisms evolved by cyanobacteria to cope with UV radiation are protection, repair and avoidance [3,4]. Cyanobacteria perform oxygenic photosynthesis using water as an electron donor. Therefore, they release molecular oxygen into the environment, which can be accumulated and converted into potentially harmful reactive oxygen species (ROS). The interaction between UVR, oxygen and certain organic compounds can produce toxic intermediates called ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroperoxy radicals (HO_2^-) and hydroxyl radicals (OH^-). These ROS can cause extensive damage to proteins, nucleic acids and other biological structures [5]. To overcome these ROS cyanobacteria has developed various enzymatic and non-enzymatic antioxidants like carotenoids, tocopherols (vitamin-E), ascorbic acid (vitamin-C) and reduced glutathione (GSH). Enzymatic antioxidants are superoxide dismutase (SOD), catalase, and glutathione peroxidise, glutathione reductase (GR) [6,7]. There is significant evidence that cyanobacteria when exposed to oxidative stress results in increase in the activities of ROS scavenging enzymes [8]. This indicates that higher and more stable antioxidant enzyme activities are associated with high stress tolerance in cyanobacteria. Thus the antioxidant defence mechanism against ROS is important for the survival of cyanobacteria under stress conditions. Apart from anatomical alterations in plants, the accumulation of UV-B absorbing compounds found in species of natural ecosystems, as well as UV-B reflecting waxes, may contribute to the protection of photosynthesis in nature [9,10] UV radiation effect on cyanobacteria results in increase of two types of sunscreen pigments scytonemin and MAAs [11]. These are also called as secondary metabolites. These secondary metabolites are thought to play multiple roles against several environmental stresses such as UV-radiation and desiccation [12]. MAAs are ultraviolet absorbing molecules having absorption maxima between 320-360 nm. These are one of the pigment molecules produced in cyanobacteria and algae [13]. According to Wildman, nutraceuticals include isoprenoid derivatives (i.e. carotenoids and tocopherols); phenolic compounds (tannins, anthocyanins, flavonoids) etc. Phenols protect against oxidation in both plants and cyanobacteria by various mechanisms. Flavonoids are the UV absorbing compounds. These protect photosynthetic tissues by acting as sunscreen pigments absorbing UV light. Flavonoids also possess free radicals scavenging activity, which offer additional protection to the cells against toxic radicals [14]. Anthocyanins are the water soluble natural pigments responsible for the colour of most fruits and vegetables and are the main polyphenols belonging to the class flavonoids [15]. Anthocyanins are synthesized within the plants from flavanol derived structures called anthocyanidins.

MATERIALS AND METHODS:

The cells of cyanobacterium, *Spirulina platensis* were grown axenically in the Zarrouks medium at 25 ± 2^0 C under continuous illumination of light 15 Wm^2. The log phase cells were harvested into the fresh growth medium and further analysis was done suspending it to the UV-B treatment of different intensities (0.7-2.8 Wm^{-2}) for 30 min of time interval. The control and treated cell suspension were used for the non enzymatic and enzymatic antioxidant assays.

Estimation of carotenoids:

The non enzymatic antioxidants like total content of carotenoids estimation is done by using the method of Semenenko and Abdullaev ,1980 **[16]**. Carotenoids concentration calculated by using formula:

$$C = \frac{A_{450} \times V \times f \times 10}{2500}$$

C=Total amount of Carotenoid (mg/ml)
V=Volume of extract (ml)
F = Dilution factor

Extraction and analysis of MAAs:

Effect of UV-B radiation on cyanobacteria results in the formation of certain sunscreen pigments/protective pigments like mycosporine like aminoacids. The UV-B stress responsive/protective pigments can be identified using spectroscopic method as described by Tartarotti and Sommaruga (2002) [17]. The methanolic extracts were scanned between 250 - 700 nm by using UV-Vis spectrophotometer.

To determine the MAAs content, maximum absorbance and corrections were made according to the following expression [18] (Gracia-Pichel and Castenholz, 1993).

Determination of total phenolic content:
Total phenolic content was determined by Folin-Ciocalteau method Singleton *et al.,* 1999[19]. Gallic acid was used as standard and the total phenolics were expressed as mg/g gallic acid equivalents (GAE). The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. Total phenolic content values are expressed in terms of gallic acid equivalent (mg/g).

Total flavonoid content:
To quantify total flavonoids, 125 µL of the sample supernatant prepared was combined with 37.5 µL of 5% (w/v) sodium nitrite and 0.625 mL of nanopure water according to Adom and Liu (2002) **[20]**. After allowing the mixture to react for 4-6 min at room temperature, 75 µL of 10% (w/v) aluminum chloride was added to each sample, followed by 0.25 mL of 1.0 M sodium hydroxide. Nanopure water (0.4 mL) was added after allowing the sample to mix by 5-7 min. The mixture was than vortexed and than measured spectroscopically at 510 nm. Total flavonoids were expressed as mg/ g.f.w of *Spirulina*.

Total anthocyanin content:
Water-soluble pigments (anthocyanins) were extracted from cyanobacterial cell sample at the end of the UV-B treatment using the method of Jordan *et al.*,(1994) **[21]**with some modifications. Cells were freezed and thawed before extraction in 10 cm^3 of acidified methanol (HCl: methanol, 1:99, v/v). Absorption spectra of the extracts were determined using a Cary 210 spectrophotometer (Varian, Palo Alto, CA, USA), and the anthocyanin contents were estimated from absorbance at 530 nm. The results obtained are expressed in the terms of mg/g.f.w.

Assays of enzymatic antioxidants:
Assay of SOD activity was done according to the method of Ginnopolitis and Ries (1977) [22] . Catalase enzyme activity was assayed as described by the method of Hans- Luck (1970) [23].

RESULTS AND DISCUSSION:

Protection mechanisms of cyanobacteria against UV radiation majorly include avoidance, protection and repair. Generally, under stress conditions like UV-B radiation, there will be generation of superoxide and peroxide radicals. To scavenge the generated toxic oxygen species there will be enhancement in the activities of antioxidant system. Several organic and inorganic compounds present in the natural environment are known to play an additional role in the protection against UV-B radiation. The antioxidant machinery includes the lipophilic antioxidants like carotenoids, hydrophilic antioxidants like GSH and enzymatic antioxidants like SOD, CAT, peroxidase, ascorbate peroxidase.

There is a relationship between the exposure of UV-B radiation and the total carotenoid concentration as shown in the Fig :1a. Control cells contain less concentration of total carotenoids concentration whereas in UV-B treated cells showed increase in the concentration with increase in the intensity of UV-B radiation. The fate of total carotenoids on further raise in the intensity of UV-B radiation to 2.8 Wm^{-2} results in the decrease of the total carotenoid concentration. This sudden decrease in the concentration is due to the damage of the cell due to high intensity of UV-B radiation.

In any living organism in response to stress it is going to produce or synthesise protective pigments and some secondary metabolites to overcome the stress conditions. Particularly under UV-B radiation most of the algae including cyanobacteria are going to synthesize some protective pigments like MAAs and scytonemin etc. When natural defences fail to protect against oxidative stress, balance may be restored by the consumption of nutraceuticals that have antioxidant properties. To know the synthesis of protective pigments in this cyanobacterium, we have gone for the spectrophotometric screening of the protective pigments. For this treatment has given to the sample of UV-B radiation of intensity 2.1Wm^{-2} for 30 min. After the treatment both treated samples and control samples were extracted with methanol and scanned from 250- 700 nm of wavelength which showed extra peaks at 310 and 334 nm at UV region (Fig: 2a) Due to the absorption peaks at 310 and 334 nm , the peaks a and b are an assumption can be made that the present MAAs in the sample can be mycosporine like glycine which has the maximum absorption at 312 nm and porphyra 334 which has the maximum absorption at 334 nm. These are the special sunscreen pigments which can trap the UV-B rays and protect the cells. The total content of the MAAs present in the cell also determined. From Fig: 2b it is clear that control cells contain less concentration of MAAs. The UV-B treated cells (2.1Wm^{-2}) for 30 min results in the increase in the concentration of the content of MAAs. This increase in the content of MAAs during stress condition is also an indication for the protection of cell by the sunscreen pigments like

MAAs. Several workers reported the protective pigments under UV-B radiation in different cyanobacterial species **[24,25,26].**Realising the importance of phenolic compounds in the scavenging of harmful free radicals we have planned a simple experiment to know the concentration of total phenolic compounds present in the cyanobacterium in untreated control samples and treated samples are done by which enhancement of total phenolic compounds was observed.

In the Fig : 1b it can be observed that total phenolic compounds in the control sample is less. The total phenolic content in the UV-B treated (2.1 Wm^{-2} for 30 min) sample is increased. Total phenolic content has been shown to increase in *Spirulina platensis* under UV-B stress conditions. This indicates that UV-B stress can also stimulate an increase in the synthesis of secondary metabolites. On the other hand as flavonoids, i.e flavonols,flavones,isoflavones and anthocyanins function in protection against UV light. They are also major antioxidants scavenging ROS under stress conditions. So the concentration of total flavonoid and anthocyanis are measured. From the Fig: 1c, the vertical bar C denotes the total flavonoid content of the control sample is 3 mg/g.f.w. Another vertical bar denotes UV treated sample (2.1Wm^{-2}) for 30 min is 9 mg/g.f.w. This surprisingly denotes the enhancement in the total flavonoids concentration in treated samples. Increase in the flavonoid concentration indicates the protection against UV-B radiation by secondary metabolites.

Another pigment compound and secondary metabolite present is anthocyanins which also has the antioxidant property. *Spirulina* consists of blue coloured anthocyanins so the name blue green algae. Anthocyanins are also one of the secondary metabolites which are involved in the protection against UV-B. From the Fig : 1d the vertical bar C denotes the total anthocyanin content of the control sample 0.3 mg/g.f.w. Another vertical bar denotes UV treated sample (2.1Wm^{-2}) for 30 min which is measured as 0.7 mg/g.f.w. Here also the enhancement in the total anthocyanin concentration in treated samples is observed. Increase in the total anthocyanin concentration indicates the protection against UV-B radiation by secondary metabolites. The increase in UV-B absorbing compounds, mainly like flavonoids and anthocyanins are recognised as the general response against UV-B radiation damage to cyanobacteria **[27]**.

After knowing about antioxidant compounds, the role of antioxidant enzyme activities was measured. All enzymes showed a same pattern of increased activity under UV-B stress. SOD and catalase are known to be involved in scavenging of toxic oxy radicals. To verify the above properties an attempt has been made to measure the SOD and catalase enzyme activity under UV-B stress. In control samples the activity of enzyme is equal to 1.9 U/mg protein (Fig :3a). Upon exposing to UV-B radiation increase in the intensity leads to raise in the enzyme activity of about 19-20%. This is due to the induction of enzyme to neutralise the formed super oxide radicals under UV-B stress.

To identify role of another antioxidant defense enzyme catalase, which scavenge free radicals, the enzyme activity has been measured after giving same intensities of UV-B radiation to the cyanobacterium sample. The activity of control sample is 0.0184 U/mg protein (Fig : 3b) Upon exposing to UV-B radiation , increase in the intensity by 2.1 and 2.8 Wm^{-2} leads to raise in the enzyme activity of about 19-23%. When the above two enzyme activity induction is compared it is clear that UV-B radiation promotes the generation of peroxy radicals than super oxy radicals. Thus UV-B radiation can cause the production of free radicals which can be scavenged by the antioxidant enzymes. The increase in the SOD, CAT activities are frequently observed under stressful conditions [28].

CONCLUSIONS:

The increase in the concentrations of secondary metabolites like phenols, flavonoids, anthocyanins clearly represents their protective role against UV-B damage. In response to UV-B radiation there is an increase in the antioxidant enzymes like SOD, CAT which are helpful in scavenging of free radicals. The increased concentrations of MAAs clearly demonstrates that they are acting as protective sunscreen compounds against the UV-B damage. Absorption of methanolic extracts at 312 and 334 nm clearly demonstrates the presence of new UV-B screening pigments like mycosporine like aminoacids under UV-B stress. By the peaks obtained at 312 and 334 nm it can be assumed that the presence of mycosporine like aminoacids like mycosporine like glycine and porphyra- 334.

Thus the above study clearly indicates that UV-B is able to cause structural and functional alterations in this cyanobacterium. The organism is able to show the possible protection mechanisms such as enzyme induction, synthesis of new sunscreen pigments, secondary metabolites like phenolic compounds, flavonoids, anthocyanins against UV-B mediated damage in the cyanobacterium, *Spirulina platensis* to withstand against the above stress conditions.

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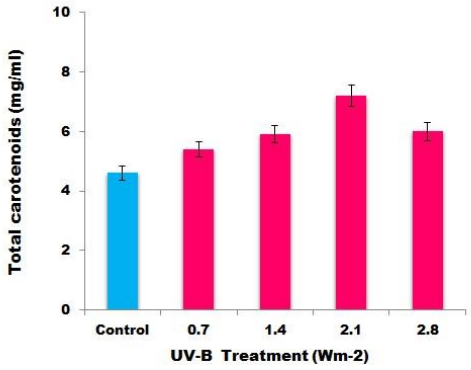

Fig : 1a Effect of different intensities of UV-B radiation on total carotenoid content in the cyanobacterium,*Spirulina platensis*.

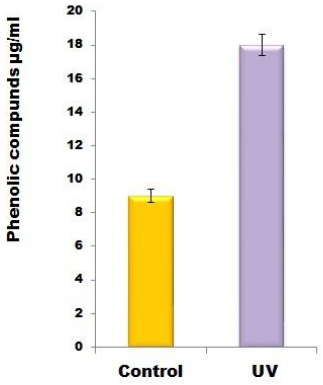

Fig : 1b Effect of UV-B radiation (2.1 Wm^{-2}) for 30 min on the concentration of total phenolic content.

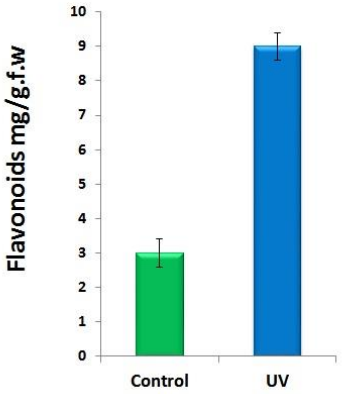

Fig : 1c Effect of UV-B radiation (2.1 Wm^{-2}) for 30 min on the concentration of total flavonoid content.

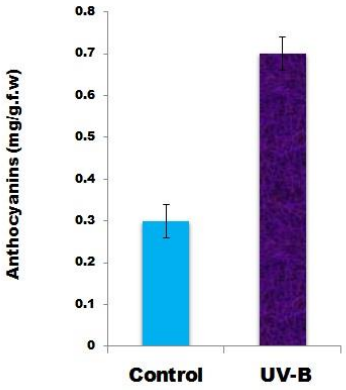

Fig : 1d Effect of UV-B radiation (2.1 Wm^{-2}) for 30 min on the concentration of anthocyanin content.

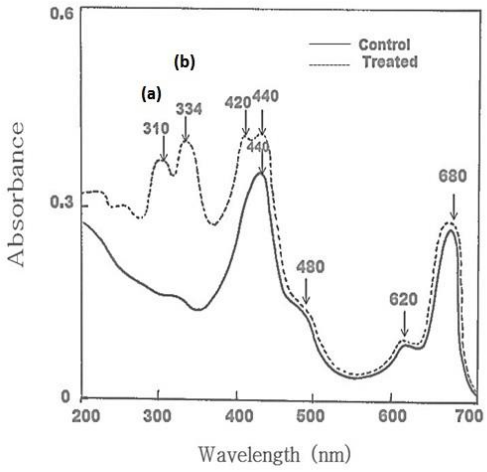

Fig: 2a Absorption spectra of *Spirulina* methanolic extracts isolated from control and UV-B treated samples (2.1 Wm^{-2}) for 30 min.

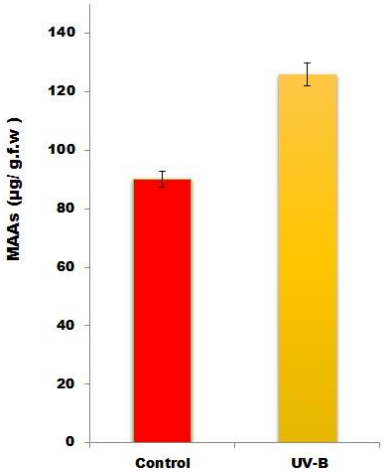

Fig : 2b Effect of UV-B radiation (2.1 Wm^{-2}) for 30 min on the concentration of total content of MAAs.

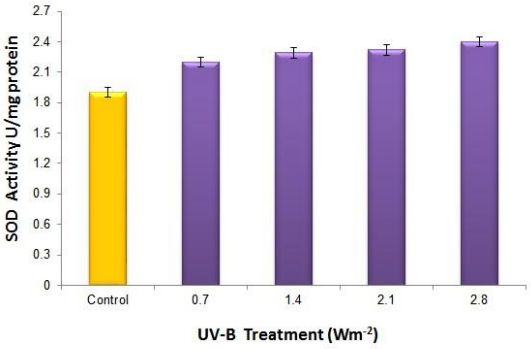

Fig : 3a Effect of different intensities of UV-B radiation on SOD activity in the cyanobacterium, *Spirulina platensis*.

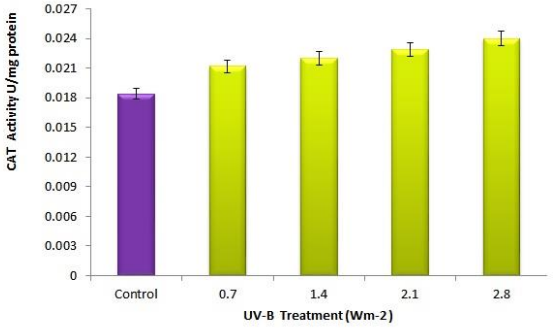

Fig: 3b Effect of UV-B radiation on CAT activity in the cyanobacterium, *Spirulina platensis*.