

Ultraviolet Radiation-absorbing Mycosporine-like Amino Acids in Cyanobacterium *Aulosira fertilissima*: Environmental Perspective and Characterization

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Abstract: The aim of this study is to screen cyanobacterial strains for the high yield of Mycosporine-like amino acids and further the effect of various physicochemical conditions were also observed for its highest yield. Cyanobacteria are one of the most primitive organisms capable of MAAs synthesis. The UV screening compounds MAAs are usually accumulated intracellularly in cyanobacteria. Among the 18 strains, *Aulosira fertilissima*, showed the presence of highest amount of MAAs hence selected for the exposure of various physicochemical conditions (pH, light quality, UV-light, photoperiod and temperature). MAAs content was highly increased by UV exposure. 20 min exposure of UV-light induces three times highest amount of MAAs as compared to control. In the presence of pH stress also the content of MAAs was approximately three times higher than control while light quality and temperature had very little effect on MAA concentration. The high-performance liquid chromatographic analysis of water-soluble compounds reveals the biosynthesis of two MAAs, porphyra-334 ($\lambda_{\text{max}} = 334$ nm) and shinorine ($\lambda_{\text{max}} = 334$ nm), with retention times of 3.5 and 2.3 min, respectively. Spectrophotometric analysis also showed absorption maxima at 334 nm.

Key words: Absorption spectrum, *Aulosira fertilissima*, HPLC, MAAs, physicochemical stress, Porphyra-334, shinorine

INTRODUCTION

There is growing interest in cyanobacterial species to explore the bioactivity of various cyanobacterial compounds associated with human life. A variety of cyanobacterial natural products with their specific activities, such as antimalarial, antituberculosis, anticancer, antifoulants, anti-inflammatory, anti-HIV, etc., have been reported from diverse cyanobacterial species Blunt *et al.* (2007) and Burja *et al.* (2001).

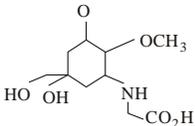
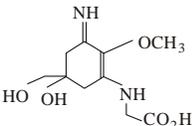
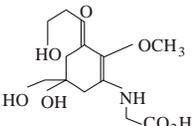
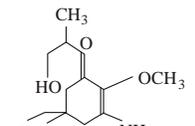
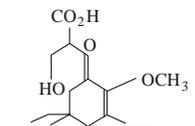
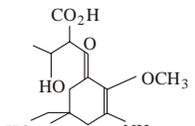
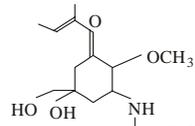
UVR is one of the most harmful exogenous agents and may affect a number of biological functions in all sunexposed living organisms. Solar radiation exposes the organisms to harmful doses of UV-B and UV-A (315-400 nm) radiation in their natural habitats. In response to intense solar radiation, organisms have evolved certain mechanisms such as avoidance, repair and protection by synthesizing or accumulating photoprotective compounds, such as MAAs (Table 1). Furthermore, MAAs is the most common compounds with a potential role as UV sunscreens in marine organisms. Mycosporine-like amino acids have been reported in diverse organisms; they are a family of secondary metabolites that directly or indirectly absorb the energy of solar radiation and protect organisms exposed to enhanced solar UVR Haider *et al.* (2007). MAAs are intracellular, small (400 Da), colorless and water-soluble compounds that consist of cyclohexenone or cyclohexenimine chromophores

conjugated with the nitrogen substituent of amino acids or its imino alcohol Singh *et al.* (2008b). In general, MAAs has a glycine subunit at the third carbon atom, although some MAAs contains sulphate esters or glycosidic linkages through the imine substituents Wu Won *et al.* (1997). MAAs are favored as photoprotective compounds because they have maximum UV absorption between 310 and 362 nm, high molar extinction coefficients ($\epsilon = 28,100\text{-}50,000$ per M \cdot cm), the capability to dissipate absorbed radiation efficiently as heat without producing Reactive Oxygen Species (ROS), and photostability and resistance to several abiotic stressors Conde *et al.* (2000) and Whitehead and Hedges (2005). It has been found that MAAs provides protection from UVR not only for their producers, but also to primary and secondary consumers through the food chain Helbling *et al.* (2002). MAAs has been reported extensively from taxonomically diverse organisms, including many marine groups such as heterotrophic bacteria Arai *et al.* (1992), cyanobacteria and micro/macroalgae (Table 1).

Aims and objective:

- To screen mycosporine-like amino acids from various cyanobacterial strains.
- To observe the synthesis of mycosporine-like amino acids under various stress conditions.

Table 1: Molecular structure, extinction coefficients and molecular weights for typically occurring MAAs

MAAs with their molecular structures	λ_{\max}	Extinction coefficient (per mol.cm)	Molecular weight (g/mol)	References
<p>Mycosporine-glycine</p> 	310	28100	245.23	Ito and Hirata (1977)
<p>Palythine</p> 	320	36200	244.24	Takano <i>et al.</i> (1978a)
<p>Asterina-330</p> 	330	43500	288.30	Gleason <i>et al.</i> (1993)
<p>Palythanol</p> 	332	43500	302.32	Takano <i>et al.</i> (1978b)
<p>Shinorine</p> 	334	44668	332.31	Tsujino <i>et al.</i> (1980)
<p>Porphyra-334</p> 	334	42300	346.33	Takano <i>et al.</i> (1979)
<p>Palythene</p> 	360	50000	284.31	Takano <i>et al.</i> (1978b)

- To characterize mycosporine-like amino acids by spectrophotometric techniques and chromatographic (HPLC).

MATERIALS AND METHODS

The strains used in the study were procured from Centre for Utilization and Conservation of Blue Green Algae, Indian Institute of Agriculture and Research Institute New Delhi, India and were maintained in BG11 media Stanier *et al.* (1971), except *Spirulina platensis* which was grown in CFTRI medium Venkatraman *et al.* (1982). Cultures were grown in BOD at 30±1°C in 12:12 light: Dark (L:D) period and illuminated in the culture cabinet at photon fluence rates of 25 µmol per m²s. 18 cyanobacterial strains were taken for the screening of mycosporine-like amino acids (MAAs). Their fresh biomass was harvested for the extraction of MAAs. Screening was followed by finding out different environmental stress (pH, light quality, UV-light, photoperiod and temperature) for the yield of MAAs. This research work was conducted from March 2008 till August 2009.

MAAs extraction and measurement: For determination of MAAs content, cells were extracted from harvested cyanobacterial biomass and washed twice with distilled water. Dried Cells were suspended and homogenized in 20% (vol/vol) aqueous methanol at 45°C in water bath for 2 h. After centrifugation supernatant is filtered through whatman filters. The absorbance of filtrate was measured spectrophotometrically at the wavelength of MAAs maximum absorbance and corrections were made according to the following expression (Garcia-Pichel and Castenholz, 1993):

$$A_{\lambda}^* = A_{\lambda} - A_{260}(1.85 - 0.005_{\lambda})$$

where,

A_{λ}^* is the corrected value of absorbance at the maximum.
 A_{λ} is the measured value of absorbance at the maximum.
 λ is the wavelength (nm) of maximal absorbance.

HPLC analysis of MAAs: Extraction of 10 mg of dried *Aulosira fertilissima* in 2 mL 20% Methanol (gradient grade) at 45°C for 2h. Centrifugation if necessary (10 min 10000 U). 1.5 mL of the supernatant was lyophilised. 2 mL 100 % Methanol was redissolved in the residue further vortexing followed by centrifugation was done. 1.5 mL of the supernatant was evaporated at 45°C. 1.5 mL H₂O was redissolving in the residue, again vortexing and centrifugation was done. Spectroscopic analysis of the supernatant was taken from 200 to 750 nm. Filtration through 0.2 µm pore sized filters. Waters HPLC (Waters 990), LiCrospher RP 18 column with precolumn (5 µm

packing; 250 x 4 mm I.D.) was used. Wavelength range for detection: 280-400 nm with a flow rate of 1.0 mL/min and a mobile phase of 0.02% acetic acid was taken for the experiment.

Effect of physicochemical conditions on the production of MAAs: After screening of cyanobacterial strains for MAAs content, *Aulosira fertilissima* was selected for finding out possibilities of increasing their yield through alterations in physiochemical environments eg. Temperature (20, 25, 30, 35 and 40°C), pH (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), light quality (white red, blue, green and yellow), UV-light (4, 8, 12, 16 and 20 min) and photoperiod (00:24, 08:16, 10:14, 14:10, 16:08 and 24:00 L/D).

Statistical analysis: All analysis was conducted using Graphpad Prism version-5.0 (Graph Pad software, San Diego, CA, USA). Stastical analysis of three replicates was done by one way analysis of variance (ANNOVA). Dunnett's multiple comparison test was used in experimental setup with control in which significant difference at a level of significance of 0.01, 0.001 and 0.0001 (p<0.01, p<0.001, p<0.0001) and 'ns' for non significant are represented.

RESULTS

All the cyanobacterial strains showed presence of water soluble, UV-absorbing substance MAAs (Table 2). Environmental factors it was observed that the synthesis of MAAs was directly proportional to temperature. With increase in temperature the synthesis of MAAs increases (Fig. 1). During the pH stress it was observed that MAAs was less than control (pH 7.8) in all pH except pH 9. The effect of increasing acidity and basicity on MAAs was not similar. With increasing acidity it decreased gradually but with increasing basicity its content initially decreased but increases at pH 9 (Fig. 2). Light quality stress showed that white light induces the synthesis of MAAs. Blue and yellow also showed significant increase in the synthesis of MAAs as compared to red and green light (Fig. 3). UV-light induces the synthesis of MAAs. The synthesis of MAAs was highest at 20 min UV light exposure (Fig. 4). Light has been found to be essential for MAAs synthesis. The concentration of MAAs was higher in light period as compared to dark. At 16 h light exposure the concentration of MAAs was highest further it showed decline. HPLC analysis of *Aulosira fertilissima* showed a chromatogram showing two prominent peaks at 334 nm (Fig. 5). Peaks were obtained at retention time of 3.5 min (for Porphyra-334) and 2.3 min (for Shinorine) the results were further compared with the absorption spectrum of elute (separated by HPLC) at 334 nm (Fig. 6).

Table 2: Screening of Cyanobacteria for Mycosporine-like amino acids

Strains	Heterocystous/Non-Heterocystous	MAAs
NCCU- 09 <i>Anabaena</i>	Heterocystous	0.0904
NCCU- 16 <i>Anabaena ambigua</i>	Heterocystous	0.0620
NCCU- 441 <i>Anabaena variabilis</i>	Heterocystous	0.1011
NCCU- 443 <i>Aulosira fertilissima</i>	Heterocystous	0.1600
NCCU- 65 <i>Calothrix brevisseima</i>	Heterocystous	0.0870
NCCU- 207 <i>Chroococcus</i>	Non-Heterocystous	0.0149
NCCU- 272 <i>Cylindrospermum</i>	Heterocystous	0.0989
NCCU- 430 <i>Gloeocapsa gelatinosa</i>	Non-Heterocystous	0.0625
NCCU- 339 <i>Haplosiphon Fontinalis</i>	Heterocystous	0.0540
NCCU- 102 <i>Lyngbya</i>	Non-Heterocystous	0.0586
NCCU- 342 <i>Microchaete</i>	Heterocystous	0.1193
NCCU- 442 <i>Nostoc muscorum</i>	Heterocystous	0.0452
NCCU- 369 <i>Oscillatoria</i>	Non-Heterocystous	0.0488
NCCU- 104 <i>Phormidium</i>	Non-Heterocystous	0.1460
NCCU- 204 <i>Plectonema</i>	Non-Heterocystous	0.0570
NCCU-12 <i>Scytonema</i>	Heterocystous	0.1229
S-5 <i>Spirulina platensis</i>	Non-Heterocystous	0.0924
NCCU-112 <i>Tolypothrix tenni</i>	Heterocystous	0.0873

mg: milligram; g: gram; DW: dry weight; MAAs concentrations are given as mg/g DW

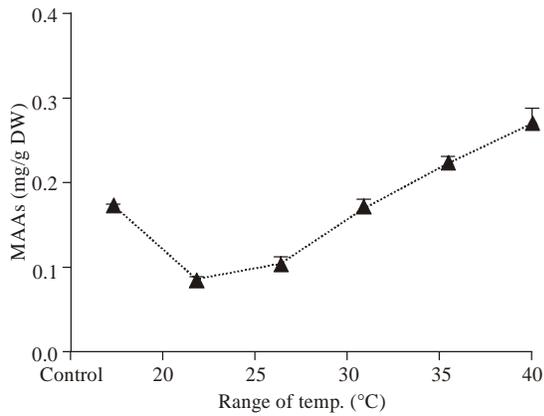


Fig. 1: Effect of temperature on MAAs in *Aulosira fertilissima*

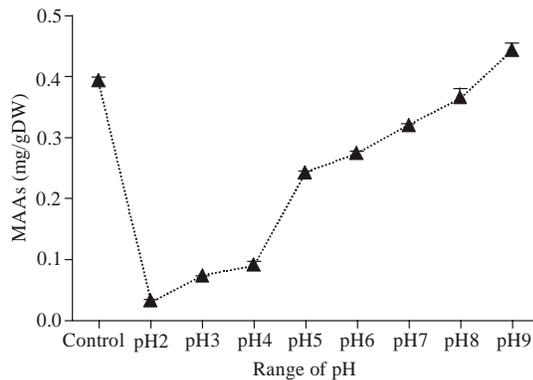


Fig. 2: Effect of pH on MAAs in *Aulosira fertilissima*

DISCUSSION

During present study water soluble, UV-absorbing substances MAAs could be detected in all tested cyanobacterial species (Table 1). In our isolates the

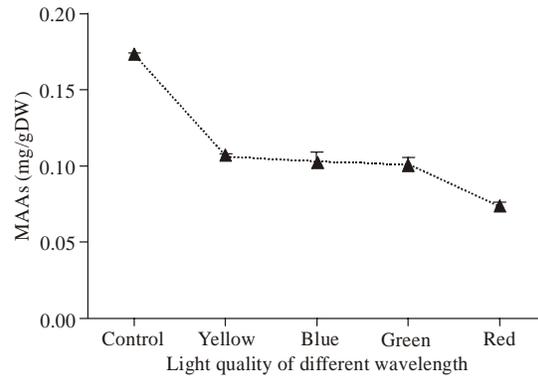


Fig. 3: Effect of different light quality on MAAs in *Aulosira fertilissima*

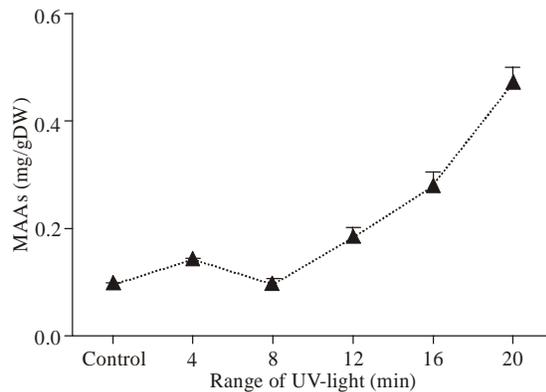


Fig. 4: Effect of UV-light on MAAs in *Aulosira fertilissima*

highest concentration of MAAs was found in (0.1600 mg/g DW) *Aulosira fertilissima* while the lowest concentration of MAAs was found in *Chroococcus* (0.0149 mg/g DW). Garcia-Pichel and Castenholz (1993)

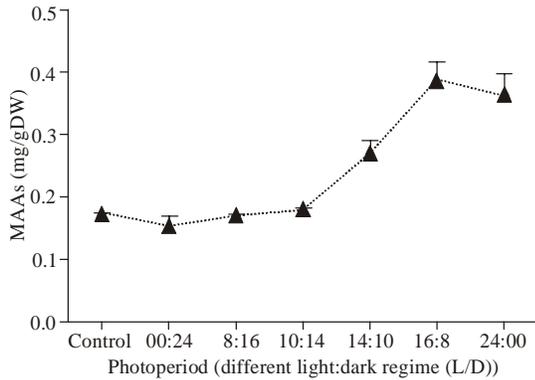


Fig. 5: Effect of photoperiod on MAAs in *Aulosira fertilissima*

reported maximum amount of MAAs in *Calothrix* sp. (0.32 mg/g DW), followed by *Lyngbya aestuarii* (0.17 A* mg/drywt) and in *Scytonema* sp. (0.01 mg/g DW). This variation in the values of MAAs may be due to the different conditions of culture maintenance. Their cultures are grown on polycarbonate Nuclepore filters receiving white light at photon fluence rates of 50 $\mu\text{mol}/\text{m}^2\text{s}$, while our cultures were grown in BG11 media receiving white light at photon fluence rates of 25 $\mu\text{mol}/\text{m}^2\text{s}$.

Effect of physicochemical conditions on the production of MAAs: Several environmental factors such as temperature, different wavelength UVR, pH, different light qualities and light as well as dark periods have been found to affect the production of mycosporine-like amino acids. The synthesis of MAAs is directly proportional to increasing temperature. The concentration of MAAs is higher at higher temperatures of 30-40°C.

Karsten *et al.* (1998) also demonstrated that the concentrations of MAAs in Rhodophyceae from polar (Spitsbergen) and cold-temperate (Helgoland, North Sea) regions are usually only half of those in species from warm-temperate (Spain) localities. MAAs was less than control (pH 7.8) in all pH except pH 9. The effect of increasing acidity and basicity on MAAs was not similar. With increasing acidity it decreased gradually but with increasing basicity its content initially decreased but increases at pH 9 (Fig. 2). Zhaohui *et al.* (2005) also found that Porphyra-334 [which is a sort of MAA] also increased with increase in pH. In present study maximum MAAs could be detected in white, yellow and blue light respectively while other lights (green and red light) did not play any significant role in the synthesis of MAAs. Korbee *et al.* (2005) reported that the shinorine was found to accumulate under white, green, yellow and red light in *Porphyra leucosticta* isolated from the intertidal zone of Lagos, Ma'laga, Southern Spain while blue light accumulates MAAs porphyra-334, palythine and asterina-330, Franklin *et al.* (2001). The MAA concentrations were significantly higher when cultures were grown with UV in comparison with samples grown without UV. The Concentration of MAAs was highest at 20 min UV-light exposure. For *Gloeocapsa* sp., the presence of MAAs led to higher growth rates under UV stress (Garcia-Pichel and Castenholz, 1993). Klisch and Ha"der (2002) reported in *Gyrodinium dorsum*, a nontoxic dinoflagellate, MAA was found to increase when induced by 310 nm radiation and also by UV-A radiation. Kra"bs *et al.* (2004) observed that similarly, a monochromatic action spectrum for photoinduction of the MAA shinorine was found in the red alga *Chondrus crispus* under UV-A radiation. Riegger and Robinson (1997) and Taira *et al.* (2004) reported that Photoinduction of MAAs by UVR has been reported in

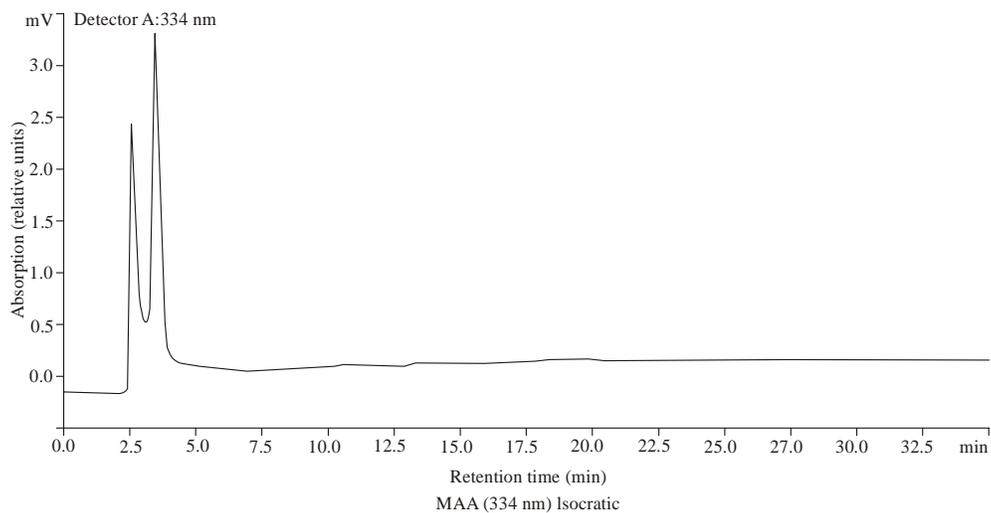


Fig. 6: HPLC chromatogram of *A. fertilissima*, showing the peaks for shinorine (2.3 min), porphyra-334 (3.5 min)

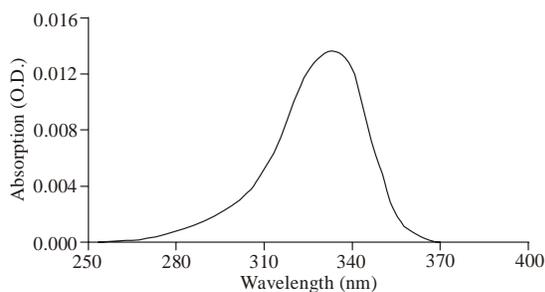


Fig. 7a: Absorption spectra of purified shinorine [as separated by HPLC] in *Aulosira fertilissima*

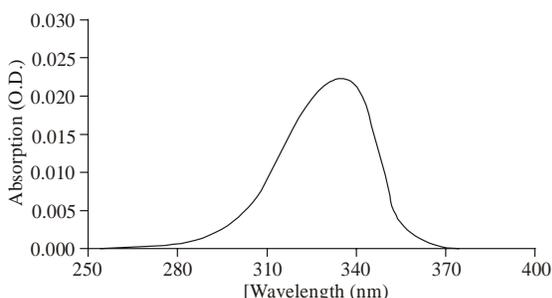


Fig. 7b: Absorption spectra of purified porphyra-334 [as separated by HPLC] in *Aulosira fertilissima*

the marine dinoflagellate *Scrippsiella sweeneyae* and diatoms. Sinha *et al.* (2001) and Sinha and Haider (2002) observed that the photo protective compound, MAAs in cyanobacteria is highly responsive to UV-B radiation. During the present study it was observed that light is essential for MAA synthesis in cyanobacteria and algae. The concentration of MAAs was higher in light period as compared to the dark. The highest amount of MAAs was observed in 16 h light exposure. In 24 h dark period the synthesis of MAAs was even less than control. Sinha *et al.* (2001) reported in an experiment, that the circadian induction in MAAs (i.e., increasing during the light period and decreasing during the dark period) was found under alternating light and dark conditions. Furthermore, under natural solar radiation, increasing concentrations of the photo protective compound shinorine, a bisubstituted MAA, were found only during the light periods, whereas more or less constant values of shinorine concentrations were found during and at the end of the dark period. This suggests that synthesis of MAAs is an energy dependent process and depends on solar energy for its maintenance in natural habitats. Purified form of MAAs was obtained through HPLC analysis. Hence HPLC chromatogram of *Aulosira fertilissima* was seen in which at 334 nm porphyra-334 and shinorine was observed with retention times 3.5 and 2.3 min, respectively. Singh *et al.* (2008a) also reported the HPLC chromatogram of cyanobacterium *A.doliolum* showing peaks of porphyra-334 and shinorine at 334 nm with

retention time 3.5 and 2.3 min, respectively (Fig. 6). The absorption spectra of the methanolic extracts (as separated by HPLC) of *A. fertilissima* showed absorption maxima for MAAs at 334 nm (Fig. 7).

CONCLUSION

During screening of cyanobacterial strains for MAAs *Aulosira fertilissima* appeared to be the best candidate. It produced highest amount of MAAs (0.1600 mg/g DW). While optimizing culture conditions for increasing yield of photoprotective pigments, it was found that 20 min UV exposure could increase MAAs content from 0.1600 to 0.472633 mg/g DW while second highest increase was observed under pH 9 where the increase in the content was 0.443133 mg/g DW.

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