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# BIOCHEMICAL CHARACTERIZATION OF MYCOSPORINE-LIKE AMINO ACIDS FROM DESICCATION TOLERANT CYANOBACTERIA *SCYTONEMA GEITLERI* AND *LYNGBYA ARBORICOLA*

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**ABSTRACT**
*Scytonema geitleri* and *Lyngbya arboricola* are desiccation tolerant cyanobacteria growing on the walls of building and bark of the trees respectively facing almost loss of water and heat during the summer season. Methanolic extract of dry mat of *Scytonema geitleri* in HPLC analysis revealed the presence of two MAAs i.e. Mycosporine-glycine ($\lambda_{max}$ 310 nm) and astrina-330. Further precipitate obtained after ammonium sulphate fractinaiton of methanolic extracts when evoporated to drynes and disolved in 0.2 % aqueous trifluoro-acetic acid and HPLC analysis using gradient of two solvents (mobile phase A and B) revealed that absorption maxima of first peak shift from 310 nm to 330 nm in addition to the astrina-330 (second peak) in the sample saturated by 0-20 % ammonium sulphate. As the saturation increased from 20-40 % to 40-60 %, absorbance maxima of first peak ($\lambda_{max}$ 330 nm) shifts to 332 nm which is of Mycosporine-2-glycine ($\lambda_{max}$ 332 nm). There was no such change in the second peak which is of the astrina-330. In *Lyngbya arboricola*, HPLC analysis showed the presence of only one MAAs i.e. Palythine ($\lambda_{max}$ 320 nm) in all the samples, irrespective of the extraction procedure.

**KEYWORDS**: desiccation, mycosporine-like amino acids, high performance liquid chromatography.

## INTRODUCTION

Cyanobacteria inhabiting terrestrial habitats are most commonly subjected to extreme of desiccation. In other words, most of the terrestrial cyanobacteria mainly occupying the subaerial habitats such as surfaces of rock, bark of trees, buildings and building material, and soil *etc*. are subjected to extremes of desiccation mainly due to uncertainty in availability of water *i.e*. frequent drying of the habitats. Besides this, these desiccation-tolerant cyanobacteria are also characteristically exposed to extremes of light and enhanced level of oxygen as compared to aquatic cyanobacteria. Desiccation-tolerant cyanobacteria are known to posses a variety of mechanisms to overcome the extremes of desiccation that includes the presence of either mucilaginous or firm sheath around their cells or trichomes. Certain water and partially lipid soluble compounds namely phycobili proteins, mycosporine like amino acids (MAAs) and their derivatives and scytonemin respectively have been reported to play an important protective role in desiccation-tolerant cyanobacteria against extremes of light and oxygen in their terrestrial habitats.

MAAs are water-soluble compounds of low molecular weight (<400 Da), composed of either an aminocyclohexenone or an aminocyclohexenimine ring, bearing nitrogen or aminoalcohol substituents, (Carreto *et al*., 1990; Karentz *et al*., 1991; Dunlap and Shick, 1998; Karsten *et al*., 1998; Rozema *et al*., 2002; Rezanka *et al*., 2004). Their absorption maximum ranges from 310 nm to 362 nm (Nakamura *et al.* 1982). So far more than 20 MAAs have been identified (Cardozo *et al*., 2007) and some unknown or partially characterized MAAs have also been reported, and many more are still to be identified. MAAs are colourless and have high molar absorptivity ($\varepsilon$=28100-50000 $M^{-1}$ $cm^{-1}$) for UV-A and UV-B. MAAs have been reported in a number of taxonomically diverse organisms such as fungi (Favre-Bonvin *et al*., 1976), marine heterotrophic bacteria (Arai *et al*., 1992), cyanobacteria (Garcia-Pichel *et al.*, 1993; Karsten and Garcia- Pichel, 1996), eukaryotic algae (Carreto *et al*., 1990; Karentz *et al.*, 1991a; Karsten *et al.*, 1998), marine invertebrates (Karentz *et al.*, 1991b; Shick *et al.*, 1992), fish (Dunlap *et al.*, 1989) and a wide variety of other fresh water and marine organisms (Karentz *et al*., 1991a; Dunlap and Yamamoto, 1995; McClintock and Karentz, 1997; Carefoot *et al.*, 1998; Dunlap and Shick, 1998). Cyanobacteria exposed to drought stress, contains high concentrations of MAAs (Tirkey and Adhikary, 2005; Wright *et al.*, 2005). MAAs provides protection from UV-induced damage by absorbing UV radiation and dissipating excess energy in the form of harmless heat (Takano *et al.*, 1978). MAAs prevents three out of ten photons from hitting cytoplasmic targets and provides protection from UV radiation (Sinha *et al.*, 1999). Besides, providing protection against UV radiation, MAAs plays additional function as 'multipurpose secondary metabolites' especially in microbial world (Oren and Nina, 2007). MAAs resists the desiccation related stress by the formation of extracellular matrix or sheath around the microorganisms in which glycosylated MAAs are embedded. MAAs alone do not provide the sufficient protection under high UV radiation, an active photo protective and / or repair mechanism is essential for survival of cyanobacteria (Gracia-Pichel *et al*., 1993,

Singh *et al*., 2009). Some MAAs has been reported to have antioxidants potential that might prevent cellular damage resulting from UV-induced production of toxic oxygen species (Dunlap *et al*., 2000). MAAs also helps microorganisms in adaptating to high salt concentration. Mycosporine-2-glycine with the maximum absorbance at 331nm (Kedar *et al.*, 2002) and a novel compound ($\lambda_{max}$ 362 nm) (Volkmann et al., 2006) was reported in the cyanobacterial cells cultured from the gypsum and Oren (1997) established that these two MAAs were involved in osmotic regulation of cyanobacteria.

So far many methods have been developed for extraction and separation of MAAs from the cultured and naturally growing cyanobacteria in water system. The aim of our present study is to develop an extraction procedure for cyanobacterial mat facing natural desiccation condition and to separate the maximum possible MAAs available in the system.

## MATERIALS AND METHODS

A). **Chemicals**

All the chemicals used were of HPLC grade.

B). **Selection of cyanobacteria**

Filamentous, desiccation-tolerant cyanobacteria *Scytonema geitleri* (heterocystous) and *Lyngbya arboricola* (non-hetrocyctous) has been selected for the present study. Dried mat of *Scytonema geitleri* and *Lyngbya arboricola* were collected from roof top of the Department of Botany and bark of the mango trees respectively from the campus of Banaras Hindu University after the rainy season. Both cyanobacteria are facing extreme desiccation condition during the summer season of May-June.

C). **Extraction of MAAS**

Five gm (dry weight) of dry mats of *Scytonema geitleri* and 2.5 gm (dry weight) of *Lyngbya arboricola* were taken and cut into small pieces and crushed in 45 ml 30 % Methanol (v/v) with the help of mortar-pestle; the crude extract was further sonicated (130 W, 20 kHz; Sonic & Materials, USA) on ice for 30 sec at 45 % amplitude followed by centrifugation at 4 $^0$C for 30 min at 10000 rpm. 1 ml of supernatant was subjected to absorption spectroscopy with a Hitachi U-2910 UV-Vis double beam spectrophotometer having 1-cm path length. 4 ml of supernatant was evaporated to dryness and re-dissolved in 1.5 ml of solvent A (0.2 % TFA, pH 3.15) for HPLC analysis. 40 ml of supernatant was left for ammonium sulphate fractionation.

D). **Ammonium Sulphate fractionation of extract**

40 ml of supernatant was precipitated by adding solid ammonium sulphate to obtain 20 % saturation, after overnight incubation, precipitate was collected by centrifugation at 4$^0$C for 60 min at 8000 rpm and supernatant was further precipitated by adding solid ammonium sulphate to obtain 20-40 % saturation, after overnight incubation, precipitate was collected by centrifugation at 4 $^0$C for 60 min at 8000 rpm. This process was repeated with the supernatant to obtain the 40-60 % ammonium sulphate saturated precipitate. No precipitation occurred on the addition of ammonium sulphate for 60-80 % saturation. All the precipitate was stored at 4 $^0$C.

The precipitates were mixed with 1.5 ml 30 % methanol and centrifuged at 4 $^0$C for 30 min at 6000 rpm. Supernatant obtained after centrifugation was evaporated to the dryness and re-dissolve in 1.5 ml of solvent A (0.2 % TFA, pH 3.15) for HPLC analysis after filtration.

E). **HPLC analysis**

The adopted method for separation of MAAs in present study is developed by the Carreto *et al*. 2005. Solvent A was 0.2% aqueous trifluoro-acetic acid (TFA) (pH 3.15 maintained by ammonium hydroxide) and solvent B was a solution containing aqueous trifluoroacetic acid (0.2%) and ammonium hydroxide at pH 2.20: methanol: acetonitrile (80:10:10, v:v:v). The aqueous 0.2 % TFA solutions were prepared as follows: 2.0 ml of TFA were added to 900 ml of water in a one liter flask and mixed using a stirrer. Ammonia solution was then added drop wise until the desired pH was obtained. The mixture was diluted to 1000 ml with water and the pH was rechecked. HPLC analysis was performed using C-18 Reverse Phase column and a Photo diode Array detector (Waters 2998) using Empower 2 software with injection volume 50.00µl, run time 51.0 minutes, using gradient of 100 % mobile phase A from zero to 2 minutes, 80 %A + 20 % mobile phase B from 3 to 15 minutes, 50 % A + 50 % B from 15 to 30 minutes and 50 % A + 50 % B for remaining 30 to 50 minutes, with a flow rate of 1 ml/ min. Detection was monitored at 330 nm and absorption spectra of MAAs were recorded at the wavelength range 200-700 nm.

## RESULTS

Absorbance spectrum of *S. geritleri* (natural mat) in 30 % methanolic extracts (Fig. 1.1 A) and methanolic extract after precipitation with 60 % ammonium sulphate (Fig. 1.1 B) showed the absorbance maxima at 330 nm and 620 nm corresponding to the MAAs and phycocyanin. Absorbance spectrum of *L. arboricola* (natural mat) in 30 % methanolic extracts (Fig. 1.2 A) and methanolic extract after precipitation with 60 % ammonium sulphate (Fig. 1.2 B) showed the absorbance maxima at 320 nm corresponding to MAAs and absorbance maxima at 560 and 500 nm corresponding to phycoerythrin. Absoption spectrum of 80% ammonium sulphate saturated precipitate of *L. arboricola* evoporated to dryness and dissolved in mobil phase A shows the partially purified MAAs at 330 nm and disappearance of photosynthetic pigments in visible range (Fig. 1.3). Results indicate that methanolic extract from the natural mat of cyanobacteria after fractionation precipitation with ammonium sulphate and dissolved in mobile phase-A gives the more purified extraction for MAAs. HPLC chromatogram of methanolic extract of *Scytonema geitleri* (dry mat) reveals the presence of two MAAs P-1 and P-2 (Fig. 2 A). In inset of the Fig. 2 A, absorbance peak maxima of P-1 at 310 nm (RT 3.7 min) and P-2 at 330 nm (RT 4.8 min) represents the two MAAs namely mycosporine-glycin and astrina-330 respectively. Methanolic extract further precipitated with 0-20% Ammonium sulphate (Fig. 2B), again two peaks appears, but now absorbance maxima of P-1 at 330 nm (RT 3.5 min) and P-2 at 330 nm (RT 4.9 min) (Fig. 2 B inset). When sample saturated with 20-40% ammonium sulphate (Fig. 2 C), absorption maxima of P-1 were found at 332 nm (RT 3.2 min) and P-2 at 330 nm (RT 4.7 min)

(Fig. 2 C inset). In sample saturated with 40-60% ammonium sulphate (Fig. 2 D), P-1 again showed a shift in absorption maxima at 332 nm while absorption maxima of P-2 remain the same at 330 nm (Fig. 2 D inset). HPLC analysis of *Lyngbya arboricola* (dry mat) extract in 30 % methanol (Fig. 3 A) and further fractionation precipitation with ammonium sulphate (Fig. B-D) reveals the presence of only one MAA i.e. Palythine having absorbance maxima at 320 nm (Fig. 3 A-D inset) but there was slight change in the retention time of the peak at 2.9 min in 30 % methanolic extract (Fig. 3 A), 2.7 min RT in 0-20 % (Fig. 3 B) and 20-40 % saturated extracts (Fig. 3 C) and 2.6 min RT in the 40-60 % saturated extract (Fig. 3 D).

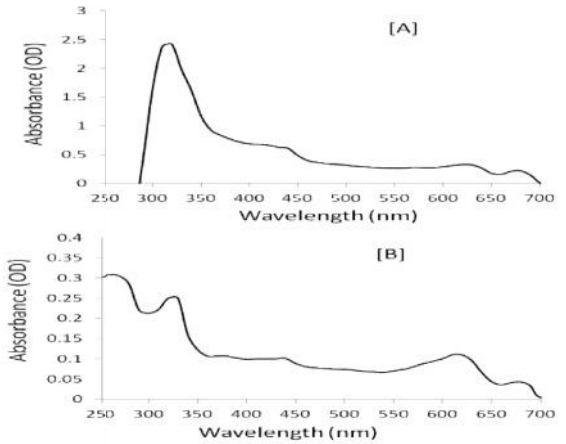


**FIGURE** 1.1 Absorption spectrum of *S. geitleri* (natural mat); [A] 30 % methanolic extract and [B] methanolic extract precipitated with 60 % ammonium sulphate showing peaks of MAAs (330 nm) and phycocyanin (620 nm).

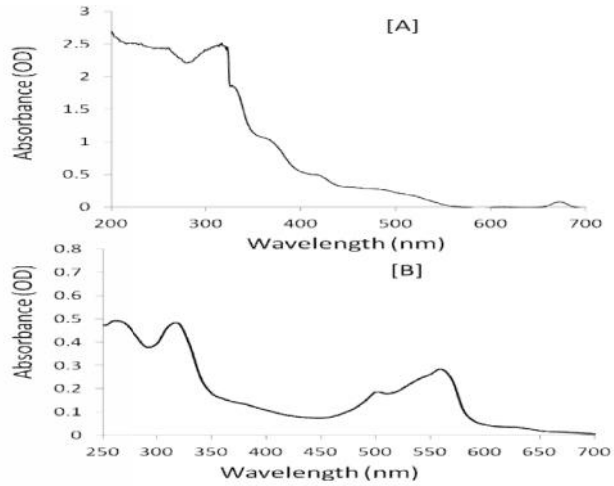


**FIGURE** 1.2 Absorption spectrum of *L. arboricola* (natural mat); [A] 30 % methanolic extract and [B] methanolic extract precipitated with 60 % ammonium sulphate showing peaks of MAAs (320 nm) and phycoerythrin (560 and 500 nm).

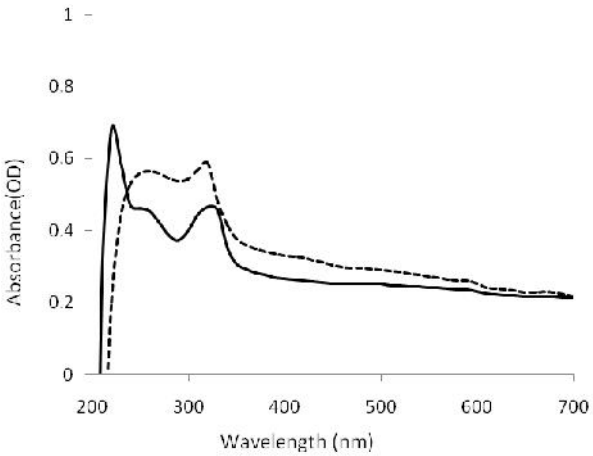

**FIGURE** 1.3 Absoption spectrum of sample of *S. geitleri* (dotted line) and *L. Arboricola* (solid line) obtained after ammonium sulphate fractination (60% saturation) precipitate mixed with 30% methanol, centrifuged and supernatent evoporated to dryness and dissolved in 0.2 % TFA (pH 3.15, solvant A) showing partialy purified MAAs peak in UV range at 330nm, other photosynthetic pigments in visible range is almost eliminated.

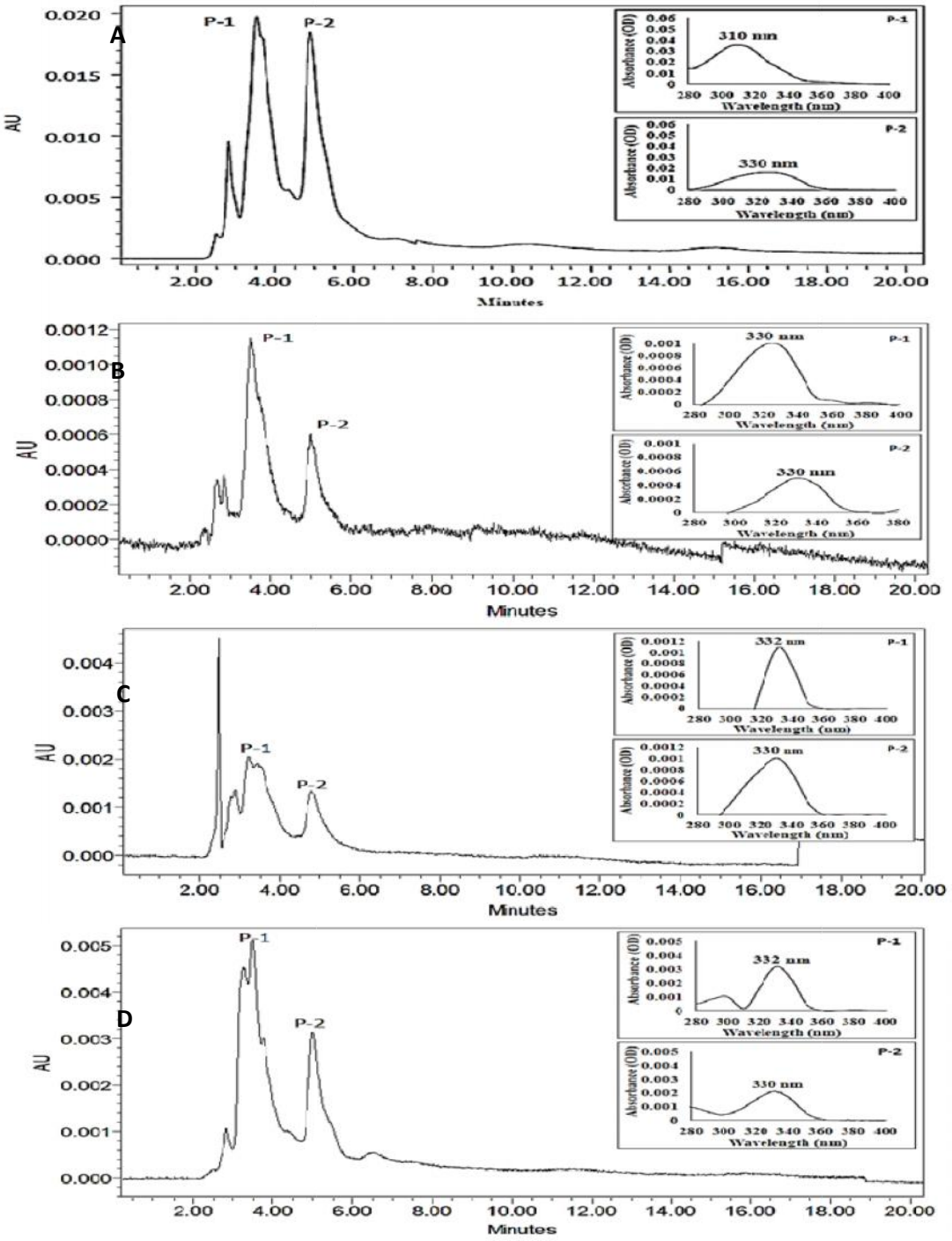


**FIGURE 2**: HPLC chromatograms represents peaks (P-1 and P-2) of mycosporine-glycine and astrina-330 at retaintion time of 3.7 and 4.8 min in 30 % methenolic extract (A), 20 % ammonium sulphate (B), 40 % ammonium sulphate (C) and 60 % ammonium sulphate (D) precipitation of *Scytonema geitleri* (dry mat). In inset showing absorbance peak maxima of P-1 and P-2.

It is found that retention time is continuously decreasing as we proceeds from 30 % methalolic extract to ammonium sulphate precipitated extract of *Lyngbya arboricola* (dry mat). In *Scytonema geitleri* retention time of Peak 1 was at 3.5 min in the 30 % methanolic extract and 20 % ammonium sulphate saturated sample, while RT of 40 %

and 60 % saturated sample was 3.2 min and 3.4 min respectively. Here also RT is decreasing from 30 % methanolic extract to 40 % ammonium sulphate saturated samples (Fig. 2 A to C, P-1) and then increased in 60 % ammonium sulphate saturated sample (Fig. 2 D, P-1). Retention time of Peak-2 in *Scytonema geitleri* (dry mat) is 4.8 min in 30 % methanolic extract (Fig. 2A), 4.9 min in 0.20 % ammonium sulphate saturated sample (Fig. 2B), 4.7 min in 20-40 % ammonium sulphate saturated sample (Fig. 2 C) and 4.9 min in 40-60 % saturated sample (Fig. 2D).

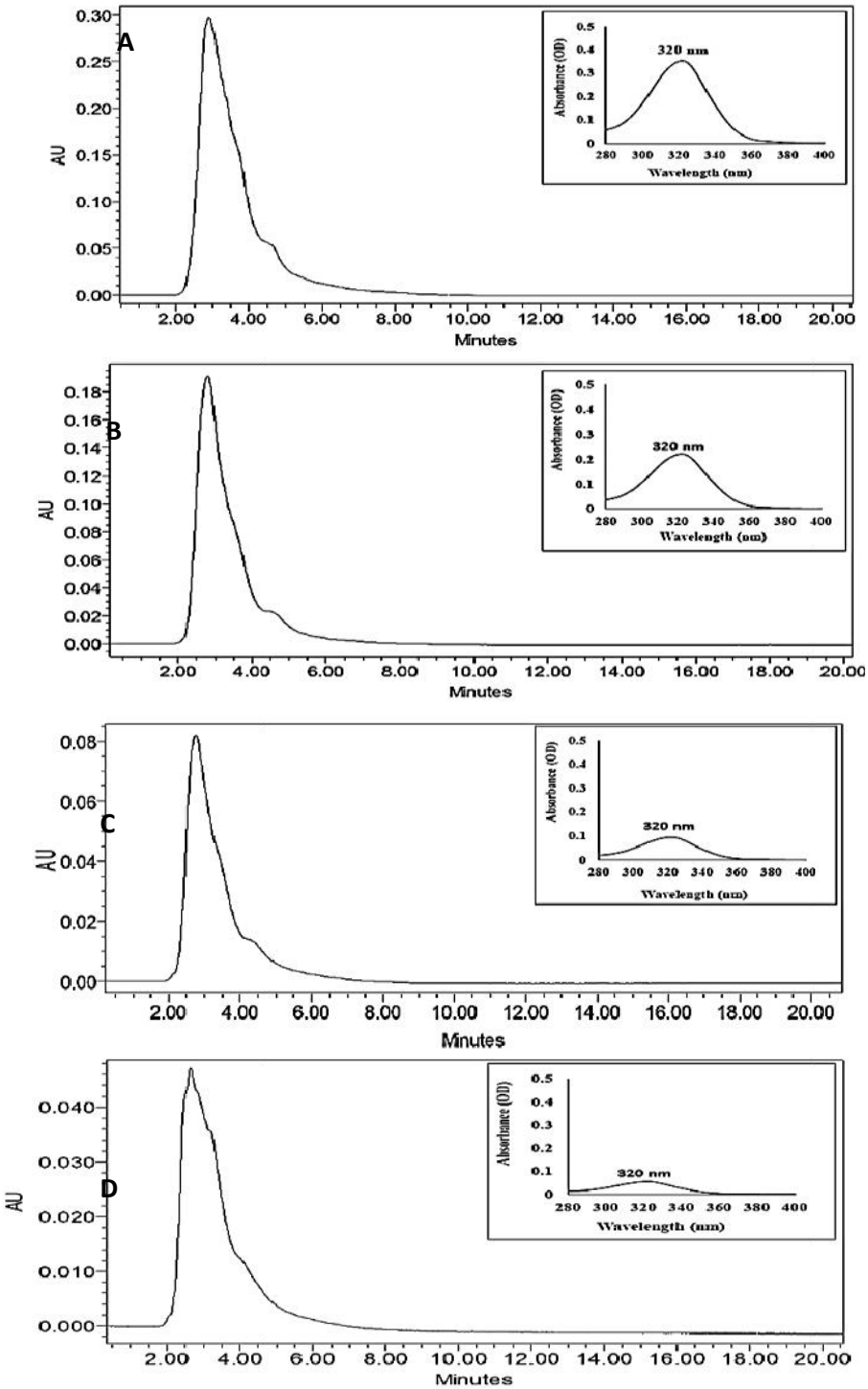


**FIGURE 3** HPLC chromatogram of MAAs in 30 % methenolic extract (A), 20 % ammonium sulphate (B), 40 % ammonium sulphate (C) and 60 % ammonium sulphate (D) precipitation fraction of *Lyngbya arboricola* (dry mat) reveals the presence of one MAA i.e. Palythine ($\lambda_{max}$ 320 nm). ). In inset showing continious decline in absorbance (OD) of peaks.

Concentration of peak-1 and peak-2 changes with the extraction procedure taking in to the optical density (OD) of each peak in to the consideration. OD of P-1 and P-2 has declined up to 96% in the sample saturated with 20 % ammonium sulphate (Fig. 2B inset) with respect to the concentration of P-1 found in the 30 % methamolic

extract. Further, P-1 and P-2 OD of 40 % ammonium sulphate saturated sample (Fig. 2C inset) was found to be declined up to 97 % and 93 % respectively with respect to sample extracted in 30% methanol (Fig. 2A inset) but optical density of P-2 (Fig. 2C inset) has found to be enhanced up to 50 % in comparison to the sample B (Fig. 2 B inset). In the sample saturated with 60% ammonium sulphate (Fig. 2D inset) P-1 and P-2 concentration was declined up to 91 % and 86 % respectively in comparison to the P-1 and P-2 of 30 % methanolic extract (Fig. 2 A inset). There was a 66 % increase in P-1 and 50% increase in P-2 concentration of sample saturated with 60% ammonium sulphate (Fig. 2D inset) in comparison to P-1 and P-2 concentration in the sample saturated with 40 % ammonium sulphate (Fig. 2C inset). In case of *Scytonema geitleri* (dry mat*)* the MAAs concentration was declined in the samples precipitated with ammonium sulphate in comparison to the sample extracted in 30 % methanol but the MAAs concentration was enhanced as the ammonium sulphate saturation increases. In case of *Lyngbya arboricola* the concentration of MAA decreased in the samples saturated with ammonium sulphate in comparison to the sample extracted in 30 % methanol which was same as found in *Scytonema geitleri* whereas OD of MAA has shown a decline as the ammonium sulphate saturation increases which is in contrast to the result found in *Scytonema geitleri.*

## DISCUSSION

MAAs extract of *S. geitleri*, first peak was showing a shift in the absorption maxima i.e. 310 nm in the sample extracted in 30 % methanol and 330 nm in the sample saturated with 20 % ammonium sulphate and 332 nm in 40 % and 60 % ammonium sulphate saturated sample. According to the absorption maxima of P-1 (Fig. 2 A-D), it appears that mycosporine-glycin (λmax 310 nm) is trasforming in to M-330 ($\lambda_{max}$ 330 nm) and M-330 is trasforming in to Mycosporine-2-glycine (λ max 332 nm). There was no change in the absorption maxima of second peak asterina-330 (Fig. 2A-D, P-2). There was a slight change in the retention time of the peaks in each samples. High diversity of MAAs found in the marine organism is due to the transformation of mycosporine-glycine, porphyra-334, shinorine and other MAA bisubstituted by amino acids (Carreto *et al.,* 2001, 2002, 2005; Shick, 2004). These compounds (mycosporine- glycine, porphyra-334 and shinorine are referred as primary MAAs, and the other MAAs, probably derived from these primary MAAs, will be referred to as secondary MAAs (Shick, 2004). *Alexandrium excavatum* culture when transferred from low to high intensity conditions, a sequential change in UV absorption spectrum with the synthesis of MAAs was found (Carreto *et al.*, 1990) that shows that all the MAAs are structurally interconvertable. Singh *et al* (2010) reported that *Anabaena variabilis* PCC 7937 under normal condition synthesises shinorine, which is a primary MAA, while in sulphur deficient culture a secondary MAA palythine-serine was appeared suggesting bioconversion of a primary mycosporine-like amino acid (MAA) into a secondary MAA is regulated by sulfur deficiency.

It has been found that with increase in the photosynthetically active radiation (PAR), biotrans formation of mycosporine like amino acids (MAAs) in the toxic dinoflagellate *Alexandrium tamarense* has been induced (Alan *et al*., 2006). In their report, Alan *et al.* (2006) has described that induction MAAs synthesis involves the two stage process; first stage involves the net synthesis of MAAs bi-substituted by the amino acids, then transformation of compounds in to the secondary MAAs, being the second stage of net MAAs synthesis.

There are many reports that these MAAs are playing important roles, helping cyanobacteria to survive in adverse conditions. Antioxidant activity of Mycosporine-glycine has been reported against peroxyl-radical initiated lipid peroxidation (Dunlap and Yamamoto 1995; Suh *et al*. 2003). Salt stress induces the synthesis of Shinorine in *Microcoleus chthonoplastes* (Karsten, 2002) and *Anabaena variabilis* PCC 7937 (Singh *et al.,* 2008). Shinorine was reported in the three filamentous and heterocystous nitrogen fixing cyanobacteria *Anabaena* sp., *Nostoc commune* and *Scytonema* sp. and UV-B induced the synthesis of shinorine in all three cyanobacteria (Sinha *et al*, 2001). M-Gly has shown highest antioxidant activity in terms of scavenging of hydro soluble radicals at all pH tested followed by combination of Asterina-330 + palythine while Porphyra-334 +Shinorine and Shinorine alone showed scarce activity of scavenging of hydrosoluble free radicals in purified aqueous extracts of the mycosporine-like amino acids (MAAs) isolated from the red alga *Porphyra rosengurttii* (porphyra-334 +shinorine), *Gelidium corneum* (asterina-330+ palythine), *Ahnfeltiopsis devoniensis* (shinorine), and mycosporine-glycine isolated from the marine lichen *Lichina pygmaea* (De la Coba *et al.,* 2009).

*Scytonema geitleri* and *Lyngbya arboricola* selected for our study is facing extreme of desiccation in the month of May-June and average day temperate of 28-32 $^0$C reaching up to 48 $^0$C in mid-day during this period followed by rainy season during the month of July-September and winter season in November-January. Dry mat of cyanobacterium is collected after the rainy season having the growing conditions after facing the almost desiccation condition shown the presence of four mycosporine like amino acids *i.e*. Mycosporin-glycine, astrina-330, Mycosporin-2-glycine and palythine in the extraction procedure given in this paper shows that these MAAs must have halped the cyanobactria to withstand to dessication condition and heat generated due to rise in temperature. The extraction procedure given in this paper help to seperate the MAAs present in the cyanobacteria specialy in mat forming cyanobecteria. Using gradient of mobile phase A containing 0.2 % TFA with ammonium hydroxide solution at pH 3.15 and mobile phase B containing aqueous trifluoroacetic acid 0.2 % and ammonium hydroxide (pH 2.20) : methanol: acetonitrile (80: 10: 10, v: v: v) provides best separation of peaks (Carreto *et al.,* 2005).

## CONCLUSION

*Scytonema geitleri* and *Lyngbya arboricola* growing under desiccation condition has shown the presence of MAAs which must have helping the cyanobecteria to tolerate the

desiccation phase and provide protection from the UV damage by their antioxidant activity. *Scytonema geitleri* after different level of ammonium sulphate fractionation shows the presence of Mycosporine-glycine and astrina-330, while in *Lyngbya arboricola* Palythine was found.
Ammonium sulphate fractionation precipitation applied for the extraction and separation of maximum numbers of MAAs present in the cyanobacteria. In present study MAAs are showing transformation in *Scytonema geitleri* extract. This may be due to the reason that MAAs is separated while transformation process was occurring in the system or fractionation precipitation with Ammonium sulphate, itself may be a reason of transformation. The transformation activity was completely absent in the case of *Lyngbya arboricola*. The process presented in this paper is also able to extract the all most all MAAs present in the Cyanobecteria. As the percent of saturation (ammonium sulphate) increases from 20 % to 60 %, concentration of Mycosporine-glycine decreases and concentration of Mycosporine-2-glycine increases at 60 % saturation, in *Lygbya arboricola* concentration of Palythene gradually decreases as the percent of ammonium sulphate saturation increases. However method presented in this paper provides gradual elimination of photosynthetic pigments and enhanced level of MAAs purification after each level of ammonium sulphate precipitation evaporated to dryness and dissolved in Solvent A containing 0.2 % aqueous trifluoro-acetic acid. Extraction procedure presented in this paper is an efficient method for extraction of purified MAAs from mat forming cyanobecteria.

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