Effects of ultraviolet-B radiation on a hot-spring cyanobacterium *Nostoc* sp. strain HKAR-2

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ABSTRACT

The effects of ultraviolet-B (UV-B; 280-315 nm) radiation on growth, survival, pigmentation and nitrate reductase enzyme have been studied in a N_2-fixing cyanobacterium *Nostoc* sp. strain HKAR-2, isolated from hot-springs of Rajgir, India. Compared to photosynthetically active radiation (PAR) and ultraviolet-A (UV-A), UV-B radiation was found to have more inhibitory effects on the growth and survival of the organism. Complete killing of *Nostoc* sp. strain HKAR-2 occurs after 240 min of UV-B radiation. Phycocyanin pigment was severely bleached and was found to be more susceptible to damage by UV-B irradiation in comparison to phycoerythrin in the test organism. *In vivo* NR activity following UV-B exposure was found to increase in *Nostoc* sp. strain HKAR-2. Screening of the organism for the presence of UV-absorbing compounds revealed the occurrence of mycosporine-like amino acids (MAAs). Absorption spectroscopy and HPLC analysis showed the presence of two MAAs, namely shinorine and P-334 in *Nostoc* sp. strain HKAR-2.

Keywords: cyanobacteria, hot-spring, mycosporine-like amino acids (MAAs), *Nostoc*, ultraviolet-B radiation

INTRODUCTION

Cyanobacteria, the primitive group of Gram-negative prokaryotes having higher plant-type oxygenic photosynthesis and cosmopolitan distribution ranging from hot springs to Arctic and Antarctic regions, were probably the first organism to release oxygen into the oxygen-free atmosphere. Thus, cyanobacteria were responsible for a major global evolutionary transformation leading to the development of aerobic metabolism and the subsequent rise of higher plant and animal forms [1]. They play an important role in successional processes, global photosynthetic biomass production, nutrient cycling [2] and as natural biofertilizers [3]. Cyanobacteria can be used as a model organism for studying the adverse effects of UV-B radiation because they were exposed to higher UV-B fluxes early in their evolution [4]. They are considered to be a rich source of novel metabolites of a great importance from a biotechnological and industrial point of view [5]. The utilization of cyanobacteria in food production and in solar energy conversion may hold immense potential for the future and could be exploited for human's economy. Any adverse effects on these ecologically and economically important organisms may adversely affect the agro-ecosystem.

Now a days, the continued depletion of stratospheric ozone layer, mainly due to anthropogenically released atmospheric pollutants such as chlorofluorocarbons (CFCs), which is responsible for the increase in solar ultraviolet radiation, mainly UV-B (280-315 nm) reaching the Earth's surface [6-10] has become a subject of much concern and debate. Chlorofluorocarbons (CFCs) seem to be the main culprit of ozone depletion and are stable in lower atmosphere, where it

Research Article, Acta Biologica Indica 2013, 2(1):265-276

exists for up to 100 years. Each CFC molecule can destroy about 100,000 ozone molecules [11]. The process of ozone depletion has been reported at mid latitudes and especially in the Antarctic where ozone levels have been reported to decline by more than 70% during late winter and early spring in the polar vortex [12].

Light is an essential factor that determines the growth of photosynthetic organisms. Harvesting of solar radiation for photosynthesis and nitrogen fixation exposes cyanobacteria simultaneously to harmful doses of UV-B (280-315 nm) and UV-A (315-400 nm) radiations in their natural habitats. Effective doses of UV-B and UV-A radiations are capable of penetrating the water column to an ecologically-significant depth [13], thereby adversely affecting the productivity of aquatic organisms [14]. The high-energetic UV-B has the greatest potential for cell damage caused by both direct effects on DNA and proteins and indirect effects via the production of reactive oxygen species (ROS) [15,16]. There are several targets for the potentially toxic ROS including lipids, DNA and proteins. Moreover, damage to the photosynthetic apparatus is also partially mediated by ROS resulting in inhibition of photosynthesis [17]. Morphology, cell differentiation, survival, growth, pigmentation, motility and orientation, N_2 metabolism, phycobiliprotein composition, protein profile, DNA and $^{14}CO_2$ uptake have been reported to be affected by UV-B [18-20].

Cyanobacteria which are simultaneously exposed to photosynthetic active radiation (PAR; 400-700 nm) and ultraviolet radiation have evolved the capacity to synthesize certain UV-absorbing compounds like mycosporine-like amino acids (MAAs) and scytonemin to counteract the damaging effects of UV-B radiation. MAAs are small (<400 Da), colorless, water-soluble compounds characterized by a cyclohexenone or cyclohexenimine chromophores conjugated with the nitrogen substituent of an amino acid or its imino alcohol [21-25]. Mycosporines were first identified in fungi as having a role in UV-induced sporulation [26-29]. MAAs has also been characterized in Antarctic and halophilic cyanobacteria [30,31], heterotrophic bacteria [32], red algae [33] and lichens [34]. The strong UV absorption maxima between 310-362 nm, high molar extinction coefficient (ε=28,100-50,000) and photostability in distilled and sea water in presence of photosensitizers [35], support the contention that MAAs have a photoprotective role [36-39]. These compounds are capable of effectively dissipating absorbed radiation without producing reactive oxygen species [40]. Differences between the absorption spectra of MAAs are due to variations in the attached side groups and nitrogen substituents. Based on their retention times (HPLC analysis) and characteristic absorption maxima more than 20 different MAAs have beenfound in various organisms [21,41]. The biosynthesis of MAAs has been suggested to occur via the first part of the shikimate pathway, details of which has been recently published by Singh et al. [42]. 3-dehydroquinate acts as a precursor for the synthesis of fungal mycosporines and MAAs *via* gadusols [43-46]. This view is supported by the inhibition of MAAs synthesis in *Stylophora pistillata* by the application of glyphosate which is a specific shikimate pathway inhibitor. The primary MAA mycosporine-glycine thus synthesized by shikimate pathway is then transformed by chemical and/or biochemical conversions into other secondary MAAs [47,48]. The synthesis of MAAs occurs in bacteria, cyanobacteria, phytoplankton and macroalgae but not in animals, because they lack the shikimate pathway. MAAs have also been shown to be highly resistant against abiotic stressors such as temperature, UV radiation, various solvents and pH [49].

MATERIALS AND METHODS

Sampling and cultivation

The test organism *Nostoc* sp. strain HKAR-2 [50] was isolated from a hot spring of Rajgir, India, by using standard microbiological techniques as described earlier [51]. *Nostoc* sp. strain HKAR-2 (Figure 1) is a member of *Nostocales* characterized by frothy thallus, gelatinous, circinate trichomes, sheath absent, cells ellipsoidal and heterocystous. Culture was grown under axenic

conditions in autoclaved liquid medium [52] without a nitrogen source in an air-conditioned culture room at a temperature 20 ± 2 °C and illuminated by cool white fluorescent light at an intensity of 12 ± 2 W/m^2. Culture was hand shaken five times daily. To avoid contamination, culture was sub-cultured at regular intervals and all the experiments were conducted from an exponentially growing culture having an initial dry wt. of approximately 0.15 mg ml^{-1}.

Growth Measurements

Growth was measured by estimating protein content at desired time intervals. All the growth experiments were performed under identical conditions. The stock culture of dispersed, exponentially growing cells was diluted to an absorbance at 750 nm of 0.035 ± 0.005; 20 ml aliquots were then transferred to each 25 ml Petri dish and exposed to PAR, UV-A and UV-B radiations for different durations.

Irradiation Source and Treatment Procedure

Exponentially growing suspension cultures were transferred into sterile 75 mm Petri dishes covered with 395 nm (for PAR treatment), 320 nm (for PAR+UV-A treatment) and/or 295 nm (for PAR+UV-A+UV-B treatment) cut-off filter foils (Ultraphan, UV Opak Digefra, Munich, Germany) to obtain the desired radiation regimes. The cultures in the Petri dishes were transferred onto a water bath placed over a slowly rotating shaker to avoid heating from radiation and self-shading, respectively. The whole set-up was placed inside a UV-chamber and the suspension were exposed to artificial PAR, UV-A and UV-B produced from a PAR, UV-A and UV-B lamp, with the intensity of which extended from 280 - 400 nm of about 20 W/m^2, 5.0 W/m^2 and 2.0 ± 2 W/m^2 respectively.

Determination of Percent Survival and Growth Following UV-B Irradiation

For determining percent survival of the organism, 0.5 ml aliquots were withdrawn at regular time intervals during UV-B irradiation and spread on agar plates supplemented with growth medium. After a 48 h dark incubation, plates were transferred to light in the culture room. Percent survival was scored by colony counts and was plotted semilogarithmically. In parallel, samples were withdrawn at desired time intervals for measuring growth, and nitrate reductase activity.

Protein Estimation

Protein concentration was determined by the method of Bradford [53]. Bovine serum albumin was used as the standard.

Nitrate Reductase (NR) Activity

The estimation of *in vivo* nitrate reductase activity (NR) activity was done by the method of Camm and Stein [54]. The activity is based on the total nitrite formed which was determined by the diazocoupling method of Lowe and Evans [55]. The cultures were supplemented with 5 mM KNO_3, pH - 7.5 overnight prior to irradiation. 1 ml of aliquots were withdrawn at desired intervals and mixed with sulphanilamide. After an interval of 15 min NR dye (α-(N-1)-naphthyl ethylenediamine dihydrochloride) was added and absorbance of pink color was estimated at 540 nm in a double beam spectrophotometer (U-2910, UV/VIS, Hitachi, Tokyo, Japan). A standard curve was prepared

with varying concentration of $NaNO_2$ in growth medium. The nitrite present in the samples was expressed in µg protein^{-1} using the standard curve of nitrite.

Native PAGE

For nitrate reductase activity, native polyacrylamide gradient gel electrophoresis was carried out in a vertical system with gels of 8 x 7 cm, 10 mm thick, using the method described by Laemmli [56], with a 5 % in the resolving gel. Gels were stained with Coomassie brilliant blue R250.

Phycobiliprotein Isolation

Exponentially growing cells were harvested by centrifugation at 1500 x g for 10 min and washed twice with 0.75 M phosphate buffer (pH - 7.0), resuspended in the same buffer and 1 mM PMSF (phenylmethylsulfonylfluoride) solution (in isopropanol) and 2 mM EDTA (ethylenediaminetetracetic acid) were added. Thereafter, cells were disrupted twice by sonication. To remove cell debris, the suspension was centrifuged at 40, 000 x g at 15 °C for 10 min in high speed cooling centrifuge (REMI C-24, India). The supernatant contained most of the free phycobiliproteins.

Extraction of MAAs

Cyanobacterial cells were harvested by centrifugation and MAAs were extracted in 2.0 ml of 100 % (v/v) methanol (HPLC-grade) by overnight incubation at 4 °C. After extraction, the aliquots were centrifuged (10 000g for 5 min) and supernatants (methanolic extracts) were evaporated to dryness at 45 °C and redissolved in 1 mL of double-distilled water and absorption spectrum was recorded. Thereafter, the samples were filtered through 0.2 µm pore-sized microcentrifuge filters and subsequently subjected to HPLC analysis.

Absorption spectroscopy

Absorption spectra of all samples were measured in a double beam spectrophotometer (U-2910, UV/VIS, Hitachi, Tokyo, Japan). The raw data were transferred to a microcomputer and peaks were analysed with software provided by the manufacturer.

High-performance liquid chromatography

Analysis and purification of MAAs was done using a HPLC system (Waters 2998, Photodiode Array, pump L-7100, USA) equipped with a Licrospher RP 18 column and guard (5 µm packing; 250 mm x 4 mm inside diameter). The samples (50 µl) were injected into the HPLC column through a Waters 717 plus autosampler. The wavelength for detection was 330 nm, the mobile phase was 0.02 % acetic acid (v/v) in double-distilled water, at a flow rate of 1.0 ml min^{-1}. The MAAs were identified by comparing the absorption spectra and retention times.

RESULTS AND DISCUSSION

Growth pattern of *Nostoc* sp. strain HKAR-2 (accession no. FJ939126) was recorded following 0-12 days of irradiation using three different cut-off filters described above in liquid medium by estimating the protein content. *Nostoc* sp**.** strain HKAR-2 showed complete inhibition of growth

after UV-B irradiation and UV-A radiation also had some sort of inhibitory effect on the growth of the organism while PAR didn't showed any such effect (Figure 2).

Figure 3 shows the survival (based on colony counts) of the test organism following light stress conditions for different durations. The survival was 70 %, 50 %, 25 % and complete killing after 60 min, 120 min, 180 min and 240 min of UV-B irradiations respectively but UV-A radiation also had some sort of inhibitory effect on the survival of organism. PAR had more or less no effect on the survival of *Nostoc* sp. strain HKAR-2.

Figure 4 shows the effects of UV-B on nitrate reductase (~ 82 kDa) activity on the test organism. NR activity increases with increase in durations of UV-B radiation. The culture of *Nostoc* sp. HKAR-2 irradiated under 295 cut-off filter showed increase in NR activity compared to the culture irradiated under 320 and 395 cut-off filters. Even after 150 min of UV-B irradiation, there was an increase in NR activity in the selected test organism. Therefore, UV-B radiation has more pronounced effect on stimulation of nitrate reductase activity. This was confirmed by nitrate reductase bioassay experiment of *Nostoc* sp. strain HKAR-2, intense pink bands after 3 h of UV-A and UV-B irradiations showed the stimulation of NR activity while PAR had no such effects (Figure 5).

Phycobiliprotein is the major light harvesting complex of cyanobacteria; it was severely affected by UV-B radiation. UV-B radiation shows drastic effect on the bleaching of pigments especially phycocyanin. Absorption spectrum of phycoerythrin showed peak at 560 nm and that of phycocyanin at 620 nm, phycocyanin was more affected to UV-B radiation as compared to phycoerythin. The absorption spectrum of *Nostoc* sp. strain HKAR-2 showed drastic decline in absorbance at 620 nm after 30 min of UV-B irradiation, but the decline in absorbance at 560 nm was less as compare to 620 nm (Figure 6). HPLC Chromatograms of partially purified aqueous solution from *Nostoc* sp. strain HKAR-2 revealed the presence of shinorine (RT 2.2 min, λ_{max} 334 nm), porphyra-334 (RT 2.7 min, λ_{max} 334 nm) (Figure 7).

The present investigation suggests that selected cyanobacteriaum is sensitive to various irradiation conditions but to a variable extent. UV-B radiation has been shown to severely affect the growth and survival of several cyanobacteria [31,57-61]. It has been suggested that growth and survival strongly depend on the efficiency of photoreactivation or excision repair of DNA [62]. Various species differ with respect to their tolerance to UV-B radiation and even closely related strains show differential sensitivity. UV-B radiation has been found to be responsible for destruction of cellular constituents having absorption maxima in the range of 280-320 nm, which further affects cellular membrane permeability and protein damage, eventually resulting in the death of the cell [63,64]. Complete killing and loss of survival within 120-180 min of UV-B exposure has been reported for several cyanobacteria due to inactivation of a number of processes [57, 58, 65]. Strains such as *Nostoc commune* and *Scytonema* sp. whose filaments are embedded in a mucilage sheath are more tolerant to UV-B radiation than the species which do not have such coverings [66]. A phycoerythrin rich (brown) strain of *Nostoc* sp. was found to be more tolerant to UV-B radiation than the phycocyanin rich (blue-green) form of this strain [57]. Survival and growth has been shown to be decreased with selected doses of UV-B (30 and 90 min) in non-heterocystous cyanobacterium, *Plectonema boryanum* [60]. Han *et al.* [67] reported inhibition of growth in a rice-field cyanobacterium *Anabaena* sp. during exposure to PAR + UVR while it was inhibited by up to 40 % by solar UVR in *Anabaena* sp. PCC 7120 [68]. Zeeshan and Prasad [69] studied the differential response of growth of *Nostoc muscorum, Plectonema boryanum* and *Aphanothece* sp. to UV-B stress, the sensitivity to UV-B was maximum in *N. muscorum* and minimum in *Aphanothece* sp.

Although, the mechanism is not yet clear but remarkable increase of NR activity in UV-B + PAR exposed filaments might be due to monomerization and photoreactivation of nitrate reductase enzyme which coincides with the work of Tado *et al.* [70]. Stimulation of NR activity following UV-B radiation has also been recorded in an angiospermic plant *Crotalaria juncea* [71], contrary to

this inhibition of the same enzyme has been reported in marine diatoms [72]. The discrepancy in the activity might be due to caused by the changes in cellular membrane permeability following UV-B exposure. Stimulation in NR activity was also reported due to blue light exposure in *Oscillatoria princes* [73].

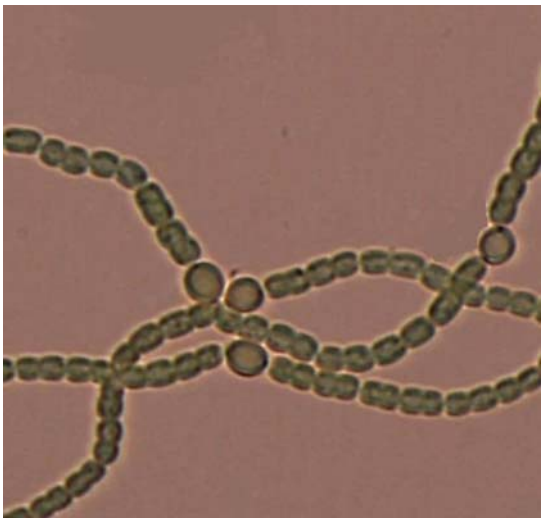


Figure 1. Photograph showing the filaments of *Nostoc* sp. strain HKAR-2.

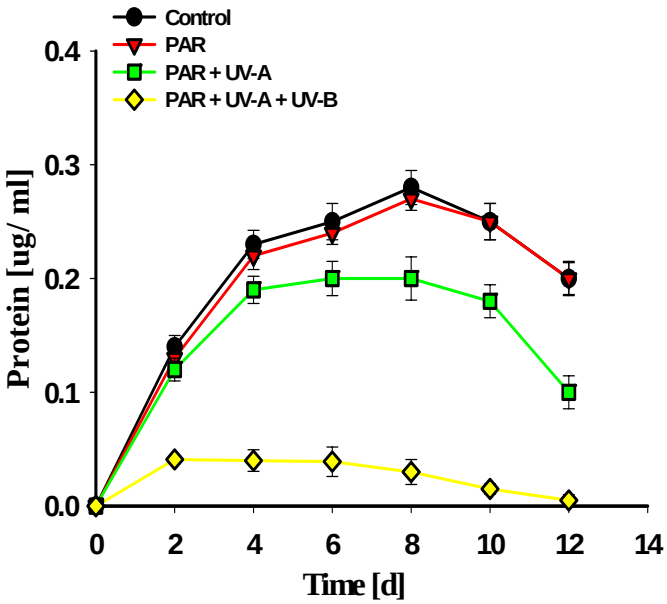
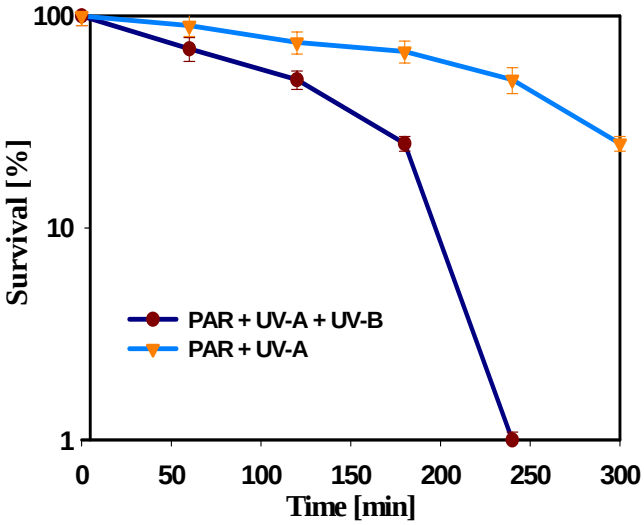


Figure 2. Growth of *Nostoc* sp. strain HKAR-2 in liquid medium. Exponentially growing cultures were exposed to the indicated stress (under PAR, PAR + UV-A, PAR + UV-A + UV-B radiations) and thereafter incubated in fluorescent light (12 ± 2 W/m^2). Growth was measured by estimating protein content at regular intervals for 12 days.

A



B

Figure 3. Percent survival of *Nostoc* sp. strain HKAR-2 under UV-A and UV-B radiations. Equal numbers of cells of all strains were plated on agar plates. After 24 h of dark incubation plates were transferred to light in the culture room. Results are based on colony count and were plotted semilogarithmically.

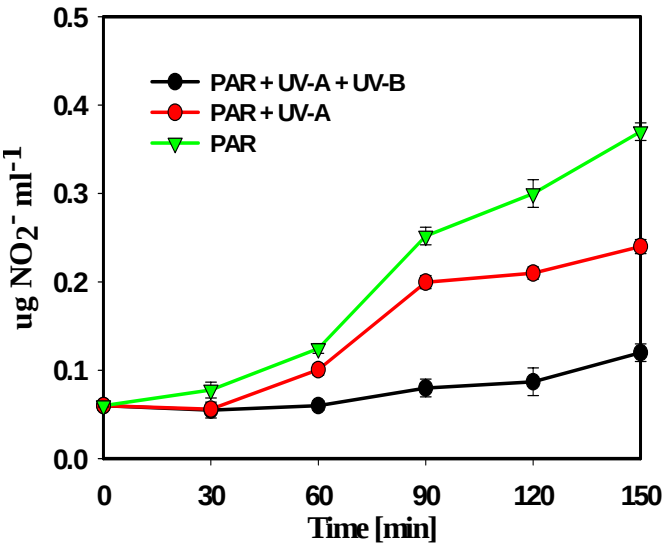


Figure 4. Nitrate reductase activity of *Nostoc* sp. strain HKAR-2 after light stress conditions (under PAR, PAR + UV-A, PAR + UV-A + UV-B radiations). Cultures grown in NO_3^- (2.5 mM) supplemented medium were exposed to irradiation for the desired time intervals.

Degradation of phycobiliproteins by UV-B radiation may be due to its proteinaceous nature suggesting that proteins may be preferred target of UV-B [74]. The protection of UV-B damage might be due to lowering in radiation amplification factors (RAF) value by visible light [75]. The results on phycobiliprotein indicate that even short exposure to UV-B bleaches the phycocyanin

more as compared to phycoerythrin. It has been reported that strong UV irradiation photo-oxidizes and bleaches all types of photosynthetic pigments [76]. UV-B-induced damaging response on photosynthetic pigments in *Nostoc muscorum, Plectonema boryanum* and *Aphanothece* sp. varied significantly; phycocyanin was severely affected by UV-B in *N. muscorum* followed by *P. boryanum* and *Aphanothece* sp. Suppression in emission peak (λ_{max} 651 nm) of phycocyanin revealed that UV-B altered the energy transfer efficiency of phycocyanin to PS II reaction center [69].

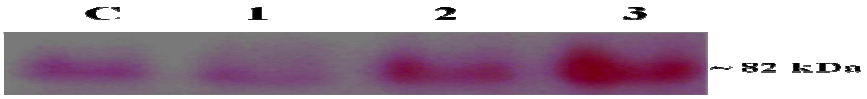


Figure 5. Native PAGE of *Nostoc* sp. strain HKAR-2 showing nitrate reductase activity under various cut-off filters. C- Control; 1- PAR; 2- PAR + UV-A; 3- PAR + UV-A + UV-B.

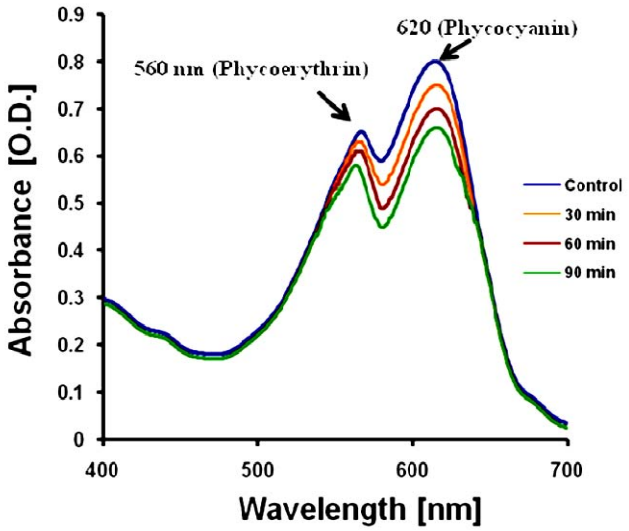


Figure 6. Absorption spectra showing the effects of UV-B radiation on phycobiliproteins of *Nostoc* sp. strain HKAR-2.

After all these deleterious effects, cyanobacteria still survive in the changing environment due to the biosynthesis of certain photoprotectant such as mycosporine-like amino acids (MAAs), having strong absorption in the UV-A/ UV-B region of the spectrum, thereby playing an important role in allowing these organisms to grow and survive in habitats exposed to strong irradiation [77]. In a terrestrial *Nostoc commune* strain, subjected to regular cycles of desiccation and rewetting, UV-absorbing compounds play a key role as UV-protectants [78,79]. *Nostoc* sp. HKAR-2 revealed the biosynthesis of shinorine and porphyra-334. The photodegradation and photophysical characteristics of MAAs have shown that these compounds are capable of effectively dissipating absorbed radiation without producing reactive oxygen species [40]. As these compounds provide photoprotection in organisms having shikimate pathway, certain marine animals such as arthropods, rotifers, molluscs, fishes, cnidarians, tunicates, eubacteriobionts, poriferans, nemertineans,

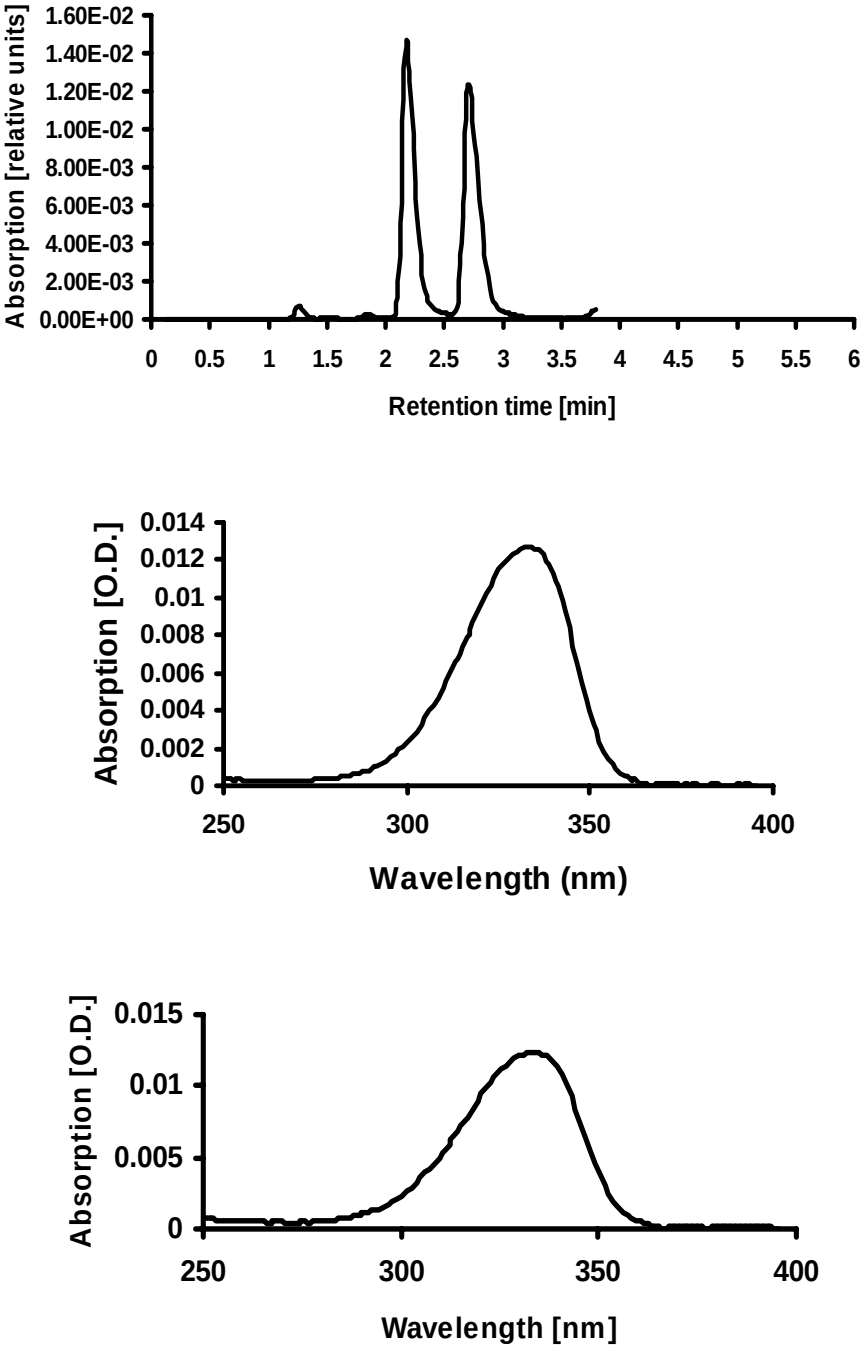


Figure 7. HPLC chromatogram and absorption spectra obtained from the HPLC analysis of partially purified aqueous solution from *Nostoc* sp. HKAR-2. A. HPLC chromatogram of MAAs; B. Absorption spectrum of shinorine (RT 2.2 min, λ_{max} 334 nm); C. Absorption spectrum of porphyra-334 (RT 2.7 min, λ_{max} 334 nm).

echinodermates, platyhelminthes, polychaetes, bryozoans and protozoans have also been reported to protect themselves from UV radiation by MAAs. However, studies have shown that in these shikimate pathway lacking animals these compounds are derived from their algal diet [80,81]. Thus MAAs provide protection from UV radiation not only in their producers but also to primary and secondary consumers [82] through food chain. MAAs are osmotically active compounds and their accumulation in the cell is regulated by osmotic mechanism [83]. This is reflected by the fact that field populations of halotolerant cyanobacteria accumulate high concentration of MAAs [30]. It seems that MAAs not only plays a significant role as photoprotective compounds but also act as an osmoregulator in certain cyanobacteria. The gene for the shikimate pathway in the metazoan *Nematostella vectensis* has been disclosed by Starcevic [84]. These genes were transferred from cyanobacteria to dinoflagellates and finally to metazoans by lateral gene transfer events. Recent report suggests that the MAA shinorine in *Anabaena variabilis* PCC 7937 is synthesized under various abiotic stressors with or without UVR, indicating their additional role. The synthesis of MAAs in cyanobacteria is also dependent on available nitrogen [85], and growth media with a nitrogen source support the highest MAA synthesis in comparison to others without a nitrogen source [86].

We conclude that continued depletion of ozone layer resulting in enhanced level of UV-B radiation might have an adverse effect on ecologically and economically important cyanobacterial populations which in turn may affect the primary productivity of the ecosystems. This would also result in imbalance in the stability of the ecosystems. However, the ability of these prokaryotic organisms to synthesize UV-absorbing compounds helps them to cope up with the deleterious effects of ultraviolet radiation.

Acknowledgements: This work was partially supported by a project grant (No. SR/WOS-A/LS-140/2011) sanctioned to Richa from the Department of Science and Technology, New Delhi, India. Part of the work was supported by UGC and Jawaharlal Nehru Memorial Fund Scholarships, New Delhi, India, sanctioned to Sunita Kumari.

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