

Effect of UV-B radiation and desiccation stress on photoprotective compounds accumulation in marine *Leptolyngbya* sp

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Abstract

Increased awareness regarding the harmful effects of ultraviolet (UV)-B radiation has led to the search for new sources of natural UV-B protecting compounds. Mycosporine-like amino acids are one of such promising compounds found in several organisms. Cyanobacteria are ideal organisms for isolation of these compounds due to their compatibility and adaptability to thrive under harsh environmental conditions. In the following investigation we report the production of shinorine in *Leptolyngbya* sp. isolated from the intertidal region. Based on the spectral characteristics and liquid chromatography-mass spectrometry analysis, the UV-absorbing compound was identified as shinorine. To the best of our knowledge this is the first report on the occurrence of shinorine in *Leptolyngbya* sp. We also investigated the effect of artificial UV-B radiation and periodic desiccation on chlorophyll-a, total carotenoids and MAAs production. The UV-B radiation had a negative effect on growth and chlorophyll concentration whereas it showed an inductive effect on the production of total carotenoids and MAAs. Desiccation along with UV-B radiation led to an increase in the concentration of photoprotective compounds. These results indicate that carotenoids and MAAs thus facilitate cyanobacteria to avoid and protect themselves from the deleterious effects of UV-B and desiccation.

Keywords: Cyanobacteria, Desiccation, Mycosporine-like amino acids, Photoprotection, UV-radiation.

Running title: Marine cyanobacterial photoprotectant

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Introduction

The ozone layer is in its primary recovery stage after the implementation of the Montreal Protocol, but the harmful ultraviolet (UV) radiations still continue to reach our earth surface (1,2). The UV-B radiation penetrates several meters into the water column depending on the amount of dissolved and particulate organic matter (3). They have the potential to cause severe damage to key physiological processes such as photosynthesis in aquatic phototrophs thus causing photoinhibition and photodamage. This physiological stress is mainly due to modified protein configuration, impaired DNA and damage to other biologically relevant molecules caused by UV radiation (4). In recent past, cyanobacteria have gained much importance due to the biosynthesis of valuable bioactive secondary metabolites which includes potent UV-absorbing compounds (5,6).

Cyanobacteria are Gram-negative bacteria possessing the ability to perform oxygenic photosynthesis and some of them can also fix atmospheric nitrogen. They are one of the most ancient organisms living on earth with oldest fossil records dating back to 3.5 billion years ago which is much before the formation of the ozone layer (7). Due to the photoautotrophic nature they have to position themselves in the photic zone thus exposing them to UV radiation. To protect themselves from the harmful effects of UV, cyanobacteria have developed several mechanisms which include stress avoidance by gliding movements, defense by the production of UV-absorbing compounds like scytonemin and mycosporine-like amino acids (MAAs) and repair mechanisms including DNA and protein repair (8).

MAAs are intracellular secondary metabolites having a molecular weight of about <400 Da. They are colorless and water soluble in nature. They are chemically composed of cyclohexenone or cyclohexenimine chromophores conjugated with amino acids or their imino alcohol (9). Due to their strong UV absorption and high molar extinction coefficient ($\epsilon = 28100-50000 \text{ M}^{-1}\text{cm}^{-1}$) MAAs are regarded as one of the potential UV-absorbing compounds. The first report of MAAs was in 1969 when Shibata observed a compound (S320) from corals and cyanobacteria of Great Barrier Reef with an absorption maximum at 320 nm (10). Since then, MAAs have been reported in diverse groups of organisms including prokaryotes such as heterotrophic bacteria and cyanobacteria, eukaryotic organisms like microalgae, macroalgae and higher animals such as mollusks, fish and tunicates. Till date more than 20 MAAs have been isolated and identified from different organisms. MAAs most commonly studied function is UV absorption. Studies have indicated that MAAs may also function as osmolytes, antioxidants, accessory pigments and intracellular nitrogen storage molecules (11).

The occurrence of MAAs is widespread in morphologically different cyanobacteria varying from colonial forms like *Aphanothece* sp. (12) to filamentous heterocystous *Nostoc* sp. (13) isolated from various environmental niches with each strain having a unique set of MAAs. Cyanobacteria living in continuously fluctuating environments such as intertidal zones need to adapt not only to high irradiance but also adjust to frequent cycles of hydration/desiccation. Desiccation leads to cell damage by affecting both cytoplasmic components including DNA and proteins as well as the cell membrane fluidity. Synthesis of exopolysaccharides, accumulation of compatible solutes and production of anti-oxidant compounds are some of the techniques employed by the desiccation tolerant cyanobacteria to overcome the consequences of drying (14-16)

Several studies have been conducted to demonstrate the inductive effect of UV-A and UV-B on the production of MAAs in cyanobacterial strains. Most of the investigations show a positive inductive effect of UV radiation on MAAs; there are also reports wherein MAAs producing cyanobacteria have no survival advantage over non-producer when exposed to UV (17-19). There are only a few documented studies conducted to assess the effects of desiccation on the levels of MAAs and carotenoids in cyanobacteria. In the following study, we have investigated the presence of MAAs from an intertidal cyanobacterial species of *Leptolyngbya* and examined the effect of UV-B and desiccation on the photoprotective pigments.

Material and methods

Isolation and growth conditions of the isolate

The experimental organism was isolated from water and sediment samples collected at Vagator beach (15°35'59.6"N 73°44'00.1"E), Goa, India. The samples were diluted in a 24-well plate with sterile artificial seawater nutrients III (ASN III) media to obtain a unialgal culture. Cycloheximide (30 µg/ml) was added to prevent the growth of eukaryotic organisms. The 24-well plate was regularly observed under the microscope (Nikon eclipse TS100F) to check for cyanobacterial growth. The unialgal strain obtained after several dilutions was then transferred to ASN III liquid media as well as maintained on slants. The strain was grown at 25±2 °C under the fluorescent white light with an intensity of 10±2 Wm⁻². The photo regime of 12 h light/dark cycle was maintained throughout the culture growth.

Identification of the cyanobacterium

The isolate was identified by studying morphological characteristics using a light microscope and scanning electron microscope (SEM, Joel JSM-6360A, Japan) (20, 21). The identity was confirmed by 16s rRNA partial gene sequencing. In brief, the genomic DNA was isolated from cell pellet using

DNeasy kit (Qiagen) and 16S rRNA gene was PCR (Applied biosystems, Veriti) amplified using cyanobacteria-specific primers CYA106F and CYA781R (22). The PCR product was run on a gel to confirm the amplification and then purified by QIAquick gel extraction kit (Qiagen). The purified product was then sequenced by the 3130xl genetic analyzer (Applied Biosystems, USA) at National Institute of Oceanography. The obtained sequence was subjected to the BLAST analysis for identification of strain.

Extraction and spectrophotometric analysis of MAAs

The exponentially growing cyanobacterial culture was harvested by centrifugation at 8000 rpm for 10 min. The pellet was further suspended in 100% HPLC grade methanol and refrigerated overnight at 4 °C. The suspension was centrifuged and the supernatant was scanned using UV-visible spectrophotometer (Cary 300 series) from 200 to 800 nm (23).

Partial purification of MAAs

The methanolic extract of the cyanobacterial pellet was evaporated to dryness by rotary vacuum concentrator (8763.RVO, Roteva). The dried extract was re-dissolved in sterile double distilled water followed by the addition of few drops of chloroform to remove the lipophilic photosynthetic pigments and other cellular debris. The mixture was vortexed gently and centrifuged. The upper clear layer of water containing MAAs was carefully removed and transferred to a fresh vial. The water extract was filtered through 0.2 µm syringe filter and evaporated to dryness. It was stored at -20 °C before further analysis.

Characterization of MAAs by LCMS

The lyophilized samples were re-suspended in 100% HPLC grade methanol and centrifuged at 8000 rpm for 2 min and the supernatant was used for MAA analysis. The partially purified MAAs were analyzed using a Waters liquid chromatography-mass spectrometry (LC-MS) system equipped with photodiode array (PDA) detector (PDA-2996), separated in phenomenex-RP C₁₈ column (5 µm, 250X6.4 mm) using Waters separation module-2695 (24). The MAAs samples (10 µL) were injected into the LC-MS column through an autoinjector. The column oven temperature was set at 40 °C. The mobile phase was methanol:water (75:25 v/v) with 0.1% acetic acid (LC-MS grade) and run isocratically at a flow rate of 1.0 ml min⁻¹. The solvent was degassed using the degasser equipped in the LC-MS system. The detection wavelength of MAA was at 330 nm, and PDA scan wavelength was from 200 to 400 nm. Absorption spectra of UV-absorbing compounds were recorded directly on the LC-MS separated peaks.

The MS analysis was performed in positive electrospray ionization (ESI) mode with a scan range from m/z 300 to 1000. Mass spectrum of the compounds was recorded under electron impact ionization (Q-TOF Waters Ultima instrument fitted with electron spray ionization; Waters Corp., Micromass UK, Manchester, UK). Identification of MAAs was made from their UV/Vis spectra and mass spectra as described earlier (25).

Effect of UV-B on photoprotective pigments

An experiment was carried out to study the effect of UV-B on the production of MAAs and carotenoids. The exponentially growing culture of the experimental organism was exposed to photosynthetically active radiation (PAR) and PAR+UV-B at $25 \pm 2^\circ\text{C}$ in a BOD incubator (Labline, LSC-45). PAR was provided by the cool white fluorescent lamp and the intensity was measured using digital Lux meter (TES-1332). Philips 20W/01-RS was used as a source of UV-B and the irradiance was determined by digital radiometer (model VLX-312). The irradiances received by the cultures for PAR and UV-B were $10 \pm 2 \text{ Wm}^{-2}$ and $1.0 \pm 0.2 \text{ Wm}^{-2}$ respectively. The cultures were regularly swirled to avoid self-shading of cells and ensure uniform exposure to radiation. Samples were collected at 0, 24, 48 and 72 h and analyzed for chlorophyll-a, carotenoids, dry weight and MAAs.

Desiccation experiment

Modified Fleming and Castenholz (2007) (26) method was used for the desiccation experiment. A uniform suspension of *Leptolyngbya* sp. obtained by gently homogenizing the culture was used for the desiccation experiment. A known volume of culture was filtered onto 47 mm diameter, 0.2 μm pore size glass fiber filters (GF/F, Whatman) to form a uniform film of cells on the filter. The filters were placed on ASN III agar petri plates and incubated under visible light at 25°C for 2 days. The plates were periodically rotated throughout the experiment to minimize the variable light effects. After 48 h of PAR incubation, half numbers of petri plates were exposed to UV-B light in addition to PAR. Subsequently half of the plates from each set that is, only PAR and PAR+UV-B were air dried in empty petri plates under respective light conditions. The filters were desiccated for 24 h and placed back on agar plates to rehydrate for a period of 24 h. The regime of 24 h of desiccation and 24 h of rehydration was repeated five times. At the end of every desiccation cycle samples were collected for the analysis of chlorophyll-a, carotenoids, dry weight and MAAs.

Measurements of growth and pigments

The growth of cyanobacteria was measured in terms of dry weight. To determine the dry weight, a known volume of the culture was centrifuged at 8000 rpm for 10 min at 4°C in a pre-weighed

centrifuge tube. The pellet was then oven dried at 60 °C and cooled in a desiccator until constant weight was achieved (27). A known volume of culture was centrifuged at 8000 rpm for 10 min at 4 °C. The chlorophyll was extracted from the pellet with 95% methanol. The suspension was centrifuged and the absorbance of the supernatant was measured at 650 and 665 nm using a UV-visible spectrophotometer. The concentration of the chlorophyll was calculated following MacKinney (1941) (28). To determine the content of total carotenoids, a known volume of culture was centrifuged at 8000 rpm for 10 min at 4 °C. The cell pellet was suspended in 85% acetone and kept at 4 °C overnight. The suspension was centrifuged and the absorbance of the supernatant was read at 450 nm using UV-visible spectrophotometer (29).

Statistical Analysis

The experimental data given are mean values of three replicates. The data was analyzed by two sample t-test to evaluate significance using statistical analysis software SPSS 16. The alpha value was set at 0.05 for the same.

Results:

Morphological and molecular identification of the isolate

The cyanobacterial strain isolated from the coast of Goa was identified as *Leptolyngbyasp.* based on morphological characteristics and BLASTn result of the 16S rRNA partial gene sequence. The isolate is a filamentous non-heterocystous form which grows as thick, dark green floating mats in liquid culture medium. The filaments are thin, long cylindrical trichomes having average cell length and width of 1.70 and 0.89 ± 0.05 μm respectively with small constrictions observed between the two cells (Figure 1). The BLASTn result showed the highest similarity of 98% with *Leptolyngbyasp.* HBC1 (30). The sequence was submitted to NCBI with accession number KT892744. A phylogenetic relationship tree with the nearest neighbors was constructed by the neighbor-joining method using MEGA 6 as shown in Figure 2.

Characterization of MAAs

The crude methanol extract of *Leptolyngbyasp.* showed absorption peaks in the UV range 340–260 nm with absorption maxima at 333 nm (Figure 3). Similar prominent absorption peaks for the crude methanolic extract of *Lyngbyasp.* between 290 and 350 nm were observed by Rastogi and Incharoensakdi (24). Comparable peaks were also detected for *Fisherellamuscicola*, a filamentous cyanobacterium isolated from soil (6). The absorbance in UV range indicated the presence of MAAs or MAAs like compounds in the crude extract. Hence the extract was purified and subjected to LC-MS analysis for identification of the compounds. The LC-MS analysis of partially purified MAAs

from *Leptolyngbya* sample recorded three prominent peaks at 2.25 min, 3.07 min and 15.23 min (Figure 4a). To identify the MAAs in the sample extract, MS was carried out by extracting the MH^+ ion from the total ion chromatogram (TIC) scans (Figure 4b).

Electrospray-ionization liquid chromatography-mass spectrometry (ESI-LCMS) analysis of partially purified MAAs showed a prominent ion peak of protonated molecule ($[M + H]^+$) at m/z 333.92 (Figure 4c). The calculated molecular weight of the identified MAAs is 332.13. Based on the UV/Vis absorption spectra and ESI-LCMS analysis, the compound was identified as shinorine (Figure 4d). These results are consistent with the previously reported results of ESI-LCMS analysis for MAAs elucidated in phytoplankton (25). The other peaks detected in the analysis were purified for further detailed studies to elucidate the possible newer MAAs.

Effect of UV-B radiation

During 72 h of exposure, the cultures growing under PAR had normal growth with a steady increase in dry weight. However, the dry weight of samples under UV light remained nearly constant (Figure 5a). The chlorophyll-a concentration of PAR exposed samples did not show any significant variation during the experimental time period. The initial chlorophyll-a concentration of 8 mg/g dry weight reduced to a minimum of 3.24 mg/g dry weight within 24 h of UV exposure; this value slowly recovered to 4.73 mg/g dry weight by 72 h. Although the chlorophyll-a level of UV-exposed samples showed an increase after 24 h, their content was always lower than the samples receiving PAR (Figure 5b).

Total carotenoids showed an increase in its concentration during the initial 48 h of UV exposure and then remained nearly constant thereafter (Figure 5c). A significant difference in the carotenoid content was observed after 48 h between UV-B exposed and PAR samples with highest carotenoids concentration of 5.6 mg/g dry weight for UV-B samples. Other than carotenoids, UV-B also had an inductive effect on the MAAs. The concentration of MAAs increased from their initial value of 1.09 to 2.26 mg/g dry weight when exposed to UV-B after 24 h and the increase continued till 72 h of exposure reaching a maximum value of 3.70 mg/g dry weight. The increase in MAAs concentration under PAR treatment was almost negligible when compared to the UV-B exposed samples (Figure 5d).

Effect of desiccation

The cultures exposed to desiccation and UV-B independently, demonstrated reduced concentration of chlorophyll-*a* initially which was later stabilized following slight increment; whereas UV-B and desiccation combined together showed a reducing trend in chlorophyll content with value reaching a

minimum of 5.6 mg/g of dry weight by day 8(Figure 5e). On the other hand the process of desiccation brought about increase in the levels of MAAs and total carotenoids in cyanobacteria as compared to hydrated cells, with an increase in the concentration from 1.2 to 2.0 and 4.4 to 5.4 mg/g dry weight respectively.

Dual effect of UV-B and desiccation led to a rise in carotenoids concentration within 48 hours and reached the highest value of 6.3 mg/g dry weight by day 4.Total carotenoid content in desiccated cells started to decrease after the 6th day of the experiment(Figure 5f). The cyanobacterial film exposed to desiccation stress, showed an increase in MAAs level during initial 48 h when compared to the hydrated cells; the concentration of MAAs did not show significant variation thereafter(Figure 5g). MAAs production was found to be highest, 3.3 mg/g of dry weight, by the cells exposed to UV-B stress. Desiccation + UV-B treatment enhanced the production of MAAs, with the highest value of 3.0 mg/g dry weight on day 4 which subsequently displayed a reducing trend.

Discussion:

In recent years cyanobacteria have gained importance as natural sources of several novel bioactive compounds. *Leptolyngbya* is one of the most common cyanobacterial genus found in different environmental niches including habitats like soil surfaces, subtidal stromatolites (30), coastal deglaciated regions of Antarctic (31) and archaeological hypogea (32). The isolated *Leptolyngbya* sp. belongs to rocky intertidal shore which is the interface of marine and terrestrial terrain. To survive under the drastically fluctuating intertidal environment with changing sunlight, wind pattern, tidal effect and several other factors, organisms need to withstand this broad array of stresses. During low tide these organisms have to cope with stress due to UV radiation along with desiccation problems. Production of UV-absorbing compounds is one of the strategies adopted by cyanobacteria growing in the regions exposed to intense solar radiation to protect them from harmful effects of solar UV-radiation. A study has shown that MAAs, the UV-absorbing compounds, are common among several terrestrial cyanobacteria but differ in their distribution (33).

Shinorine is one of the most dominant MAAs found in several species of cyanobacteria with maximum absorbance at 334 nm. The core of shinorine molecule, 1-amino-3-iminocyclohexene moiety is mainly responsible for the absorption of UV radiation (34). Mycosporine-glutaminol (λ_{max} = 310 nm), one of the MAAs, was reported from a *Leptolyngbya* sp. isolated from granite surface in Nepal. This MAA was previously considered to be typical for terrestrial fungi. *Leptolyngbya foveolarum*, a rock-inhabiting species from tintenstrich, contained the terrestrial mycosporine-

glutaminol-glucoside and its corresponding free carboxylic acid derivative (35). As per our knowledge this is the first report of the presence of shinorine in marine *Leptolyngbya* sp.

UV-B is one of the most harmful fractions of solar radiation entering our atmosphere in a significant amount. Exposure to UV-B can have lethal effects on cyanobacteria leading to low levels of photosynthetic activity and reduced cellular biomass (18). It is also known to affect the chlorophyll-a concentration in cyanobacteria (36). In an estuarine microcosm study, Worrest et al. (1981) investigated the effect of UV-B radiation on phytoplankton community (37). Their report suggests a negative effect of UV on biomass and chlorophyll-a concentration of the phytoplanktons. In the present study, low values of dry weight of UV-B exposed samples when compared to PAR are likely due to lower chlorophyll-a values leading to reduced photosynthetic activity and growth. Bleaching caused by exposure to UV-B radiation could be a reason for reduced chlorophyll-a content within 24 h of exposure (38). Similar results were obtained with respect to chlorophyll-a concentration by Singh et al (2008b) while experimenting with shinorine producing *Anabaena* sp.(39). The results thus indicate that UV-B radiations have a negative effect on the growth of the cyanobacterial cells.

UV-B radiation had an inductive effect on photo protective compounds that is total carotenoids and MAAs. Carotenoids in cyanobacteria not only act as light-harvesting accessory pigments but they also function to protect against photo-oxidative damage caused by UV radiation (40). The high energy of UV radiation leads to the formation of reactive oxygen species (ROS) which are highly unstable and can cause damage to biomolecules like lipids and proteins. Carotenoids quench this singlet excited state oxygen and dissipate the energy in the form of heat. An increase in the total carotenoids when exposed to UV-B thus indicates their protective role against the oxidative stress caused by radiation. Induction of MAAs by UV radiation has been observed in several species of cyanobacteria (24, 41). Sinha et al in 2001 reported the production of MAAs shinorine in three cyanobacterial species namely, *Anabaena* sp., *Nostoc commune* and *Scytonema* sp. under solar UV-B (42). The increase in MAAs concentration under UV-B radiation was recorded in *Lyngbya* sp. which is in accordance with our results (24). Similar findings were also obtained with *Microcoleus chthonoplastes*, one of the dominant species of intertidal cyanobacterial mats (19).

The coastal intertidal zones are exposed to air during low tide subjecting the organisms to adverse environmental conditions especially desiccation. Drying can result in crowding of cytoplasmic components, condensation of nucleoid, and oxidation of biomolecules like proteins and DNA. Cyanobacteria manage to survive by adapting strategies and physiological features such as reduction in water permeability of membranes, decreased metabolic and developmental rates, maintenance of intracellular osmolytes and production of protective macromolecules. Although UV absorbing

function is the most studied role of MAAs, they are also known to have other roles like antioxidants and osmolytes in different organisms (11). Even though not proved by the experimental evidence, the previous study suggested that MAAs production is stimulated by the combination of desiccation and irradiation to which the organisms are exposed (43). The results of our study demonstrate that desiccation has a stimulating effect on the production of MAAs and carotenoids although the effect is lesser when compared to only UV-B or UV-B along with desiccation stress.

In aerobic photosynthetic organisms like cyanobacteria, the cycle of desiccation and rehydration in presence of light instigates the production of ROS causing oxidative stress. This inhibits the repair of photosynthetic machinery during the process of recovery after rehydration (44). The increase in MAAs and carotenoids concentration under desiccation and UV-B condition could reinforce its role not only as a UV-protective compound but also as an antioxidant. The study conducted by De la Coba et al (2009) demonstrated that shinorine, an imino-MAAs act as an antioxidant by inhibiting the process of lipid peroxidation and thus preventing the bleaching of other pigment molecules (45). Several studies have illustrated the neutralizing effect of carotenoids against the harmful oxygen free radicals. They act as antioxidants by quenching of singlet oxygen, radical scavenging and releasing excessive light energy through the xanthophylls cycle (46-48). A combined effect of UVB and desiccation demonstrated lesser production of MAAs as compared to UV-B treatment. Exposure of UV-B and desiccation may collectively increase the level of stress more when compared with UV-B. To overcome the level of stress, the organism needs to spend more energy for its maintenance and repair. This might be the reason for reduced MAAs content under simultaneous treatment of UVB and desiccation where in cyanobacteria reduces its energy investment into production of MAAs.

The increment of MAAs and carotenoids in *Leptolyngbya* sp. exposed to UV-B and desiccation may thus form a defense system along with other cell repair mechanisms to combat oxidative stress and facilitate faster recovery. Hence the production of UV-absorbing compounds forms one of the important mechanisms adopted by cyanobacteria to protect themselves from UV radiation. Furthermore, MAAs from cyanobacteria are potential metabolites for the use in sunscreen cosmetics and other pharmaceutical research.

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Figure Legends

Fig 1: Morphology of *Leptolyngbya* sp. observed under (a) light microscope and (b) scanning electron microscope (SEM).

Fig 2: A phylogenetic tree based on 16S gene sequence showing relationship of the isolated *Leptolyngbya* sp.

Fig 3: UV/visible spectrum of *Leptolyngbya* sp. in 100% methanol showing peaks of MAAs (333 nm), chlorophyll-a (432 and 665 nm) and carotenoids (471 nm).

Fig. 4: LCMS analysis (a) of the MAAs from *Leptolyngbya* sp. MAAs was identified by TIC scans (b) and ESI-MS data (c) as shinorine. The inset (d) showing the structure of the the identified MAAs shinorine.

Fig. 5: Effect of UV-B radiation and desiccation on MAAs and carotenoids accumulation in *Leptolyngbya* sp.

(a) dry weight of the cell pellet, (b) chlorophyll-a, (c) total carotenoids and (d) MAAs content under PAR and UV-B treatment in liquid culture.

(e) chlorophyll-a, (f) total carotenoids and (g) MAAs under UV-B and desiccation. DW refers to dry weight. (*) indicate significant difference between the samples. The error bars indicate standard deviation of the mean values.

Figures and Graphs

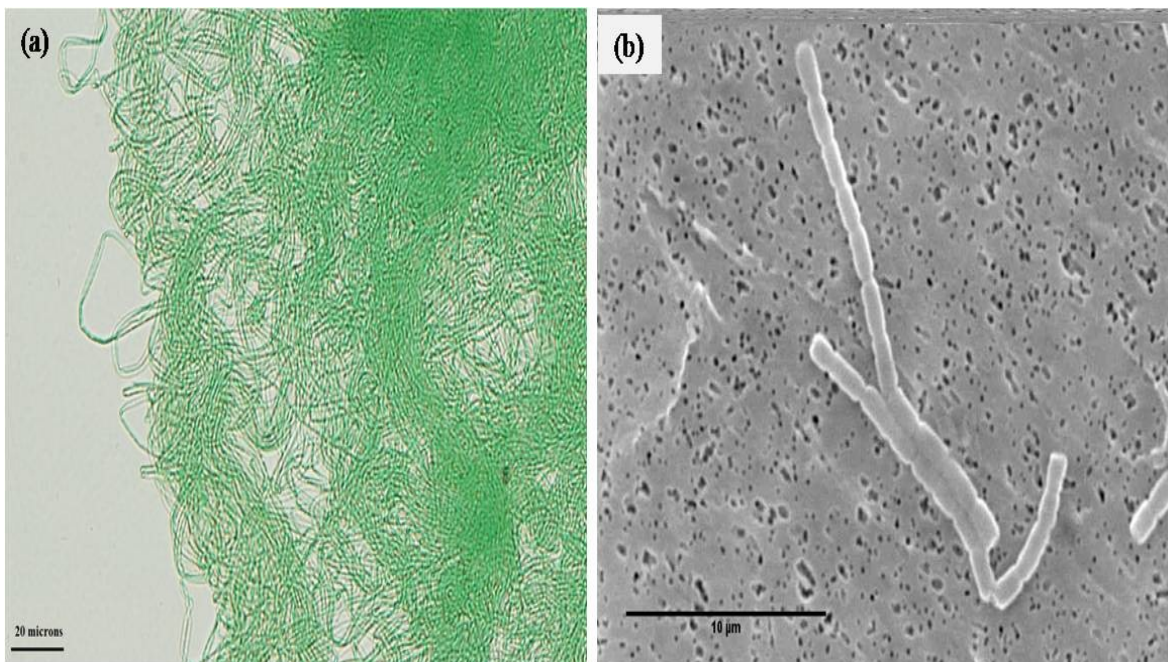


Fig. 1

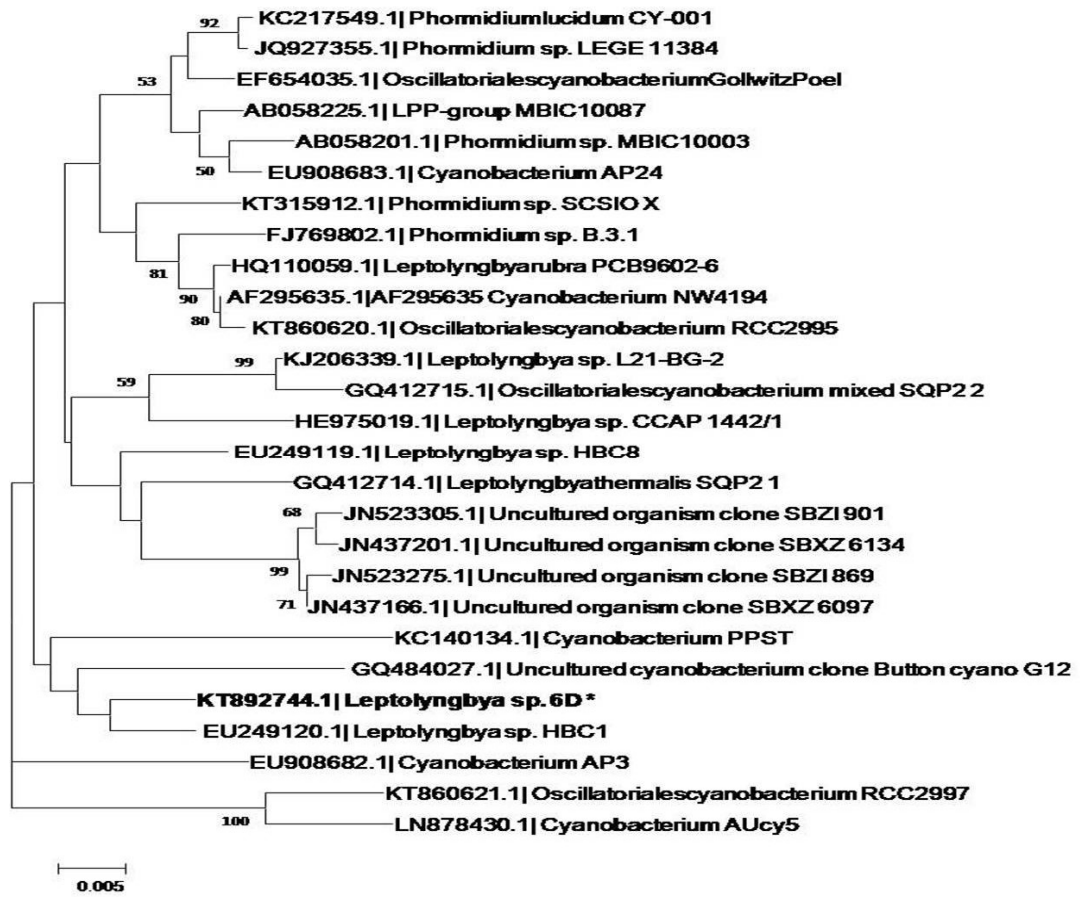


Fig. 2

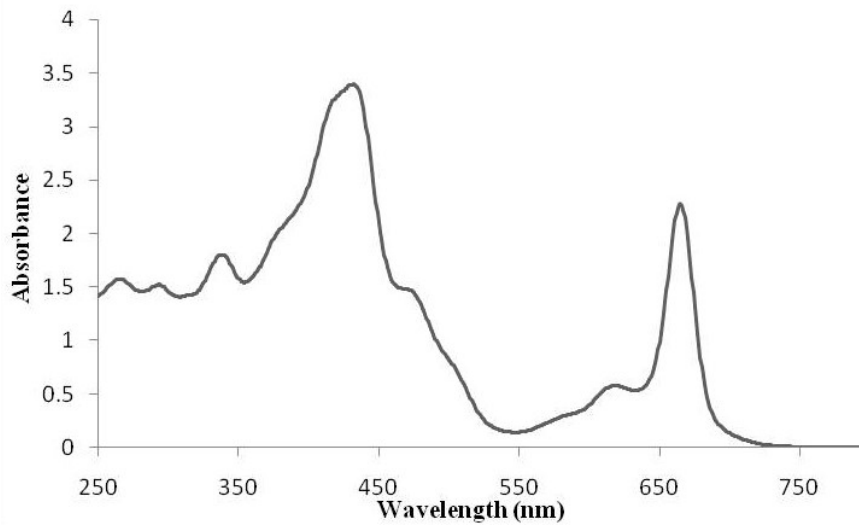


Fig. 3

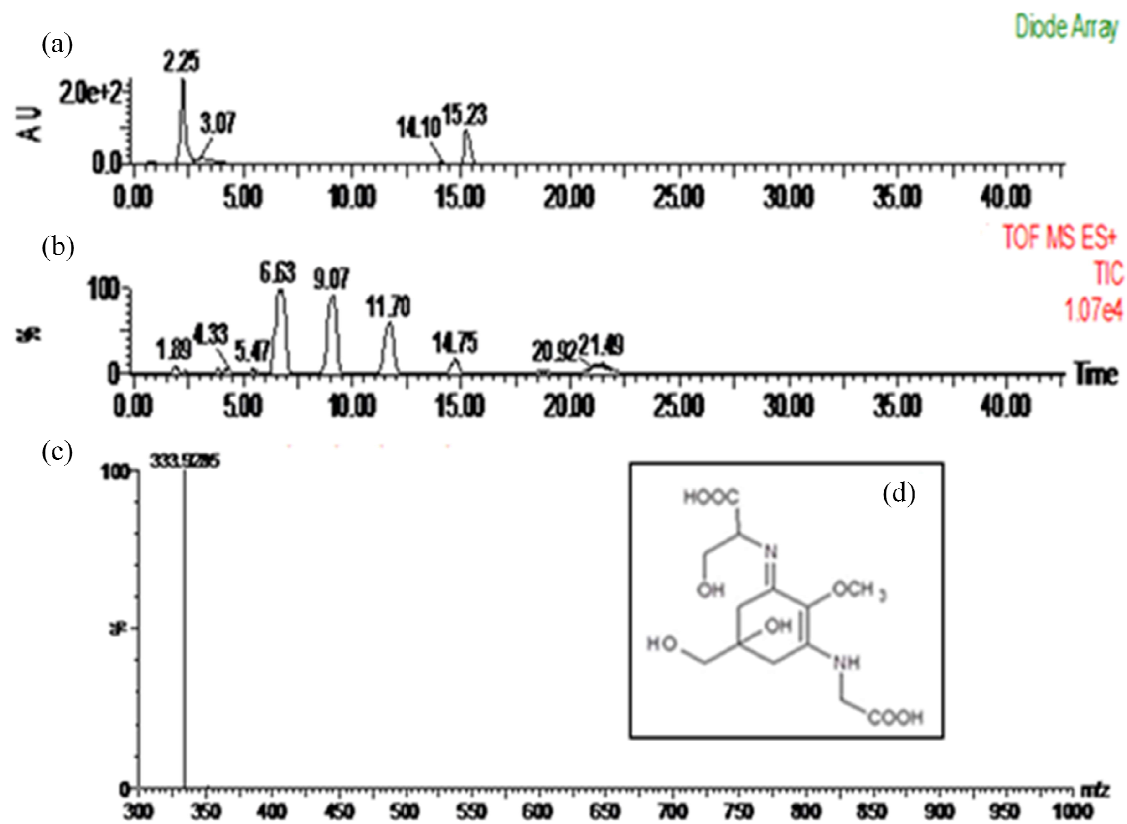


Fig. 4

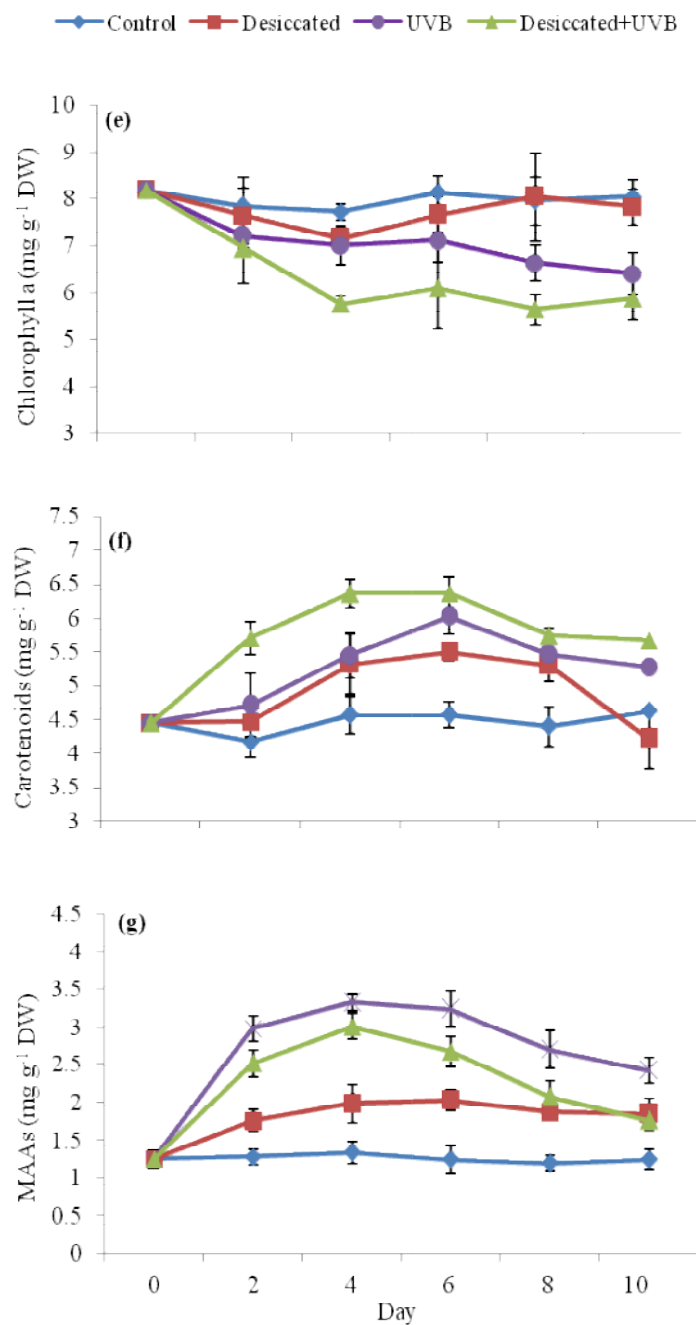
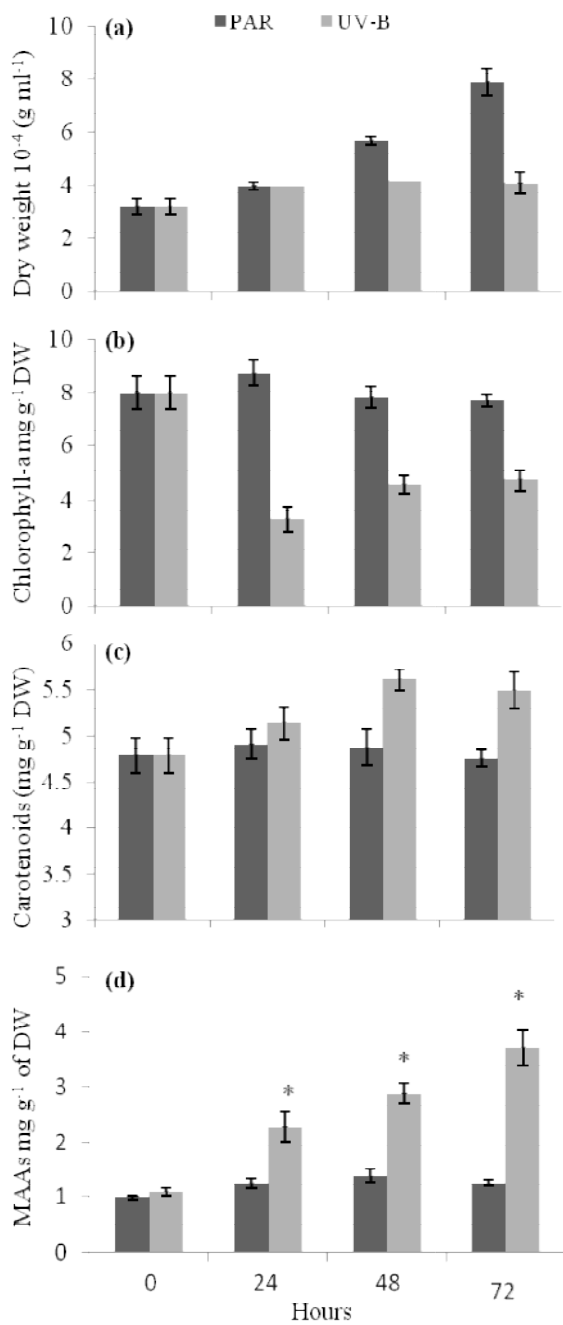


Fig. 5