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Research Paper

Phycocyanin genes, *cpcA* and *cpcB* of *Nostoc sp.* HKAR-2 and Ultraviolet-B radiation

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Abstract

Cyanobacteria engross a wide array of light due to presence of phycobiliprotein and various pigments. Phycobiliproteins are the accessory light-harvesting complex, responsible for the vivid pigmentation of cyanobacteria. Anthropogenic activities result in continuous ozone depletion as a result ultraviolet-B radiation reaches the Earth's surface distressing all the organisms particularly photosynthetic. *Cpc* and *apc* genes encode phycobilisomes. *CpcA* and *cpcB* genes code for α and β subunits of phycocyanin respectively were adversely affected following high radiations. Adverse effects of ultraviolet-B radiation on cyanobacterial phycobiliproteins were studied in *Nostoc* sp. HKAR-2. Absorption spectrum substantiate the degradation of phycobiliproteins after UV-B irradiation at desired time intervals. *Cpc* genes amplification showed that they were repressed following UV-B irradiation, UV-A radiation also has marked effect on the expression of both genes but PAR remains ineffective for *cpcA* gene expression. *cpcB* gene was more prone to high radiations compared to *cpcA* gene. Cyanobacteria have mechanisms to mitigate the high radiation impacts.

Key words: UV-B, *Nostoc* sp. HKAR-2, phycobiliprotein, absorption spectrum, *cpcA, cpcB*.

INTRODUCTION

In cyanobacteria, light is not only captured by light-harvesting complex II (LHCII), but also by large membrane-extrinsic complexes, the phycobilisomes (PBSs), which are attached to the outer surface of thylakoid membranes, it can also transfer energy to photosystem I [1,2]. These complexes are highly ordered, supramolecular assemblies of 5-20 × 10^6 Da and consist of phycobiliproteins (PBPs), which carry covalently linked bilins and linker peptides, which are required for the organization of the phycobilisome. The vivid pigmentation of cyanobacteria is mostly a consequence of the presence of the

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phycobilisome (PBS) [3-5]. While a number of structural classes of PBSs have been described [6], the most common structural form is composed of a central tricylindrical core substructure made up primarily of the phycobiliprotein, allophycocyanin (AP, λ_{max}= 650 nm). To this core are attached six peripheral rods made up of stacked hexameric disks of either phycocyanin (PC, λ_{max} = 620 nm) alone or phycocyanin in combination with phycoerythrin (PE, λ_{max} = 565 nm) or phycoerythrocyanin (PEC, λ_{max} = 590 nm). Ulrich et al. [7] suggested the high degree of wavelength-dependence phycocyanin production in *A. marina* MBIC11017, enables it to grow with yellow and green light. A number of uncoloured linker polypeptides functions to maintain the overall structure of the complex as well as direct its assembly. The chromophores that covalently bind to the apo-phycobiliproteins are the linear tetrapyrroles phycocyanobilin, phycourobilin or phycoerythrobilin. Each phycobiliprotein is composed of a specific α and β subunit that associate into heterodimers and subsequently aggregate into trimers and hexamers. Non-pigmented or linker polypeptides serve as structural elements involved in the biosynthesis and stabilization of PBS [8], but also facilitate efficient flow of excitation energy to the photosynthetic reaction centers. Monomers form disk shaped trimers ($\alpha_3\beta_3$), and hexamers ($\alpha_6\beta_6$). These oligomers are the building units for the assembly of phycobilisomes. The membrane-phycobilisome association is mediated by a large chromoprotein present within the phycobilisome core, which also has linker polypeptide features; it is referred to as the anchor protein or core-membrane linker polypeptide [9,10]. In *Synechocystis* sp. strain PCC 6803, operon contains five genes: *cpcB* and *cpcA* encode the β-PC and α-PC subunits, respectively, while *cpcC2, cpcC1* and *cpcD* encode the rod linkers L_R^{30}, L_R^{33} and L_R^{10} respectively and the two independent genes (*cpcG1* and *cpcG2*) encode the rod-core linker (LRC) that attaches the proximal PC hexamer to the core [11]. The *cpc* operon encodes several subunits of the phycobilisome and their associated linker proteins, the first gene in the operon is *cpcB* [12-14].

Cyanobacteria by reorganization of their photosynthetic centers with their associated antenna systems, respond to environmental changes [15]. Phycobilisomes are major target of UV-B radiation in cyanobacteria [11] since they absorb in UV region. At high doses, UV-B damages phycobilisome structure and function [16-18]. The effects of UV-B radiation on isolated phycobilisomes from *Synechococcus* sp. PCC 7942 showed photodestruction of both α- and β-subunits of phycocyanin [9]. Similarly, isolated α- and β-phycocyanins irradiated for various periods of time showed that both

phycobiliproteins had similar photodestruction quantum yields, although the β-PC was more affected [16]. Rinalducci et al. [19] suggested that when isolated biliproteins or entire phycobilisomes were irradiated with UV-B, the β-PC is the first and most affected biliprotein, although its aromatic amino acids content is lower than other biliproteins, probably due to the presence of two bilins as chromophore. Thus, the β-PC damage is related to an intrinsic property of this biliprotein and not to its location in the phycobilisome structure. This evidence could explain our previous results on isolated phycobilisomes and intact cells, where a destruction of the β-PC and consequently of the supramolecular organization of phycobilisome was observed and moderate UV-B intensity (1.3 W/m^2) induces an oxidative stress mediated destruction of cyanobacterial phycobilisome. This is obviously of concern, because previous consideration of absorption cross-sections and photodestruction quantum yields for UV-B photons of purified phycobiliproteins and of DNA showed that phycobiliproteins will be destroyed about 20 times faster than DNA bases [16]. PC contains two subunits α and β, which are the products of *cpcA* and *cpcB* genes respectively. Some genes coding for PC and linker polypeptides in phycobilisome rods constitute the *cpc* operon [20]. Specific rod linkers (LR) assemble the PC hexamers into rods and tune their electronic properties in order to optimize directional energy transfer [21].

MATERIALS AND METHODS

EXTRACTION OF PHYCOBILIPROTEIN

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 sonicating. 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.

GENOMIC DNA ISOLATION

DNA extraction was done by the protocol given by Sinha et al. [22]. Briefly, the pellet obtained was washed twice with 1 ml of STE buffer (50 mM NaCl + 50 mM Tris-HCl, pH - 8.0 + 5 mM EDTA) and re-suspended in a 500 µl TE (wash) buffer (50 mM Tris-HCl, pH - 8.0 + 50 mM EDTA). Thereafter the cells were broken by sonification for 3 min on ice. Subsequently the cells were treated with 100 µg/ml of proteinase K. Thereafter 750 µl of

prewarmed (55 ^{0}C) extraction buffer (3 % (w/v) CTAB + 1 % (w/v) sarkosyl + 20 mM EDTA + 1.4 M NaCl + 0.1 M TrisHCl, pH - 8.0 + 1 % (w/v) 2-mercaptoethanol) were added and incubated at 55 ^{0}C for one hour in a water bath with mixing by gentle inversion every 10 minute. The resulting suspension was allowed to cool for 1-2 min and thereafter, 750 ml chloroform: isoamyl alcohol (24:1, v/v) were added and mixed by gentle inversion until an emulsion was formed. After centrifugation (10,000 x g for 8 min at room temperature) the supernatant was transferred to sterile microcentrifuge tubes. Thereafter 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate (pH - 5.2) were added and kept at 0 ^{0}C overnight for the precipitation of DNA. Next day the precipitated DNA was centrifuged at 10,000 x g for 15 min. The pellet obtained was briefly rinsed once with ice-cold 70 % ethanol, dried and re-hydrated with 30 ml TE buffer (10 mM Tris-HCl, pH - 8.0 + 1 mM EDTA).

AMPLIFICATION OF CPC GENES OF PHYCOCYANIN

Primers with the sequences *cpcA* forward: 5'-AGA ATT TTA CCA TAT GGT TAA AAC CCC C-3'; *cpcA* reverse: 5'-GGC AGG AAG CTT TCT AGC TG-3'; *cpcB* forward: 5'-AGG AGA TTA AAG CAT ATG ACA TTA GAC GTA-3'; *cpcB* reverse: 5'-GGA CTT GTT AGA ATT CAT TAA CCA ACA GCA G-3' were used to amplify the *cpcA* and *cpcB* genes of phycocyanin.

RESULTS AND DISCUSSIONS

3.1. Morphology of the test organism

Nostoc sp. HKAR-2 [Fig. 1] was isolated from hot spring, Rajgir, Bihar and is characterized as thallus frothy, gelatinous, trichomes circinate, sheath absent, cells and heterocysts ellipsoidal. Because sheath is not present, this organism is highly prone to UV-B radiation.

3.2. Spectroscopic investigation of phycobiliproteins from *Nostoc* sp. HKAR-2 after UV-B irradiation

Fig. 2 illustrate the partially purified phycoerythrin [A] and phycocyanin [B] from *Nostoc* sp. HKAR-2. Absorption maximum of phycoerythrin and phycocyanin was at 560 and 620 nm respectively in *Nostoc* sp. HKAR-2. The absorption spectra of *Nostoc* sp. HKAR-2 showed drastic decline in absorbance at 620 nm after 30 min of UV-B irradiation and it was gradual after 60 and 90 min of UV-B irradiation, but decline in absorbance at 560 nm was less as compare to 620 nm (Fig. 3) which was inferred as phycocyanin was more

prone to UV-B radiation as compared to phycoerythrin. Fluorescence microscopy is another important tool to follow the behavior of phycobilisomes (PBPs) following high radiations. Fluorescence excitation at 620 nm of *Nostoc* sp. HKAR-2 resulted into emission at 643 nm. There were decrease in fluorescence of PBPs after 3 h of UV-B irradiation, UV-A radiation had some effect on the decrease of fluorescence of PBPs but PAR didn't show any such effects, in addition, there was also shift of fluorescence towards shorter wavelength after UV-B irradiation (Fig. 4). Purified phycobiliproteins might form a mixture of monomers, trimers and hexamers. Trimers and hexamers are interconvertible and loss of monomers resulted in dissambely of PBPs, hence impairment of energy transfer from phycobilisome complex to photosynthetic reaction centre. The earlier degradation of phycobiliprotein by UV-B radiation is due to its proteinaceous nature suggesting that proteins may be preferred target of UV-B radiation [11]. The protection of UV-B damage might be due to lowering in radiation amplification factors (RAF) value by visible light [23]. 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 [24]. In the native states, phycobiliproteins are strongly fluorescent because their tetrapyrrole chromophores are kept in rigid extended conformations, while denatured phycobiliproteins have low fluorescence yields due to the less rigid chromophores. Compared to native phycobiliproteins, the denatured ones show largely red-shifted absorption spectra because the conformation of the chromophores transforms from the extended to the cyclohelical. Absorption spectroscopic data of the phycobiliprotein signify that phycocyanin which function as accessory pigment for the operation of photosystems is one of the main targets of UV-B in cyanobacterial strains.

3.3. UV-B radiation impacts on the *cpcA* and *cpcB* genes of phycocyanin

Effect of various light sources on *cpcA* (492 bp) which codes for α subunit of phycocyanin, (Fig. 5A) and *cpcB* (522 bp) which codes for β subunit of phycocyanin (Fig. 5B) genes of *Nostoc* sp. HKAR-2 was measured by template activity in PCR reactions. Less intensity of the bands after PCR amplification inferred that both genes were repressed after 3 h of UV-B irradiation (Fig. 5A & B, Lane 5), UV-A radiation also has prominent effect on the repression of both genes (Fig. 5A & B, Lane 4) but PAR remains ineffective for *cpcA* gene even after 3 h of irradiations (Fig. 5A, Lane 3) but *cpcB* gene

was repressed after irradiation to high intensity of PAR (Fig. 5B, Lane 3). *cpcB* gene was more prone to high radiations compared to *cpcA* gene. An accumulation of PC was detected in low PAR cells compared with high PAR cells of *Synechocystis* sp. PCC7942 [25]. Loss of low molecular mass (αβ) subunits as well as high molecular mass linker polypeptides are indicative of the fact that the energy transfer from the accessory pigments to the photosystems were probably impaired by UV-B irradiation. In *Synechocystis,* there was down-regulation of most genes (*apcA*, *apcB*, *apcC*, *apcE*, *apcF*, *cpcA*, *cpcB*, *cpcC*, *cpcD*, and *cpcG*) encoding structural subunits of the phycobilisome light-harvesting antenna following UV-B irradiation. A putative operon formed by *cpcB* (*sll1577*), *cpcA* (*sll1578*), *cpcC* (*sll1579* and *sll1580*) contained some of the most highly repressed genes, with expression levels of 0.05- to 0.08-fold but the transcription levels of *cpcE* and *cpcF* were not affected by UV-B and the transcription levels of *nblA* and *nblB* were up-regulated by three- to eight-fold, contrary to the repression of phycobilisome structural genes. This suggested a coordinate slowdown of phycobilisome biosynthesis and activation of phycobilisome degradation in UV-B-treated *Synechocystis* [26]. MacDonald et al. [27] suggested that high-PAR (300 μmol PAR $m^{-2}s^{-1}$) irradiated cells of *Synechococcus* initially had fewer *cpc* transcripts encoding phycocyanin, lower phycocyanin content, and more *psbAII/AIII* transcripts encoding the D1:2 photosystem II (PSII) protein isoform while low-PAR:high-UV-B (50 μmol PAR $m^{-2}s^{-1}$, 0.75 μmol UV-B $m^{-2}s^{-1}$) irradiated cells, in contrast, suffered short-term inhibition of PSII. UV-B radiation led to phycobilin bleaching, thereby contributing to significant cell death of many filaments of *Microcoleus chthonoplastes* [28]. Rinalducci et al. [29] suggested the mechanism of photodegradation of antenna system in cyanobacteria. Exposure of isolated intact phycobilisomes to illumination with strong white light (3500 μmol/m^2s photosynthetically active radiation) gave rise to the formation of free radicals, which subsequently led to specific protein degradation as a consequence of reactive oxygen species induced cleavage of the polypeptide backbone. The use of specific scavengers demonstrated an initial formation of both singlet oxygen ($^1O_2$) and superoxide (O_2^-), most likely after direct reaction of molecular oxygen with the triplet state of phycobiliproteins, generated from intersystem crossing of the excited singlet state. 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 [30].

Fig. 1: Photographs showing the filaments of *Nostoc* sp. HKAR-2.

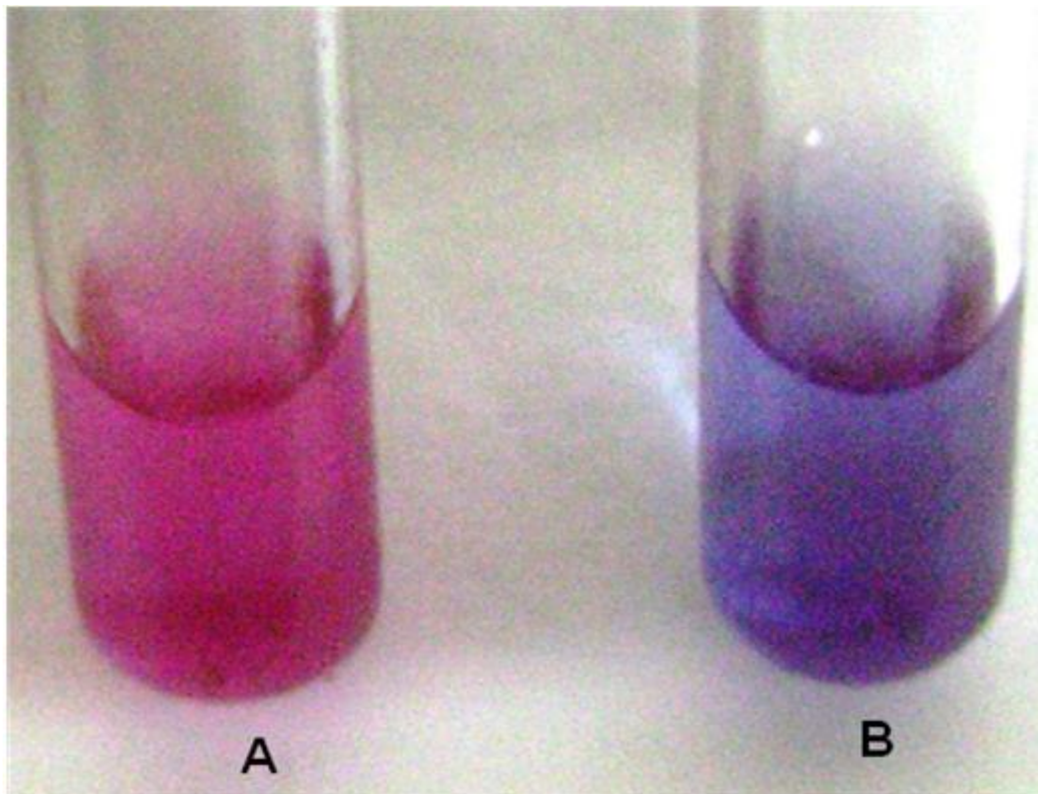

Fig. 2: Partially purified phycobiliproteins from *Nostoc* sp. HKAR-2, A - Phycoerythrin; B - Phycocyanin.

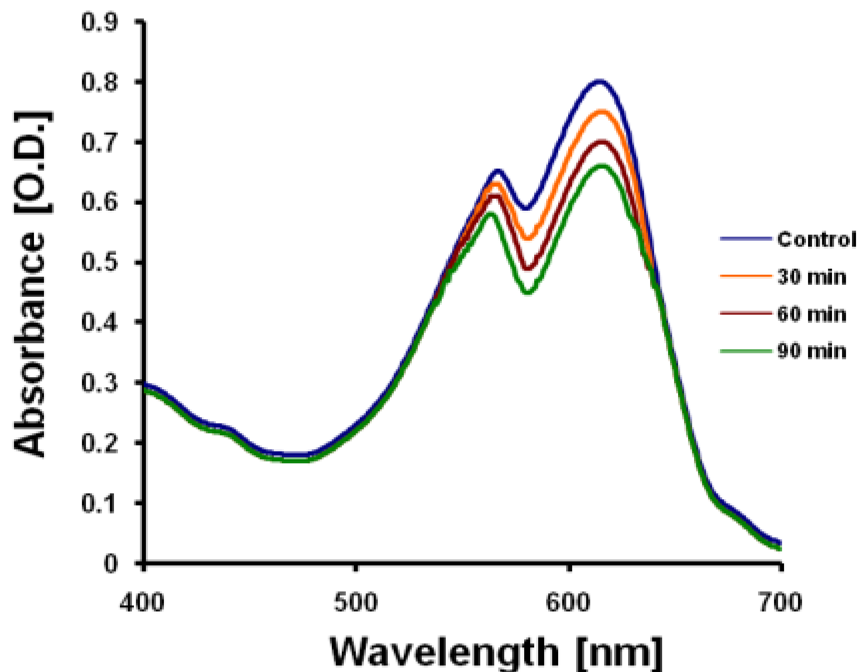

Fig. 3: Absorption spectra showing the effects of UV-B stress on partially purified phycobiliproteins from *Nostoc* sp. HKAR-2.

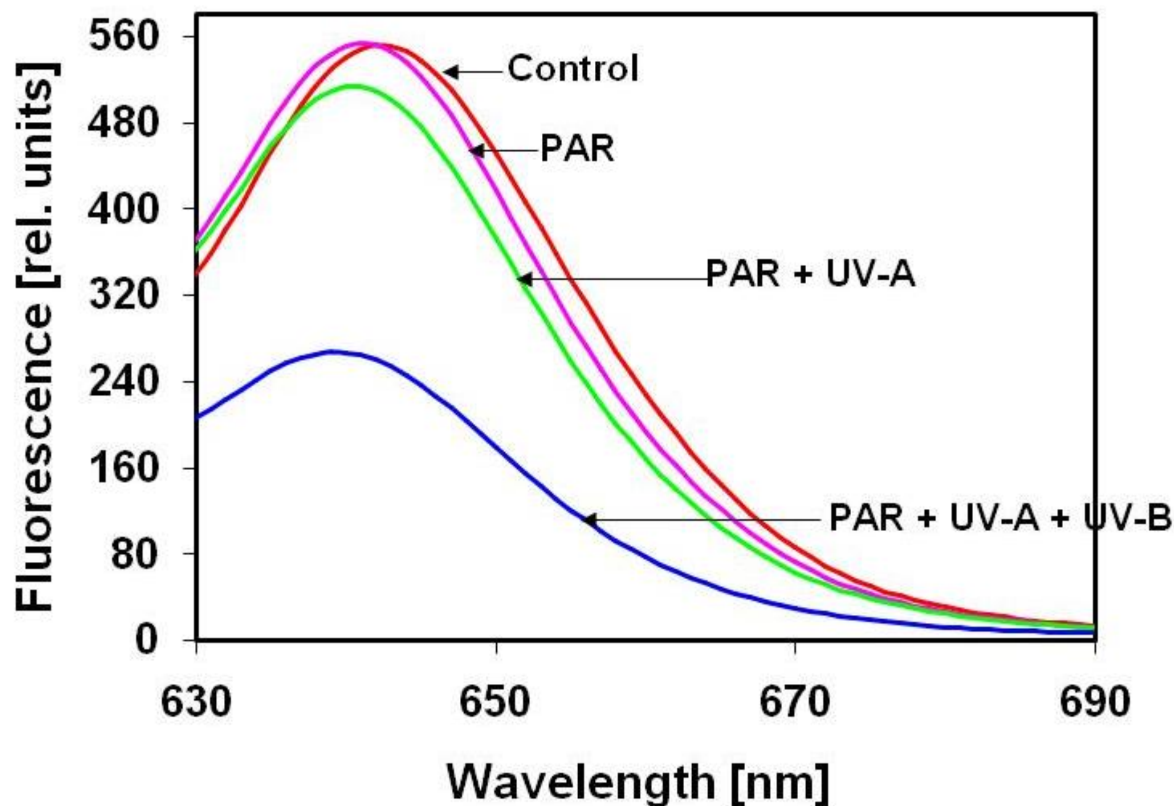

Fig. 4: Fluorescence emission spectra of phycocyanin of *Nostoc* sp. HKAR-2 under various cut-off filters when excited at 620 nm.

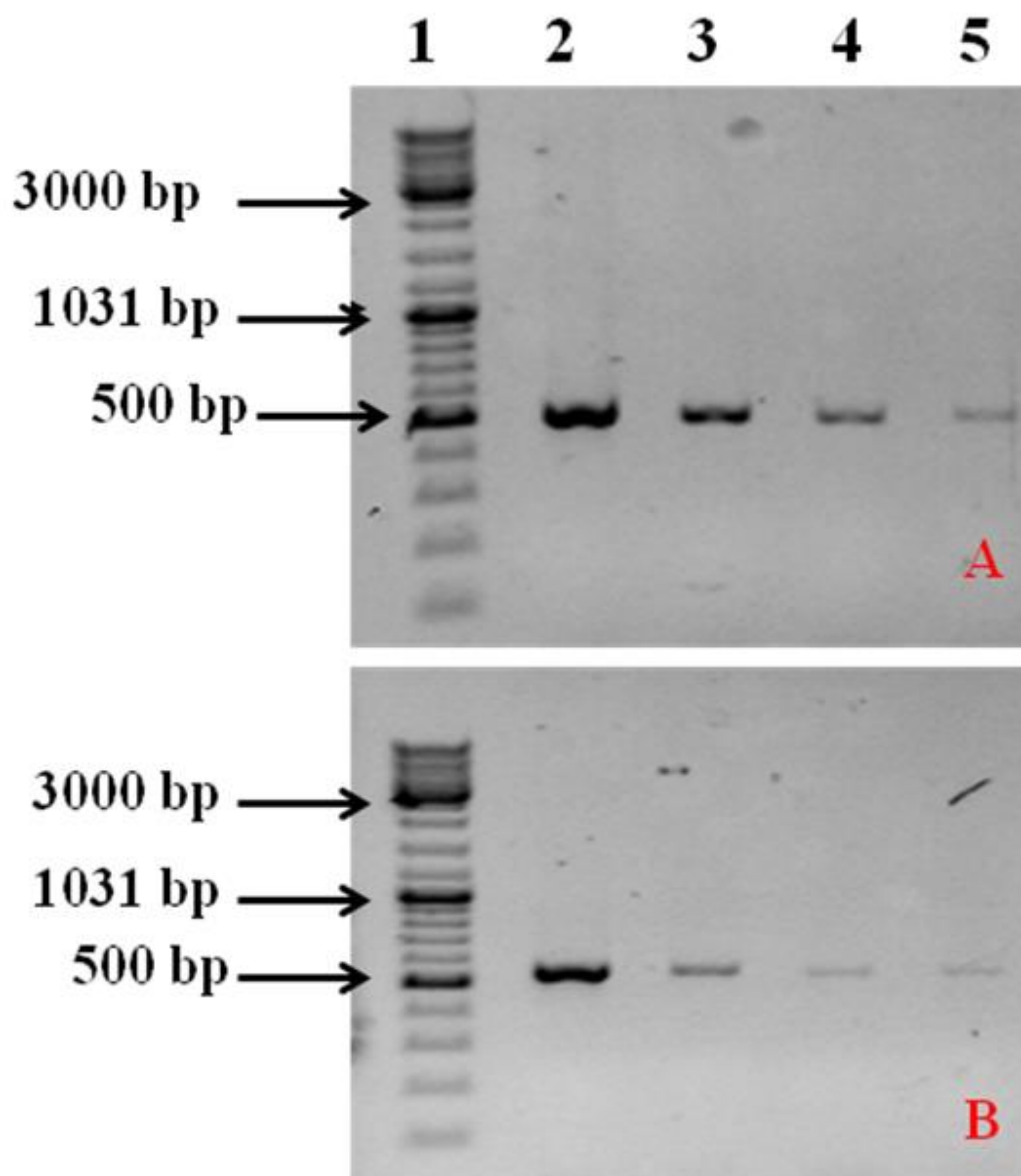

Fig. 5: Effect of various light sources on *cpcA* (492 bp) (A) and *cpcB* (522 bp) (B) genes as measured by template activity in PCR reactions. Lane 1: DNA Marker; Lane 2: Control; Lane 3: PAR; Lane 4: PAR + UV-A; Lane 5: PAR + UV-A + UV-B.

CONCLUSIONS

The adverse effects of UV-B radiation on the phycobilisomes of *Nostoc* sp. HKAR-2 is observed, despite all these, the cyanobacterial species survives in the shifting environments. Some PE-producing cyanobacteria have the capacity to maximize the absorption of available light by altering the composition of their phycobilisome rods in response to the wavelength of light under which they are grown. Growth of the cyanobacterial culture in red light ($\lambda > 590$ nm) results in rods composed of PC, while

growth in green light (λ < 590 nm) results in rods containing both PC and PE. The color of light also determines whether PE- or PC-specific linker polypeptides are produced. This phenomenon has been shown to be widespread among the cyanobacteria [31] and is termed complementary chromatic adaptation [32]. Campbell *et al.* [25] suggested UV-B induced rapid but transient *cpcBA* transcripts, associated with an accumulation of psbAII/AIII transcripts, which represent a rapid down-regulation of phycocyanin expression to lower the UV-B absorbance by the cell. A phycobiliprotein has been used as a model for refolding/unfolding studies [33]. The spectral shifting [34-36] and fluorescence recovery/quenching [37-39] could be taken as probes to monitor the refolding and unfolding of proteins.

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