Isolation, identification and characterization of novel thermotolerant Oscillatoria sp. N9DM: Change in pigmentation profile in response to temperature.

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3.1 Introduction

Cyanobacteria are the largest, most structurally diverse and widely distributed group of photoautotrophic eubacteria (Schöpf, 1993; Stanier and Cohen-Bazire, 1977). Their simple nutritional requirements – light, water, carbon dioxide, and inorganic salts – allow these organisms to occupy highly diverse ecological niches. The content and ratio of different pigments in cyanobacteria may be affected by environmental conditions such as temperature, nutrient availability, light, presence of toxic compounds, pH, etc., and their combinations (Parmar et al., 2011; Jing et al., 2007; Burns et al. 2005; Reuter and Muller 1993; Tandeau de Marsac and Houmard, 1993). General responses to temperature variation include changes in both cellular morphology and physiology.

Thermotolerant cyanobacteria are the primary producers among microbial communities in geothermal springs worldwide having temperatures up to 75°C (Castenholz, 2000) which is the maximum temperature limit for performing oxygenic photosynthesis (Ferris and Ward, 1997). Environmental stress influences the organism to inhibit or enhance the functioning and production of some physiologically important proteins. One such physiologically important group of proteins called phycobiliproteins (PBPs) situated on the outer surface of thylakoid membrane in the form of phycobilisomes (PBSs) (Soni et al., 2006). The effects of temperature and external inorganic carbon availability on cyanobacterial photosynthesis and growth have been observed to be intrinsically linked (Burns et al. 2005). This suggests that the C-phycocyanin rods, from the thermophilic alga are much more resistant to dissociation than mesophilic C-phycocyanin rods. Even though the amino-acid composition of thermophilic and mesophilic C-phycocyanin is very similar. Berns and Scott (1966) discovered that, at 49°C aggregation of C-phycocyanin was strongly increased. These properties of C-PC suggest the protein stability increases as the environmental stress increases up to certain limit. Burns et al., (2005) proposed different pathways that a thermophilic protein could be protected from adverse effects of high temperature.

PBS is composed of central core and outer rods. Different components of PBSs evolved independently from each other according to necessities of cyanobacteria under different stress conditions (Six et al., 2007a). Core of PBS evolved together
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with the main genome and hence evolutionary ancient and conservative mechanism has not allowed much phenotypic variability (Partensky, 2007; Six et al., 2007b). In contrast, rod region appears to have evolved later either via horizontal gene transfer or gene acquisition from cyanophages (Six et al., 2007b), which suggest the process of light energy transfer from PBS rods to cores has evolved according to necessities of cyanobacteria under different stress condition. It is believed that in rod region, the PC gene seems to have evolved much before the PE gene (Six et al., 2007b) to cope with different stress.

The characteristic absorbance of the biliproteins (Grossman et al., 1993; Zehetmayer et al., 2004) in the visible part of the spectrum is due to open-chain tetrapyrrole prosthetic groups, known as chromophores or bilins which includes phycoerythrobilin (PEB), phycocyanobilin (PCB), phycourobilin (PUB), phycobiliviolin (PVB) and phycobilimobilin (PΦB). Chromophores are covalently attached to α, β and sometimes γ subunits of PBPs via thioether bond. Ratio of different chromophores attached to specific PBP determines its subtype. The subtypes of phycocyanin found in cyanobacteria are C-PC, R-PCII and R-PC III contains only PCB, PEB:PCB in the ratio of 2:1 and PEB:PCB in the ratio of 1:2 respectively (Six et al., 2007b). Absorption peak of phycoerythrin at 495 nm and 545 nm or 566 nm contains low and high PEB:PVB or PUB ratio respectively and corresponds to PE II and PE I respectively. PEC is functionally similar to phycoerythrin but has some structural similarities to phycocyanin (Füglistaller et al., 1981). PEC contains isomeric PVB and PCB in the ratio of 1:2.

The ability of phycobilisomes to exhibit variability in harvesting of light is most advantageous in allowing a good match between the absorption spectra of a particular cyanobacterium and the available light. In some cyanobacteria, the colour of light influences the composition of phycobilisome and consequently they appear green in red light and red in green light. This process, known as Complementary Chromatic Adaptation (CCA), is a way for the cells to maximise the use of available light for photosynthesis. When a cyanobacterium exhibits complementary chromatic adaptation, the rods of the phycobilisomes change with the change in light. The changes include one or both biliproteins and linkers (Tandeau de Marsac and Cohen-Bazire, 1997). Red light may produce C-phycocyanin which absorbs red wavelength and green light produces
C-phycoerythrin, which absorbs green wavelength. On the basis of shift in PE and PC content during growth in red and green light (Tandeau de Marsac, 1977; Kehoe, 2010) chromatic adaptors are classified into four different groups.

Here, we report the isolation and identification of novel cyanobacterium Oscillatoria N9DM that exhibit change in pigment variation when exposed to various temperatures. An isolated culture of Oscillatoria N9DM also changes their pigmentation profile with respect to various light wavelengths. We for the first time also tried to describe that the change of pigmentation in response to temperature has very close similarity to CCA or are probably linked.

3.2 Materials and Method

3.2.1 Chemicals
Sephadex G-150 powdered matrix (bead diameter 200-300 µm and fractionation range 4-150 kDa) was purchased from GE Healthcare Limiteć (Amersham, Buckinghamshire, UK), diethyl amino ethyl (DEAE) cellulose weak anion exchange resin was purchased from Sigma (St. Louis, Missouri, USA), protein molecular mass standard from Bangalore Genei (Bangalore, Karnataka, India), bis-acrylamide from Merck (Darmstadt, Hesse, Germany) and sodium dodecyl sulphate and electrophoresis grade acrylamide from Merck (Darmstadt, Hesse, Germany). All other chemicals were ultra-pure or molecular biology grade and were used without further purification. All buffers and solutions were prepared in milli-Q water supplemented with 0.02% (w/v) sodium azide.

3.2.2 Sampling, isolation and cultivation of culture
Two thermotolerant cyanobacterial cultures namely N9DM I and N9DM II were collected from different wells of Tuwa geothermal spring (22°47'58.93"N 73°27'38.33"E) situated in Panchmahal, Gujarat, India, having temperatures of 30 ± 2°C and 55 ± 2°C respectively (Fig. 3.1). Collected cultures were plated centrally on 1.5% agar prepared in Castenholtz’s D medium containing cyclohexamide (100 µg/ml). Minute agar blocks with an individual self isolated trichome were transferred in 2L Erlenmeyer flasks containing 0.8 L of slightly modified Castenholtz’s D medium (Castenholtz, 1969) supplemented with glycine (100 mg/l) and maintained under three different temperature conditions: 30 ± 2°C (Group I), 55 ± 2°C (Group II) and intermediate temperature at 42 ± 2°C (Group
III) for 60 days. The cultures were supplied with 12/12 h of light/dark illumination (130 μmol photomn−2s−1) of fluorescent daylight lamps. Freshly inoculated culture grown at 30 ± 2°C sub-cultured from group II (temperature 55 ± 2°C) cultures was also maintained in above mentioned conditions and called reverted cultures. Group I and group II cultures were also kept under red, yellow, green and blue lights under similar conditions.

Fig. 3.1: A view of Tuwa geothermal spring, Panchmahal, Gujarat, India. This bathing area has varied temperatures ranging from ~30°C to >70°C, the latter at the centre.

3.2.3 Identification

3.2.3.1 DNA extraction

Genomic DNA extraction from group I and group II cultures was performed independently using a slight modification of the standard method of Wu et al. (2000). Pelleted culture aliquots were resuspended in 10 ml of extraction buffer [100 mM Tris-C1(pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl] along with 1ml of each 10% Sarkosyl and 10% CTAB so as to make final concentration of 1% (w/v) and incubated in water bath at 60°C for 30 minutes. The mixture was centrifuged at 10000 × g for 10 minutes at 4°C. Equal volume of phenol: chloroform: isoamylalcohol (25:24:1) was added to aqueous phase and mixed for 20 minutes and then centrifuged at 10000 × g for 10 minutes at 4°C. The pellet was discarded and the supernatant was mixed well with equal volume of chloroform and centrifuged at 10000 × g for 10 minutes at 4°C. Aqueous phase was collected and the DNA was precipitated by adding equal volume of 70% (v/v)
chilled ethanol and one tenth volume of 3.0 M ammonium acetate (pH 5.2). The DNA was pelleted down by centrifugation at 10000 × g. Finally the pellets were washed with chilled 70% (v/v) ethanol; air dried and resuspended in 10 mM Tris (pH 8.0). The extracted DNA was quantified and checked for its purity using a Nanodrop spectrophotometer (Implen Version 1.3, Munchen, Germany).

3.2.3.2 PCR amplification and sequencing of 16S rRNA gene

The 16S rRNA gene was amplified from group I and II cyanobacterial cultures by PCR amplification. Amplification was carried out in a 30 μl PCR reaction consisting of 1X buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100), 0.33 mM each of dNTPs, 100 ng of template DNA, 0.66 μmoles each of custom synthesized bacteria-specific universal primers 8F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (5'-GGT TAC CTT GTT ACC ACT-3'), and 1.5 U of Taq DNA polymerase. Amplification program was performed with initial denaturation step at 94°C for 5 minutes; followed by 30 cycles of 1 minute denaturation step at 94°C, 1 minute annealing step at 55°C, and 1 minute elongation step at 72°C and a final extension step at 72°C for 20 minutes using Biorad iCycler version 4.006 (Biorad, CA, USA). The ~1.5 kb PCR product of all three cultures was sequenced by automated DNA Analyzer 3730xl using BigDye™ Terminator 3.1 sequencing chemistry (Applied Biosystems, Foster City, CA). The full 16S rRNA gene sequences derived from this study were submitted to Gene Bank under the Accession No. JN661705.

3.2.3.3 Comparative sequence analysis

The 16S rRNA gene was sequenced using the original PCR primers with an automated DNA Analyzer 3730xl using BigDyeTM Terminator 3.1 sequencing chemistry (Applied Biosystems, Foster City, CA) sequencer. Nucleotide alignments of 16S rRNA gene sequence and several other cyanobacterial taxa were created using CLUSTALX with default gap penalties (Higgins et al., 1988; Higgins et al., 1989; Thompson et al., 1997; Tamura et al., 2007). Gaps were excluded for phylogenetic analyses. Distance matrix trees were generated by the Neighbour-Joining algorithm with Kimura 2 parameter distance in MEGA 4.0 software (Tamura et al., 2007). The NJ calculations were subjected to bootstrap analysis (1000 replicates). Escherichia coli K12 were used as the out-group for all analyses.
3.2.4 Chlorophyll a estimation

Growth of culture was measured by extracting chlorophyll a from fresh and nutrient free algal sample according to procedure described by Marsac and Houmard (1988). Chlorophyll was measured spectrophotometrically at 665 nm (Analytik Jena AG Specord® 210, Jena, Germany).

3.2.5 Extraction and purification of biliproteins

Thirty six days old cyanobacterial cultures from all groups were harvested by centrifugation at 8000 \( \times g \) for 10 minutes (Kubota 6500, Bunkyo-Ku, Tokyo, Japan) at 27°C. The cell pellet was washed with 1M Tris-Cl buffer (pH 8.1). Ten grams of washed cell mass was suspended in 50ml of 1M Tris-Cl buffer (pH 8.1) and crushed in liquid nitrogen. The cell debris was removed by centrifugation at 18000 \( \times g \) for 30 minutes at 42°C and colored supernatant containing PBP extract was collected and termed as crude extract.

The crude extracts of all three groups and reverted culture were saturated to 20% ammonium sulphate followed by 70% ammonium sulphate. Pellet of 70% ammonium sulphate precipitation was resuspended in 10 mM Tris-Cl buffer (pH 8.1). The extract thus obtained was termed as 70% ammonium sulphate cut (ASC).

The crude extracts of all three groups and reverted culture were also saturated with gradient of 40%, 50%, 60% and 70% ammonium sulphate for another set of experiment. Pellet of all ammonium sulphate precipitate was resuspended in 10mM ammonium sulphate (pH 8.1).

All three ASC were loaded on pre-equilibrated Sephaaex G-150 column (150 cm \( \times \) 1.5 cm, bed height 105 cm) and eluted with 10 mM Tris-Cl buffer (pH 8.1). The flow rate was maintained at 60 ml h\(^{-1}\) using peristaltic pump (Model P1; Pharmacia, Uppsala, Sweden). Colored elutes were collected as 0.5 ml fractions and termed GPC fraction.

For each of the group, colored GPC fractions were pooled and concentrated up to 1 ml and loaded on a DEAE cellulose weak anion exchange column (60 mm \( \times \) 12 mm, 5 cm bed height) pre-equilibrated with 10 mM Tris–Cl buffer (pH 8.1). The column was washed with 10 bed volumes of the same buffer and then elution
was carried out by step wise increase of NaCl from 0 to 0.4 M. Colored fractions were collected and termed ion exchange chromatography (IEC) fractions. IEC fractions were analysed by UV–Vis absorption spectroscopy and denaturing as well as non-denaturing gel electrophoresis.

3.2.6 Spectroscopic estimation of phycobiliproteins
Absorbance of crude extract, ASC, GPC fractions and IEC fractions for all three groups was recorded on a UV-Vis spectrophotometer (Analytik Jena AG Specord® 210, Jena, Germany). The amount of PBP in each sample was determined by the method of Lowry et al. (1951) and Bennett-Bogorad (1973) equation (Eq. 1). The fluorescence excitation and emission spectra were recorded using a Shimadzu Spectrofluorophotometer (RF-5301 PC, Kyoto, Japan) with spectral band width of the excitation and emission slits of 1.4 nm. All the measurements were taken at 20-25°C.

\[
\text{C-PC (mg ml}^{-1}) = \frac{(\text{OD}_{620} - 0.7\text{OD}_{650})}{7.38}
\]

3.2.7 Electrophoretic analysis
Different fractions were also characterised in terms of number of subunits and their molecular weight. Non-denaturing and denaturing polyacrylamide gel electrophoresis were carried out in vertical slab gel apparatus as described before (Singh et al., 2009; Parmar et al., 2010) using 10% (w/v) and 15% (w/v) polyacrylamide respectively. The gels were visualized by silver staining according to Garfin (1990). The proteins containing bilin in the same gels were also visualized by zinc-acetate staining, as described in the Brekelman and Lagarias (1986). For calibration of molecular weight, two marker proteins ranging from 6.5 kDa to 43 kDa (Aprotinin-6.5 kDa, Lysozyme-14.3 kDa, Soyabean Trypsin Inhibitor-20.1 kDa, Carbonic Anhydrase-29 kDa and Ovalbumin-43 kDa) were used.

3.3 Results and discussion
Cyanobacteria have long history of adaptation to survive in extreme or variable environments by developing specific regulatory systems in addition to more general mechanisms equivalent to those of other prokaryotes or photosynthetic
Chapter 3: Isolation, identification and eukaryotes (Castenholz and Utkilen, 1984). In the past decade, considerable progress has been made towards understanding the expression of cyanobacterial pigmentation in response to variation in the intensity and spectral quality of incident light. But none of them reported the change of pigmentation with respect to change of temperature.

3.3.1 16S rRNA gene sequence determination and phylogenetic placement

In the present study full length 16S rRNA genes of both cyanobacterial isolate N9DM I and N9DM II were sequenced and were found to be 100% similar and submitted to the NCBI GenBank (JN661705). Complete identity between both sequences confirmed that the strains isolated from different temperature wells were similar. Furthermore, sequence was aligned with other 16S rRNA gene sequences available in database. Phylogenetic clusters of strains N9DM along with other closely related cyanobacteria strains was constructed (Fig. 3.2). It was found that N9DM showed 92% similarity to Oscillatoriales, hence the culture identified as nearest neighbour of Oscillatoria and was named Oscillatoria sp. N9DM. This culture is one of the very few reported isolate of thermo tolerant Oscillatoria (Castenholz and Utkilen, 1984; Jing et al., 2005; Castenholz et al., 1991).

Fig. 3.2: Phylogenetic tree derived from 16S rRNA gene sequence of N9DM and sequences of closest phylogenetic neighbours obtained by NCBI BLAST(n) analysis. The NJ-tree was constructed using neighbour joining algorithm with Kimura 2 parameter distances in MEGA 4.0 software. Escherichia coli strain ATCC00096 has been taken as an out-group. Numbers at nodes indicate percent
bootstrap values above 50 supported by more than 1000 replicates. The bar indicates the Jukes–Cantor evolutionary distance.

3.3.2 Effect of temperature on production of cell mass and biliprotein
We demonstrated that Oscillatoria sp. N9DM which grows at high temperature can also grow at lower temperatures and vice-versa in the standard Castenholtz’s D medium supplemented with glycin (100 mg/l). After every 48 h of incubation 50 mg of cell mass was taken from the each group and analysed for its chlorophyll $a$ content and phycobiliprotein content. It was found that pigmentation prototype of Oscillatoria sp. N9DM was directly affected by change in temperature. Morphological appearance of culture of group I (30 ± 2°C), group II (55 ± 2°C) and group III (42 ± 2°C) were brick red, bluish green and army green respectively. Group I cultures achieved maximum growth in 36 days and started PE production in the log phase. Phycobiliprotein analysis of crude extract confirmed that group I culture grown at 30 ± 2°C was potent producer of PE (Fig. 3.3) but also produces PC and APC in minute quantity. After 36 days, cells entered into the declination phase of growth and the amount of PE decreased gradually (Fig. 3.3). Group II culture showed comparatively sluggish growth and achieved maximum growth in 50 days and surprisingly started production of PC in the log phase (Fig. 3.3). Irrespective of PE, group II cultures showed invariable PC content per unit dry cell mass and was found constant throughout the experiment even after the cells entered into the declination phase of growth (Fig. 3.3). Moreover group III culture showed intermediate growth pattern, achieved maximum growth in 40 days after inoculation and produced both PEC (or PE I) and PC. This culture was exceptionally susceptible to temperatures and able to produced either PE or PC according to the surrounding temperature.

Crude protein obtained from different groups (Fig. 3.4) were precipitated by ammonium sulphate and purified by combination of size exclusion and ion exchange chromatography. Total protein content and percent yield of each group after every step is shown in Table 3.1.
Table 3.1: Total protein content and % yield of each group after every step.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Types of PBP</th>
<th>Total PBP content after each purification step (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crude extract</td>
<td>70% ASC</td>
</tr>
<tr>
<td>Group I</td>
<td>PE</td>
<td>15.45</td>
<td>13.8</td>
</tr>
<tr>
<td>Group II</td>
<td>PC</td>
<td>16.1</td>
<td>14.1</td>
</tr>
<tr>
<td>Group III</td>
<td>PEC</td>
<td>16.2</td>
<td>13.9</td>
</tr>
</tbody>
</table>

Fig. 3.3: Growth pattern in terms of chlorophyll a and concurrent phycobiliprotein (PE and PC) content profile in Oscillatoria sp. N9DM from 0–60 days.

3.3.3 Characterization of biliproteins in response to temperature

3.3.3.1 UV-Vis and Fluorescence Spectroscopy

The absorption spectra of crude phycobiliproteins from all three groups as well as reverted culture were found different (Fig. 3.4). Proteins from group I culture grown for many generation at 30 ± 2°C showed peaks of very high absorbance at 570 nm, low absorbance at 620 nm and 650 nm corresponding to PE, PC and APC respectively (Fig. 3.4). Whereas group II culture grown for many generation at 55 ± 2°C showed a peak of high absorbance at 620 nm and a shoulder peak of low absorbance at 650 nm (Fig. 3.4) corresponding to PC and APC respectively.
However group I and II cultures grown for many generations at 42 ± 2°C (called group III culture) showed a peak of high absorbance at 570 nm and low absorbance at 620 nm (Fig. 3.4) corresponding to PE and PC respectively. Further reverted culture (sub-cultured from 55 ± 2°C culture) grown at 30 ± 2°C showed similar absorbance pattern as seen in group I culture (Fig. 3.4).

The absorption spectra of purified phycobiliproteins from all three groups as well as reverted culture were compared and found different. Protein purified from group I culture grown at 30 ± 2°C showed two sharp absorption peaks at 539 nm and 567 nm (Fig. 3.5a) and emission peak at 575 nm (Fig. 3.5b) corresponding to PE II and PEC (or PE I) respectively. Surprisingly protein purified from group II culture (grown at 55 ± 2°C), showed a sharp absorption peak of 611 nm (Fig. 3.5a) and emission peak at 646 nm (Fig. 3.5b) corresponding to PC. However, proteins purified from group III cultures (grown at 42 ± 2°C) showed absorption peaks at 567 nm and 611 nm (Fig. 3.5a) and emission peaks at 575 nm and 646 nm (Fig. 3.5b) corresponding to PEC (or PE I) and PC respectively. Further increase or decrease in temperature, surrounding the group III culture compelled the organism to produce either PE or PC. Hence we considered 42 ± 2°C is the critical temperature at which Oscillatoria sp. N9DM maintains itself as evolutionary plastic and able to produce PE or PEC or PC as and when required. Proteins purified from reverted culture (Freshly inoculated culture grown at 30 ± 2°C sub-cultured from group II cultures) showed absorption peaks at 567 nm and 611 nm and emission peak at 575 nm and 646 nm corresponding to PEC (or PE I) and PC respectively (Fig. 3.5a) rather than PC only. But from the second generation onwards PEC (or PE I) and PE II was produced by reverted culture, showed quick and sequential acclimation of isolated culture according to the surrounding environment.

3.3.3.2 Denaturing gel electrophoresis

Silver stained SDS-PAGE profile of group I cultures (30 ± 2°C) showed the presence of four bands having molecular masses 23, 20, 17.5 and 15 kDa corresponding to α and β subunit of PEC (or PE I) and PE II respectively (Fig. 3.6, Lane 1). Group II culture (55 ± 2°C) showed the presence of two band having molecular masses 20 and 17.5 kDa corresponding to α and β subunit of PC respectively (Fig. 3.6, Lane 3). Moreover group III cultures (42 ± 2°C) showed the
presence of three distinct bands having molecular masses 23, 20 and 17.5 kDa corresponding to α and β subunit of PEC (or PE I) (23 and 20 kDa) and PC (20 and 17.5 kDa) respectively (Fig. 3.6, Lane 2). Furthermore reverted cultures (Freshly inoculated culture grown at 30 ± 2°C sub-cultured from group II cultures) showed four bands having molecular masses of 23, 20, 17.5 and 15 kDa corresponding to α and β subunit of PEC (or PE I) and PC respectively (Fig. 3.6, Lane 4).

3.3.3.3 Zinc acetate staining
Bilins of the protein fluoresces orange in the presence of zinc ions under the UV radiation (Brekelman and Lagarias, 1986). This proved that bilin chromophore group were attached to the respective PBPs and makes them efficient for energy transduction (Fig. 3.6).

Results obtained from the growth experiment and phycobiliprotein content of group I and II culture, provide evidence that apart from affecting the physiology of photosynthesis, biliproteins especially PE which is found on the distal part of rod, is also utilised as carbon and nitrogen source in the nutrient depleted media (Parmar et al., 2011; Adir et al., 2001), hence group I culture maintained itself at stationary phase for some time. Once PE comes below the threshold limit to supply nutrients as carbon and nitrogen, cells enter in to death phase. On the other hand, PC found at the proximal part of rod, purified from group II culture was very much stable and doesn’t get degraded even in the nutrient depleted media because phycobilisome structure in thermotolerant organisms are stabilized by amino acid residue substitutions. Each substitution can form additional ionic interactions at critical positions of each associated interface (Adir et al., 2001). In addition, the number of patches of aromatic residues (Kannan and Vishveshwara, 2000) and a significant shift in the position of ring D of the B155 phycocyanobilin cofactor in phycocyanin, enables the formation of important polar interactions at both the (αβ) monomer and (αβ)₆ hexamer association interfaces could enhance thermal stability (Adir et al., 2001). Hence thermo stable PC in vivo carry out important function of energy transduction only as it is last resort to transfer energy up to core and some alternate pathway will fulfill the need of carbon and nitrogen during the nutrient depletion. To best of our knowledge, this is the first report of isolation and characterization of thermotolerant cyanobacteria, with respect to change in their
pigmentation to perform efficient photosynthesis in response to change in surrounding temperature.

3.3.4 UV-Vis Spectroscopic characterization of biliproteins in response to gradient of ammonium sulphate precipitation

Individual phycobiliprotein pigment from each group was obtained by gradient of ammonium sulphate precipitation and further purified by combination of IEC and GPC. Proteins purified from 40% ASC of group I culture showed a sharp absorbance peak at 567 nm and a shoulder peak at 539 nm corresponds to PE I and PE II respectively (Fig. 3.7a). However 50% ASC of group I culture showed a sharp absorbance peak at 567 nm and 611 nm corresponds to PE I and PC respectively (Fig. 3.7a). Further 60% and 70% ASC of group I culture showed a sharp absorbance peak at 567 nm, 611nm and a shoulder peak at 652 nm corresponds to PE I, PC and APC respectively (Fig. 3.7a).

Proteins purified from 50%, 60% and 70% ASC of group II culture showed a sharp absorbance peak at 611 nm and a shoulder peak at 652 nm corresponds to PC and APC respectively (Fig. 3.7b).

Proteins purified from 40% and 50% ASC of group III culture showed a sharp absorbance peak at 567 nm corresponds to PE I (Fig. 3.7c). However 60% ASC of group III culture showed a sharp absorbance peak at 567 nm and a shoulder peak at 611 nm corresponds to PE I and PC respectively (Fig. 3.7c) which suggests that at pigmentation changes through intermediate temperature.

Proteins purified from 60% ASC of reverted culture showed a sharp absorbance peak at 567 nm, 611 nm and 652 nm corresponds to PE I, PC and APC respectively (Fig. 3.7d).
Fig. 3.4: UV–Vis absorption overlay spectra of crude phycobiliproteins extracted from *Oscillatoria* sp. N9DM culture grown under different temperatures.
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Fig. 3.5: (a) UV–Vis absorption overlay spectra of phycobiliproteins purified from Oscillatoria sp. N9DM culture grown at different temperature in white light. Group I; Group II; Group III; 30 ± 2°C reverted culture [First generation grown at 30 ± 2°C sub-cultured from group II cultures]; (b) fluorescence emission overlay spectra of phycobiliproteins purified from different groups. Group I; Group II; Group III and Reverted culture.

Fig. 3.6: 15% (w/v) silver stained SDS-PAGE of purified PBPs. Lane M: protein molecular mass standard in kDa; Lane 1: PE I and PE II (Group I); Lane 2: PEC or PE I and PC (Group II); Lane 3: PC (Group III); Lane 4: PEC or PE I and PC (Reverted).
Fig. 3.7: UV–Vis absorption overlay spectra of phycobiliproteins (precipitated at 40-70% ammonium sulphate gradient) purified from (a) group I culture grown at 30 ± 2°C; (b) group II culture grown at 55 ± 2°C; (c) group III culture grown at 42 ± 2°C; (d) reverted culture (sub-cultured from either group I or II culture) grown at 30 ± 2°C.
3.3.5 Effect of light colour on biliprotein production

It is well known fact that some cyanobacteria can acclimatize to the quantity and quality of light present in their environment. Acclimation includes synthesis of specific polypeptides of light harvesting antenna complex or phycobilisomes and the process is called CCA. Effects of light colour on all three groups were carried out at 30 ± 2°C unless specified. Each group cultures acclimatised depending on the available wavelength and shows almost similar results as seen in CCA (Kehoe, 2010). Growth rate was found significant in white, red and yellow light.

3.3.6 Characterization of biliproteins in response to light colour

3.3.6.1 UV-Vis and Fluorescence Spectroscopy

The absorption spectra of purified biliprotein from group I culture grown under white light showed peaks at 539 nm and 567 nm \((\text{Fig. 3.8a})\) corresponding to PE II and PEC (or PE I) respectively. Whereas group I culture grown under yellow light, acclimatised itself and showed absorption peak at 565 nm and 611 nm \((\text{Fig. 3.8a})\) corresponding to PEC (or PE I) and PC respectively. Similarly group I culture grown under red light acclimatised itself and showed a sharp absorption peak at 611 nm \((\text{Fig. 3.8a})\) corresponding to PC. Group II culture grown under white light showed absorbance peak at 611 nm \((\text{Fig. 3.8b})\) corresponding to PC. Whereas group II culture sub-cultured and grown at 30 ± 2°C under yellow light showed absorption peaks at 555 nm and 611 nm \((\text{Fig. 3.8b})\) corresponding to PEC (or PE I) and PC respectively. Similarly group II culture sub-cultured and grown at 30 ± 2°C (i.e. reverted culture) under red light showed absorption peak at 611 nm \((\text{Fig. 3.8b})\) corresponding to PC. Group III culture grown under white light showed absorption peaks at 565 nm and 611 nm \((\text{Fig. 3.8c})\) corresponding to PEC or (or PE I) and PC respectively. Whereas group III culture sub-cultured and grown at 30 ± 2°C under yellow light showed absorption peaks at 565 nm and 611 nm corresponding to PEC (or PE I) and PC respectively (Fig. 3.8c).

The fluorescence emission spectra of group I culture under white light showed peak at 575 nm \((\text{Fig. 3.8d})\) corresponding to PE II and PEC (or PE I). Whereas group I culture grown under yellow light showed emission peak at 575 nm and 646 nm \((\text{Fig. 3.8d})\) corresponding to PEC (or PE I) (absorption peak at 565 nm) and PC respectively. Similarly group I culture grown under red light showed emission peak
at 646 nm (Fig. 3.8d) corresponding to PC. Group II culture grown under white light showed emission peak at 646 nm (Fig. 3.8d) corresponding to PC. Whereas group II culture sub-cultured and grown at 30 ± 2°C under yellow light showed emission peak at 575 nm and 646 nm (Fig. 3.8d) corresponding to PEC (or PE I) (absorption peak at 565 nm) and PC respectively. Similarly group II culture sub-cultured and grown at 30 ± 2°C (i.e. reverted culture) under red light showed emission peak at 646 nm (Fig. 3.8d) corresponding to PC. Group III culture grown under white light showed emission peak at 575 nm and 646 nm (Fig. 3.8d) corresponding to PEC (or PE I) (absorption peak at 565 nm) and PC respectively. Whereas group III culture sub-cultured and grown at 30 ± 2°C under yellow light showed emission peak at 575 nm and 646 nm (Fig. 3.8d) corresponding to PEC (or PE I) (absorption peak at 565 nm) and PC respectively.
Fig. 3.8: (a) UV–Vis absorption overlay spectra of PBPs purified from Group I, sub-cultured at: 30 ± 2°C, White light; 30 ± 2°C, Yellow light; 30 ± 2°C, Red light; (b) UV–Vis absorption overlay spectra of PBPs purified from Group II, sub-cultured at 55 ± 2°C, White light; 30 ± 2°C, Yellow light; 30 ± 2°C, Red light; (c) UV–Vis absorption overlay spectra of PBPs purified from Group III, sub-cultured at 42 ± 2°C, White light; 30 ± 2°C, Yellow light; (d) fluorescence emission overlay spectra of PBPs purified from cultures grown at 30 ± 2°C at different wavelengths. --- White; --- Red and --- Yellow light.
3.3.6.2 Denaturing gel electrophoresis

Silver stained SDS-PAGE of purified PE obtained from group I culture grown under white light showed the presence of four bands having molecular masses 23, 20, 17.5 and 15 kDa corresponding to α and β subunits of PEC (or PE I) and PE II respectively (Fig. 3.9, Lane 1). Whereas group I culture grown under yellow light showed the presence of three prominent bands having molecular masses 23, 20 and 17.5 kDa corresponding to α and β subunit of PEC (or PE I) (23 and 20 kDa) and PC (20 and 17.5 kDa) respectively and one faint band of molecular weight 15 kDa corresponding to gradually degrading β subunit of PE II (Fig. 3.9, Lane 2). Furthermore group I culture grown under red light showed the presence of two bands having molecular masses 20 and 17.5 kDa corresponding to α and β subunit of PC respectively (Fig. 3.9, Lane 3). Protein purified from group II culture grown under white light showed the presence of two band having molecular masses 20 and 17.5 kDa corresponding to α and β subunit of PC respectively (Fig. 3.9, Lane 4). But when the group II culture was grown under yellow light at 30 ± 2°C, showed the presence of three distinct bands having molecular masses 23, 20 and 17.5 kDa corresponding to α and β subunit of PEC (or PE I) (23 and 20 kDa) and PC (20 and 17.5 kDa) respectively and one faint band of molecular weight 15 kDa corresponding to newly synthesised β subunit of PE II (Fig. 3.9, Lane 5). Further same culture grown under red light at 30 ± 2°C, showed the presence of two distinct bands having molecular masses 20 and 17.5 kDa corresponding to α and β subunit of PC respectively (Fig. 3.9, Lane 6). Proteins purified from group III cultures (42 ± 2°C) showed the presence of three distinct bands having molecular masses 23, 20 and 17.5 kDa corresponding to α and β subunit of PEC (or PE I) (23 and 20 kDa) and PC (20 and 17.5 kDa) respectively and one faint band of molecular weight 15 kDa corresponding to either newly synthesised or gradually degrading β subunit of PE II (Fig. 3.9, Lane 7).

3.3.6.3 Zinc acetate staining

Bilins of the protein fluoresce orange in the presence of zinc ions under the UV radiation (Brekelman and Lagarias, 1986). This proved that bilin chromophore groups were attached to the respective PBPs and makes them efficient for energy transduction (Fig. 3.9).
Here we found group I and group II cultures change their pigmentation in response to temperature and shows resemblance to CCA. The newly isolated culture Oscillatoria sp. N9DM changed both its PE and PC levels in response to quality of light to perform efficient photosynthesis; hence we placed this culture in GROUP III chromatic adaptors (Tandeau de Marsac, 1977; Kehoe, 2010). Absorption (565 nm and 611 nm) and emission (646 nm) peaks of phycobiliproteins purified from cultures grown in yellow light as well as at intermediate temperature (group III culture) corresponded to PEC (or PE I) and PC. Similarly cultures grown in red light as well as at high temperature (group II culture), produced to PC only. These finding showed a strong correlation between light color and respective temperatures. Correlation in production of identical pigmentation pattern at specific wavelength and temperature provided evidence that, in some cyanobacterial species function of complementary colours can also be fulfilled by range of temperatures. On the basis of this evidence we hypothesise that CCA and
thermoadaptation may have evolved simultaneously during the course of evolution. But long term exposure of constant temperature, mesophilic cyanobacteria adapt themselves according to the respective complementary colour only. Whereas thermotolerants, like Oscillatoria conserve the phenomenon of thermoadaptation due to variable temperature of hot springs from its origin, along with CCA. The mechanism of pigmentation mediated by thermo-adaptation is not well understood but again we hypothesise that PE synthesis was halted by very similar transcriptionally attenuated pathway called Cgi (Control of green light) system (Bezy et al., 2011) and its interaction with Rca (Regulation of complementary chromatic adaptation) system (Kehoe and Gutu, 2006).

The experimental system described in this chapter has opened up an opportunity to study the molecular phenomenon that occurs during the thermo-adaptation. For the first time our results demonstrate that high temperature tolerance benefitted photosynthetic performance of Oscillatoria N9DM sp., in in vivo and in vitro by changing antenna molecule pigmentation pattern. This experimental evidence might give us insights into understanding of numerous biological changes under different stress conditions (Banerjee et al., 2012; Ehira and Ohmori, 2012).

3.4 Conclusion

In the present study two thermotolerant cyanobacterial strains N9DM I and N9DM II were collected from Tuwa geothermal spring, Panchmahal, Gujarat, India, from different wells having temperature 30 ± 2°C and 55 ± 2°C respectively. Analysis of 16S rRNA gene sequence revealed that both the strains were nearest neighbour of Oscillatoria sp. In addition to high temperature tolerance Oscillatoria N9DM sp. changes phycobilisome rod pigmentation when exposed to various temperatures. Oscillatoria N9DM sp. produces phycoerythrin (PE) and phycocyanin (PC) at 30 ± 2°C and 55 ± 2°C, respectively whereas at intermediate temperature (42 ± 2°C) it produced phycoerythrocyanin (PEC). Moreover reverted cultures (Normally grown at 55 ± 2°C but now sub-cultured and grown at 30 ± 2°C) started producing PE. Furthermore, Oscillatoria N9DM sp. produced PE, PC and PEC in white, red and yellow light respectively and it showed direct correlation to different proteins produced at various temperatures. To best of our knowledge, this is the first report describing correlation of temperature and its effect on the
Chapter 3: Isolation, identification and phycobiliprotein production. This study for the first time also tried to explain that thermo-adaptation might have been evolved along with complementary chromatic adaptation (CCA) in certain thermotolerant cyanobacteria where fluctuation of temperature was prevalent throughout the year.
3.5 References


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