2.1 Introduction

Different pigments are employed by photosynthetic organisms to efficiently harvest the spectrum of light energy that drives photosynthesis. There are many reports of cyanobacteria modulating their relative pigment content in response to changes in light quality as well as light intensity (Raps et al., 1983; Anderson et al., 1983; Tandeau de Marsac and Houmard, 1988). In cyanobacteria, light harvesting is carried out primarily by a group of pigment proteins, called phycobiliproteins, that constitute a macromolecular complex called the phycobilisome (Gantt, 1975; Glazer et al., 1983; Zuber, 1986; Bryant, 1991). As reported by Tandeau de Marsac and Houmard (1988), one observes an inverse correlation between light intensity and pigment content: the less energy available, the more photosynthetic pigments are synthesized by the cells. Positive influence of the spectral band on the pigment content of the cells, termed complementary chromatic adaptation, appears to be restricted to some cyanobacteria. In this type of adaptation, changes in cell pigmentation, in response to specific spectral illuminations, result from modifications of the relative amounts of red coloured phycoerthrin (PE) and the blue coloured phycocyanin (PC), with a predominance of PE in green light and of PC in red light grown cells.

Complementary chromatic adaptation is known to occur only in PE producing cyanobacteria. Among the PE producers, three physiological groups can be distinguished: group I, no complementary chromatic adaptation, PE and PC synthesis is independent of light wavelength, group II, unidirectional adaptation, only the synthesis of PE is regulated by light wavelength, group III, bi-directional or complete complementary chromatic adaptation (Tandeau de Marsac, 1977).

According to a review by Grossman et al., (1993) depending on the strain, cyanobacteria can tolerate direct sunlight (~ 1700 $\mu Em^{-2}s^{-1}$) or require PPFD as low as

~1-2 $\mu Em^{-2}s^{-1}$. Most cyanobacteria can adapt to 50-200 $\mu Em^{-2}s^{-1}$ and maximum growth rates (μ_{max}) are reached with a PPFD of about 50-60 $\mu Em^{-2}s^{-1}$. As a general rule, small unicellular species and filamentous strains with a narrow cell diameter have the highest μ_{max}. Because of the selective absorption of radiation above 550 nm, by the upper water layers and water turbidity, almost no radiation above 600 nm can reach a depth of 4 m; photon flux density is thus, an important parameter to consider, since it has a major influence on growth and metabolism of many cyanobacteria.

Cyanobacteria vary their pigment content and other physiological aspects in such a way, so as to optimize their performance, during exposure to different part of spectra and irradiance of light. The present study aims to test the above hypothesis by determining the growth rates and photosynthetic pigment contents of the three selected cyanobacteria, *A. indica*, *P. tenue* and *L. limnetica* cultured under different intensities of light (4, 10 and 16 $\mu Em^{-2}s^{-1}$) and different qualities of light [(white (400-750 nm), red (620-700 nm), blue (430-500 nm) and green light (500-570 nm)].

2.2 Materials and Methods

***Materials*:**

Standard protein molecular weight markers were obtained from Sigma-Aldrich Co., USA. All other reagents used were of analytical grade available from commercial sources and used without further purification.

Methods:

2.2.1 *Quantification of Chl 'a' and Phycobiliproteins*

Fresh water strain, *A. indica*, cultured in Zarrouk's medium and the two marine strains, *P. tenue* and *L. limnetica* cultured in ASN-III medium (section 1.2.1) were grown under different light intensities (4, 10 and 16 $\mu Em^{-2}s^{-1}$) and different light qualities [(white (400-750 nm), red (620-700 nm), blue (430-500 nm) and green light

(500-570nm)]. The different light qualities were provided by wrapping the culture tubes with different coloured cellophane papers. All the experiments were carried out in duplicates and the graphical data represents the mean values. Cultures were harvested every seven days for 5 weeks, by centrifugation at 10000 xg for 30 min at 4°C.

For PB extraction the harvested cell mass was suspended in 100 mM Na-Phosphate buffer (pH 7.0) and the cell mass was disrupted by sonication for 20 s. Repeated cycles of freezing (–195°C) and thawing at room temperature (23±1°C) were carried out till complete extraction was done, which was followed by centrifugation at 10000 xg for 30 min at 4°C and the clear supernatant containing PB was collected.

Estimation of PB

The absorbance values of the phycobiliprotein containing supernatant were measured on a CARY 500 Scan UV-Vis, NIR spectrophotometer at 620, 652 and 562 nm for calculating the concentrations of PC, APC and PE, respectively, with the following equations (Bennett and Bogorad 1973, Subramanian et al., 1994)

$\text{C-PC (mg/ml)} = [A_{620} - 0.474(A_{652})] / 5.34$

$\text{APC (mg/ml)} = [A_{652} - 0.208(A_{620})] / 5.09$

$\text{PE (mg/ml)} = [A_{562} - 2.41(\text{PC}) - 0.849(\text{APC})] / 9.62$

For Chl ‘*a*’ extraction the cell mass was suspended in 90% methanol and the extraction was carried out twice at 4°C for an hour, in the dark, followed by centrifugation at 10000 xg for 10 min at 4°C. Methanolic extract obtained as supernatant was collected for further estimation.

Estimation of Chl ‘a’

Estimation of chl ‘*a*’ was done as mentioned in section 1.2.2.

2.2.2 *Spectral Analysis*:

UV-VIS absorption spectroscopy

To study the spectral nature of PB, under different growth conditions, the cells were grown at different light conditions as mentioned in section 2.2.1 and were harvested in the log phase. Extraction was done as mentioned in section 2.2.1 and the spectra recorded in UV-Vis range with respect to Na-phosphate buffer as blank. All UV-Vis absorption spectra were recorded on a CARY 500 Scan UV-Vis, NIR spectrophotometer with 1 cm path length.

FT-IR Spectra

The PB extracted from the cells in the log phase was dialyzed against water at 4°C for 48 hrs and then freeze dried by Virtis Freeze mobile 8EL and the spectras were recorded on a Perkin Elmer Spectrum GX FT-IR spectrophotometer as KBr pellet.

2.2.3 *Gel Electrophoresis*:

The PB extracted from cells grown under different nitrate conditions were electrophoresed by SDS-PAGE (15%) (Sambrook et al., 1989). Samples were heated for about 5 min at 95°C with 2% (w/v) SDS, 10% (v/v) glycerol, 4.5% (v/v) β-mercaptoethanol, 0.25% (w/v) bromophenol blue and 60 mM Tris (pH 6.8) for about 5 min at 95°C. Gels were run at 20±2°C, visualized by silver staining (Wray et al., 1981) and photographic documentation was done. The molecular weight of the separated linkers and subunits were determined by calibrating the gel with molecular weight markers.

2.3 Results

2.3.1 *Growth Curves and PB content*:

The unique physiological and morphological features of cyanobacteria and their diverse habitats indicate that, they may possess strategies for photosynthetic acclimatization to fluctuations in irradiance and quality of light available to them due to environmental factors.

Higher intensity of 16 $\mu Em^{-2}s^{-1}$ seems to be detrimental for the growth of *A. indica*. The doubling time increases to ~16 hrs under 16 $\mu Em^{-2}s^{-1}$ (Table 2.1). The lowest (~ 6 hrs) was observed in cells grown under 10 $\mu Em^{-2}s^{-1}$. As can be seen in Fig.2.1a highest concentration of PC is observed in *A. indica* grown under 10 $\mu Em^{-2}s^{-1}$. But PC content decreases rapidly in cells grown under 16 $\mu Em^{-2}s^{-1}$. APC concentration also decreases under 16 $\mu Em^{-2}s^{-1}$ but is almost equal in cultures grown under 4 and 10 $\mu Em^{-2}s^{-1}$ (Fig.2.1b). Light quality does not seem to affect the growth of *A. indica* to a considerable extent, though the doubling time seems to prolong under green light with white and red light showing similar results. PB content is also not largely affected by light quality, but an increase in PC content is observed under red light (Fig.2.2a). APC/PC ratio is highest in blue light, but data analysis shows that it is not due to rise in APC content but more due to decreased PC content. Highest APC content is found in culture grown under white light (Fig.2.2b).

Table 2.1: Doubling Time* of the three strains under different light conditions.#

A. indica	***L. limnetica***	***P. tenue***
~ 13 hrs (*4 $\mu Em^{-2}s^{-1}$*)	~ 13 hrs (*4 $\mu Em^{-2}s^{-1}$*)	~ 11 hrs (*4 $\mu Em^{-2}s^{-1}$*)
~ 6 hrs (*10 $\mu Em^{-2}s^{-1}$*)	~ 6 hrs (*10 $\mu Em^{-2}s^{-1}$*)	~ 7 hrs (*10 $\mu Em^{-2}s^{-1}$*)
~ 16 hrs (*16 $\mu Em^{-2}s^{-1}$*)	~ 18 hrs (*16 $\mu Em^{-2}s^{-1}$*)	~ 20 hrs (*16 $\mu Em^{-2}s^{-1}$*)
~ 9 hrs (*white light*)	~ 13 hrs (*white light*)	~ 14 hrs (*white light*)
~ 11 hrs (*red light*)	~ 16 hrs (*red light*)	~ 13 hrs (*red light*)
~ 12 hrs (*blue light*)	~ 21 hrs (*blue light*)	~ 13 hrs (*blue light*)
~ 16 hrs (*green light*)	~ 17 hrs (*green light*)	~ 13 hrs (*green light*)

*** The doubling time is expressed as approx. values as decimals have been eliminated from the values displayed.**
 # Light conditions are shown in the bracket.

As in *A. indica*, in *L. limnetica* too, the optimum growth was observed in cells cultured under 10 μEm^{-2}s^{-1} with a doubling time of 9 hrs and 16 μEm^{-2}s^{-1} seems to have a damaging effect on the culture, showing a doubling time of 18 hrs. Light quality does not seem to influence the growth to a large extent, though white light proves to be the best, showing the lowest doubling time of ~12 hrs and blue light seems to show an increased doubling time of ~21 hrs (Table 2.1). As seen in Fig.2.3a PC concentration is not much affected within the range of 10-16 μEm^{-2}s^{-1}, though it has a pronouncing effect on the concentrations of APC and PE. 10 μEm^{-2}s^{-1} proves to be the optimum light intensity for APC production, whereas, 4 μEm^{-2}s^{-1} delivers a higher content of PE. But, both the pigments show a sharp decline in the concentration when cells are grown under 16 μEm^{-2}s^{-1} (Fig.2.3b&c). Light quality affects differentially the rates of PE and PC synthesis in *L. limnetica* to a greater extent as compared to *A. indica* and *P. tenue*. No large differences were observed in PC and APC content under different light quality (Fig.2.4), but PE content seems to greatly increase under green light and the lowest PE content was observed in white light (Fig.2.4c). Thus, also the PE:PC ratio is higher under green light as compared to the other conditions.

P. tenue when grown under 16 μEm^{-2}s^{-1} shows a high doubling time of ~20 hrs but 10 μEm^{-2}s^{-1} again proves to be favourable showing a doubling time of ~7 hrs. *P. tenue* also shows a prominent differences in the PB content when grown under different light intensities. All the three pigments showed highest concentration in the cells cultured under 10 μEm^{-2}s^{-1} and a steep downward curve is observed in all the three pigments when grown under 16 μEm^{-2}s^{-1} (Fig.2.5). No large difference in the doubling time was observed, when *P. tenue* was grown under different parts of light spectra. Though, the different quality did not induce a striking difference in PC

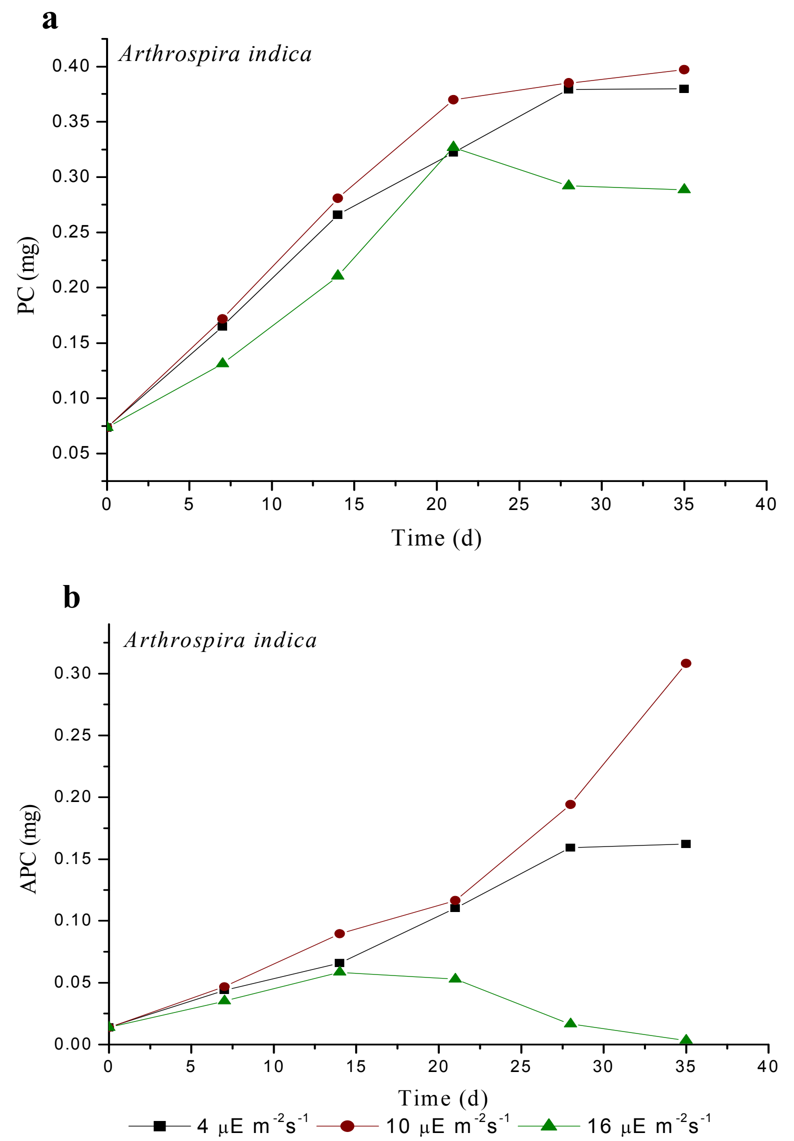

Fig. 2.1(a-b) Variation in PB content under different light intensities in *A. indica*

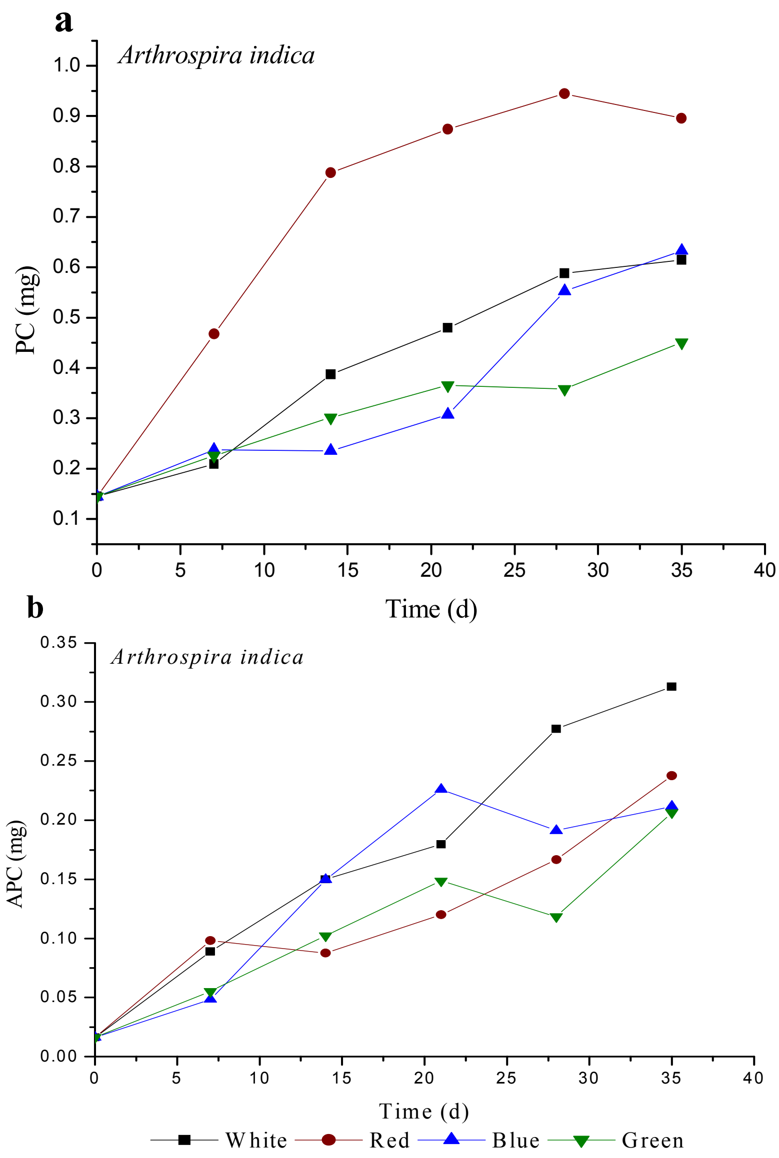

Fig. 2.2(a-b) Variation in PB content under different spectral region in *A. indica*

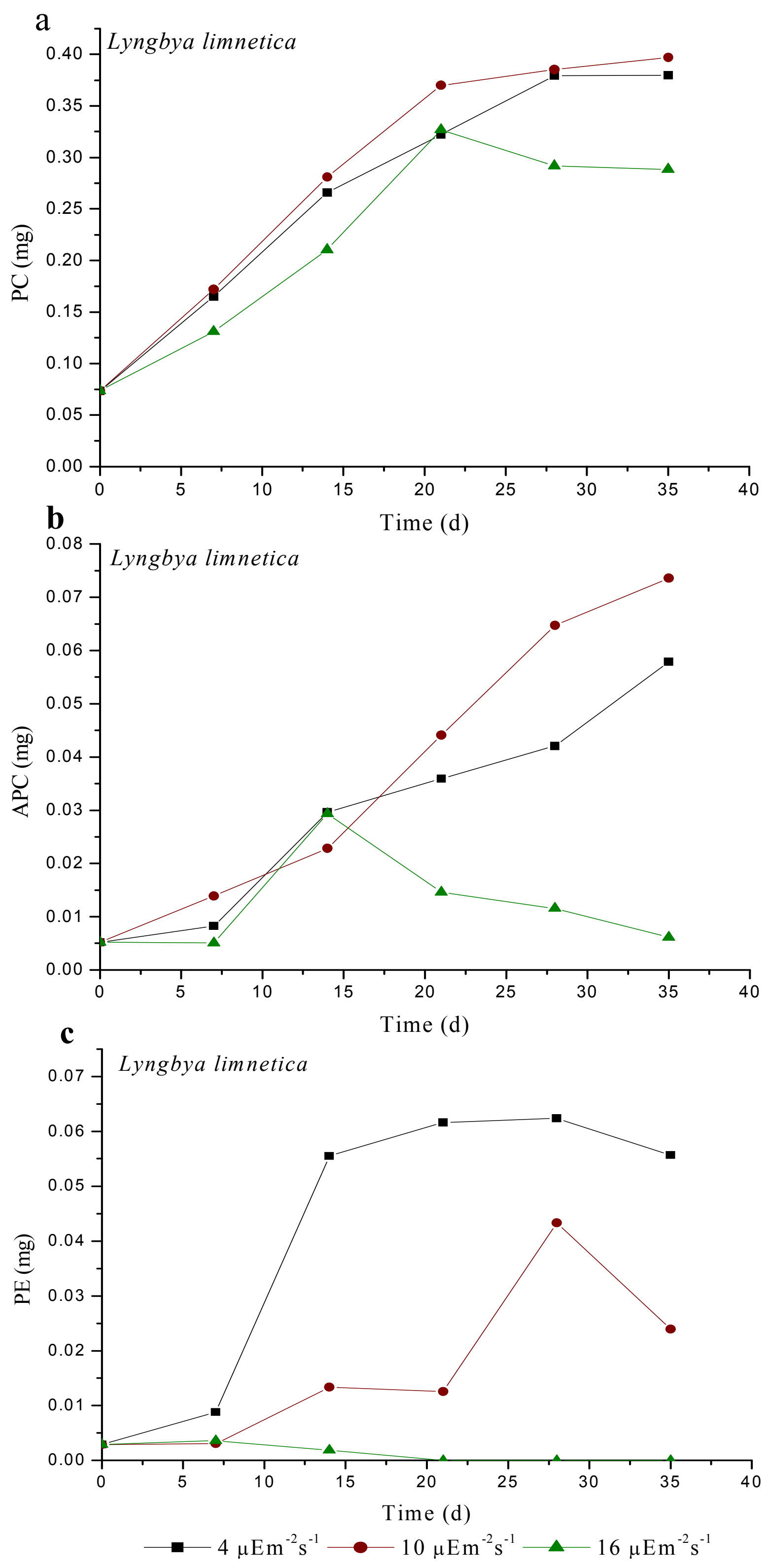

Fig. 2.3(a-c) Variation in PB content under different light intensities in *L. limnetica*

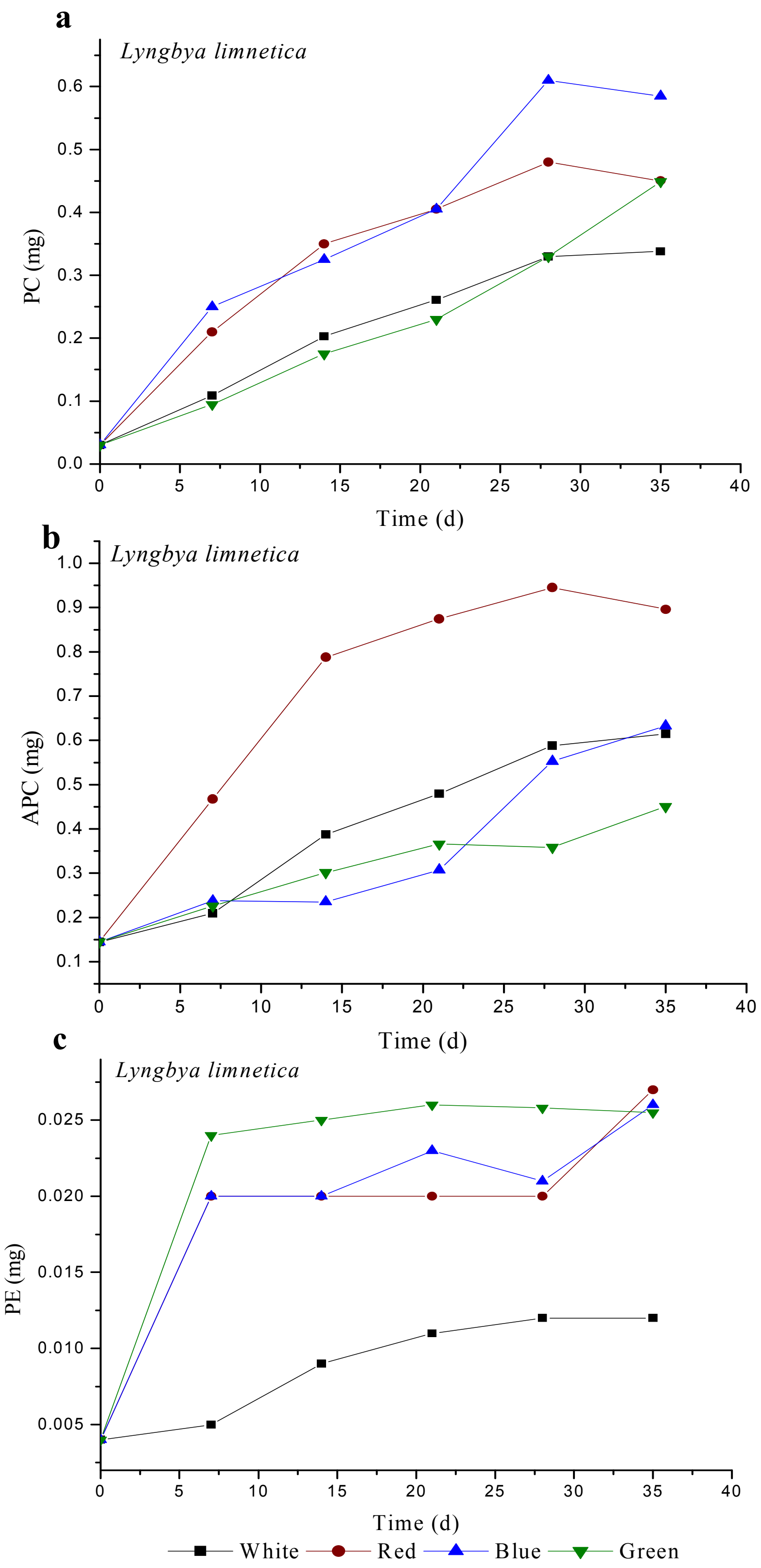

Fig. 2.4(a-c) Variation in PB content under different spectral region in *L. limnetica*

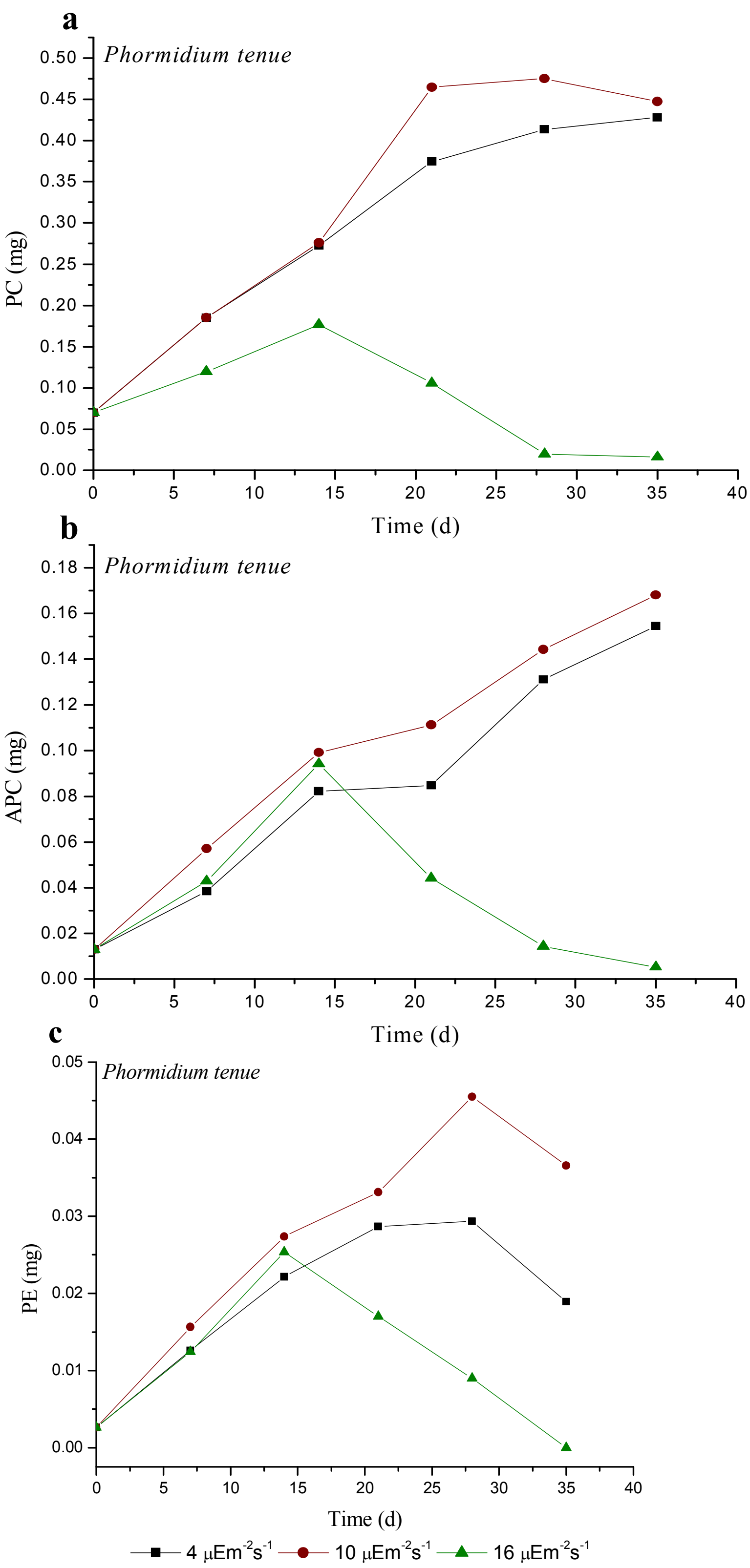

Fig. 2.5(a-c) Variation in PB content under different light intensities in *P. tenue*

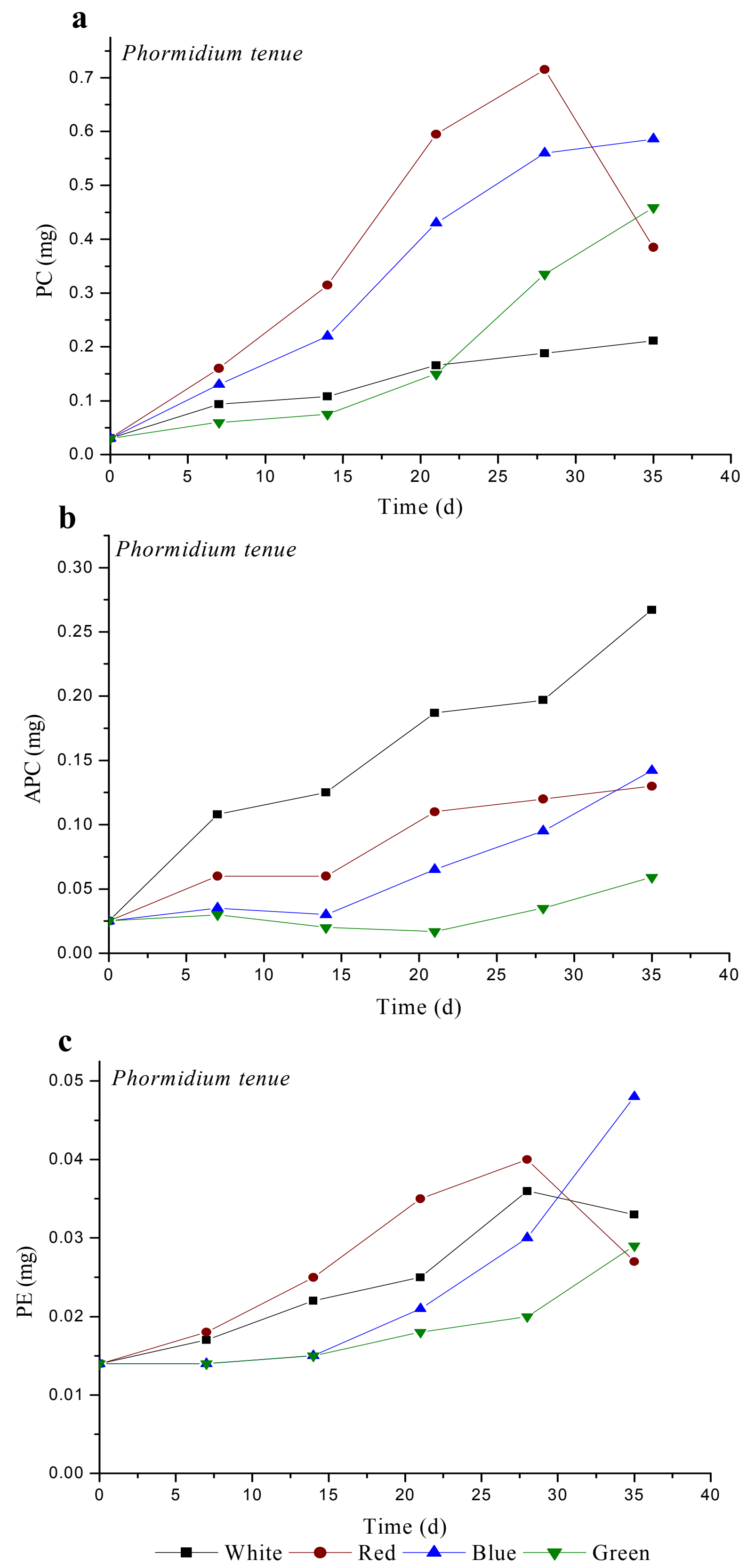

Fig. 2.6(a-c) Variation in PB content under different spectral region in *P. tenue*.

content but a significant difference was observed in APC content (Fig.2.6b). Highest APC content was observed in cultures grown under white light and least in cultures grown under green light. Light quality does not seem to have a considerable effect on PE concentration (Fig.2.6c).

2.3.2 S*pectral Studies*:

Absorption spectra of PB extract from all the three test cultures showed intense absorption peaks at ~620 nm, which is characteristic of PC, the major component of PB extract, and a relatively weak absorption peak at ~350 nm in visible region. Both of these peaks are due to the properties of phycocyanobilin chromophore (Patel, 2004). The present study reports a comparative evaluation of spectral behaviour of PB extract within a specified range of light intensity and spectral quality.

The absorption spectra of PB extract of *A. indica*, as a function of light intensity, is shown in Fig.2.7a. There is not much difference in the absorption maxima when the cultures are grown under 4 or 16 $\mu Em^{-2}s^{-1}$ but when grown under 16 $\mu Em^{-2}s^{-1}$ the cells show a blue shift of ~2 nm. Though, the PC content seems to be low in 16 $\mu Em^{-2}s^{-1}$, yet the protein content remained as high as in 10 $\mu Em^{-2}s^{-1}$, this can be interpreted by the peak, it has at ~280 nm, which is a general protein peak due to its aromatic amino acids. No large differences are observed in the absorption spectra of PB extract of *A. indica* grown under white, red and blue light, 'green light' PB extract shows a blue shift of ~5 nm. A slight shoulder at ~652 nm is observed in all the 3 absorption spectra which is characteristic of APC (Fig.2.8a).

In *L. limnetica* too, no difference in the λ_{max} is observed in absorption spectra of PB extract from cells grown under 4 and 10 $\mu Em^{-2}s^{-1}$ but in case of 16 $\mu Em^{-2}s^{-1}$ a blue shift of ~8 nm is observed which is a considerable shift with a λ_{max} at 610 nm (Fig.2.7b). Different light quality does not seem to affect the absorption spectra of PB

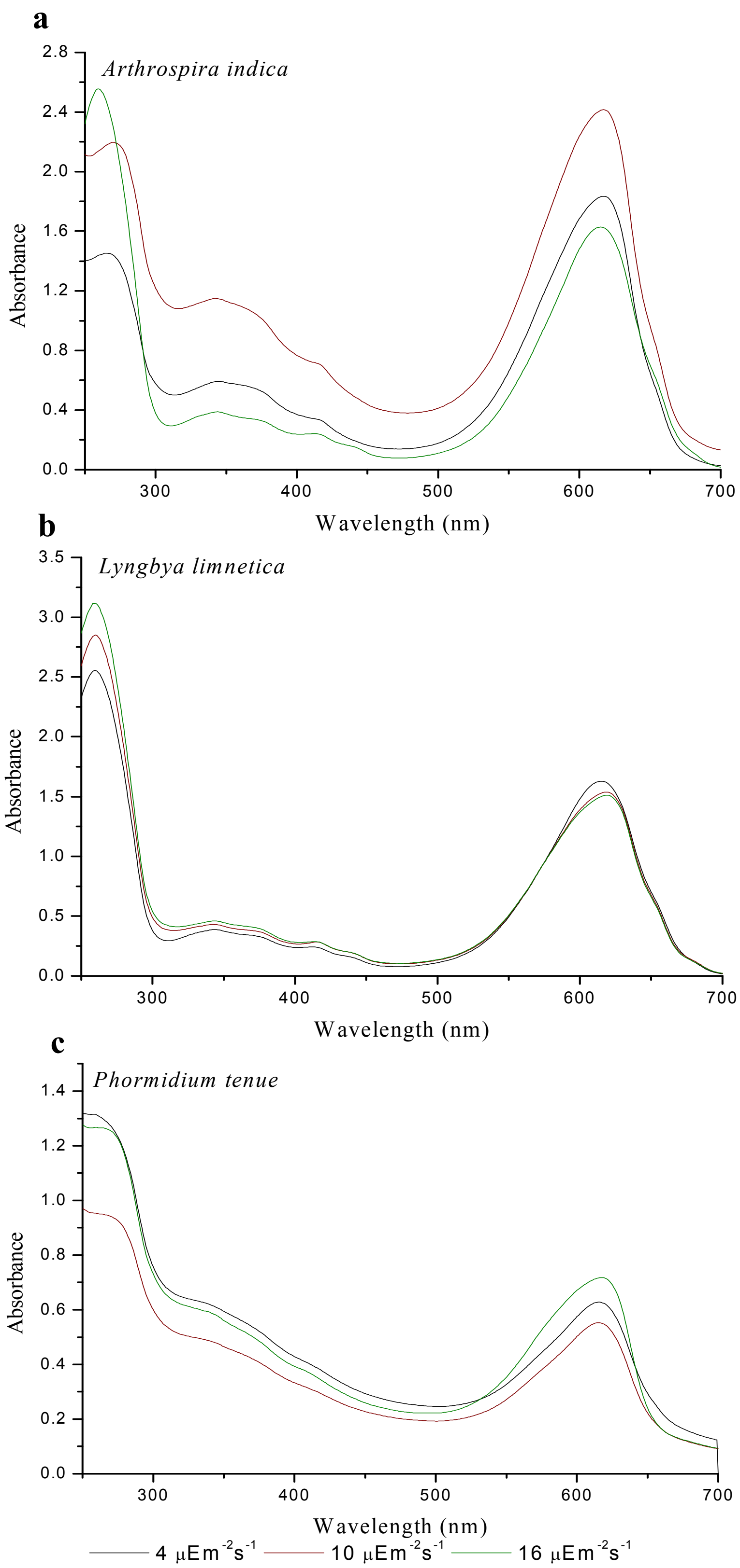

Fig. 2.7(a-c) Absorption spectra of PB extract under different light intensities.

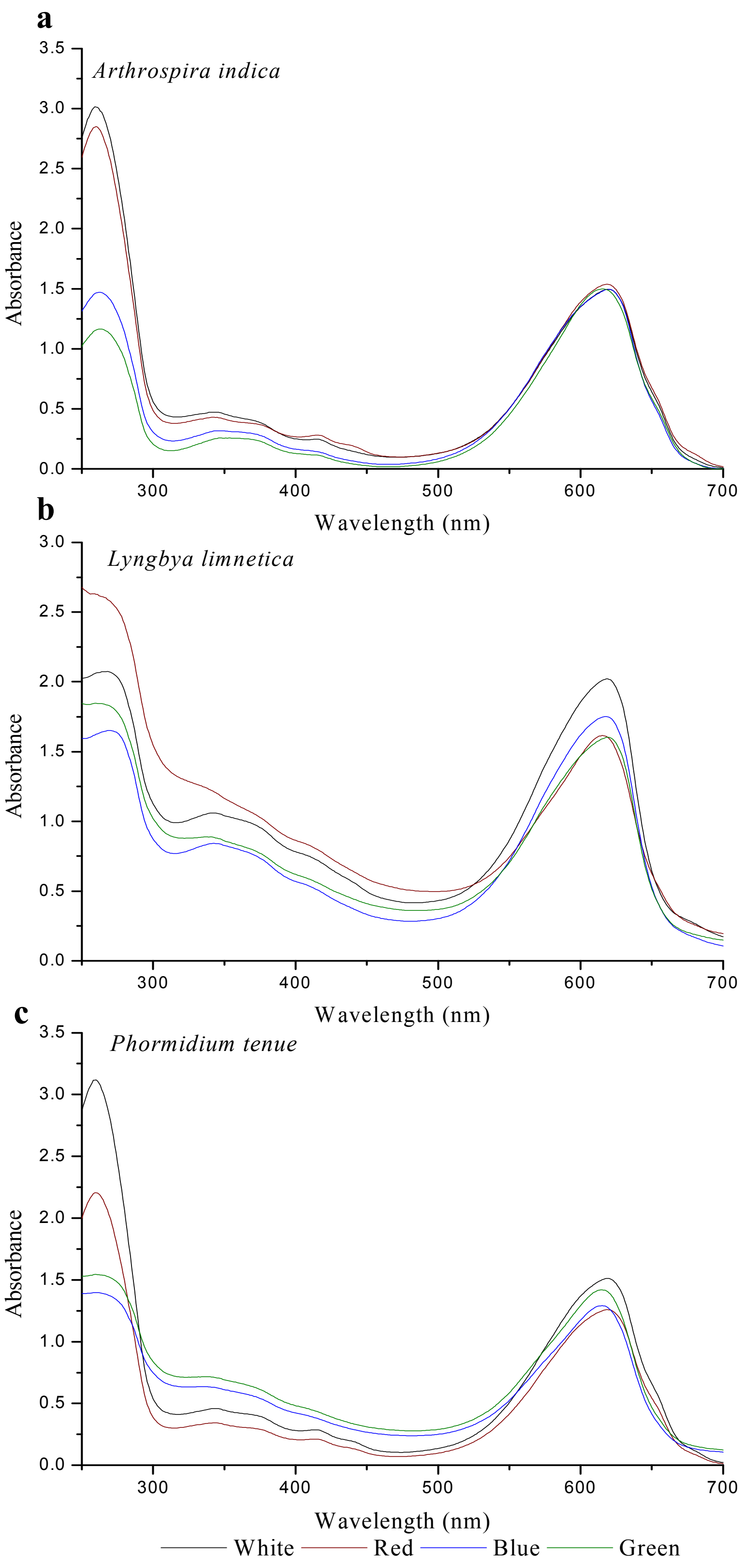

Fig. 2.8(a-c) Absorption spectra of PB extract under different spectral region.

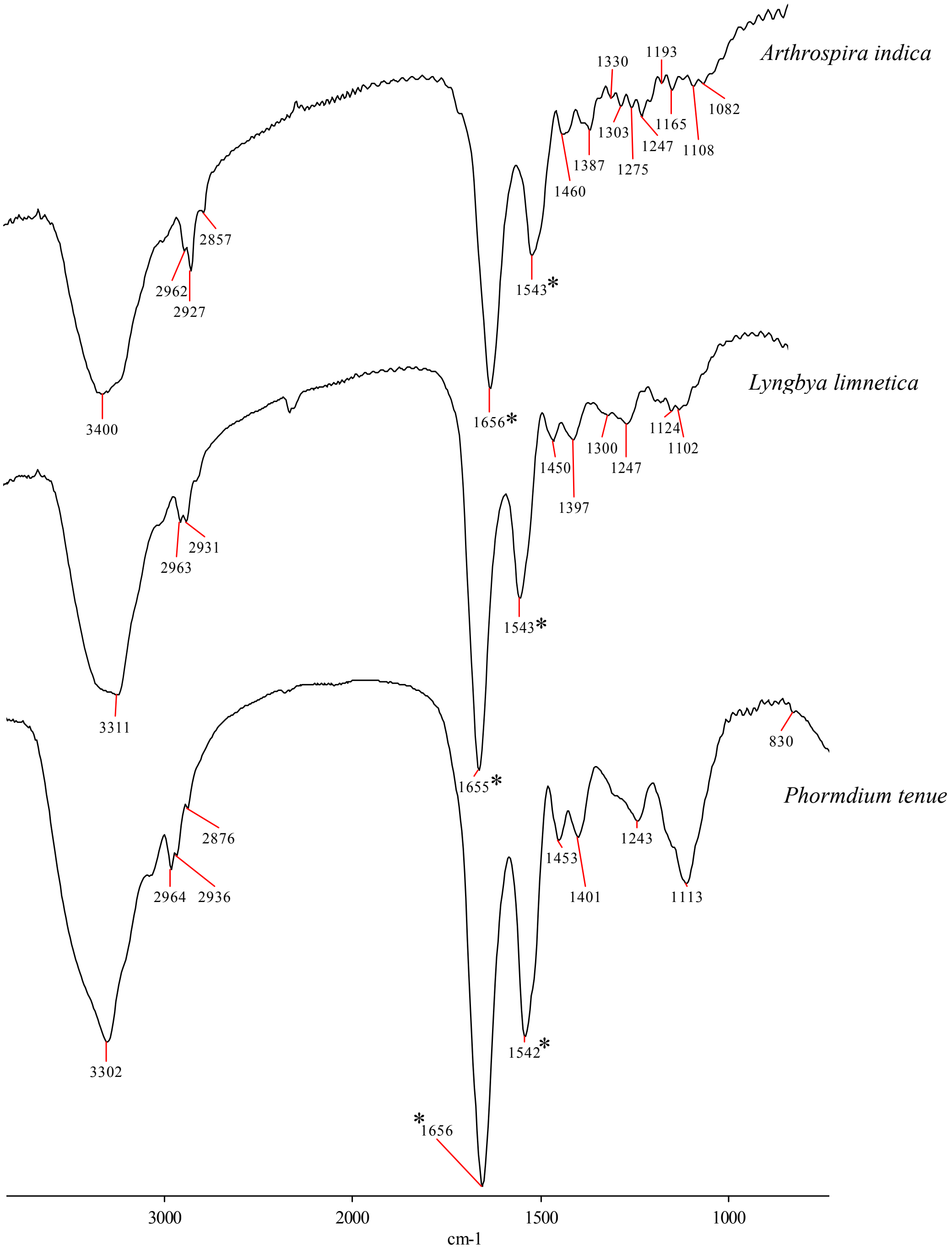

Fig. 2.9 FT-IR Spectra of PB extract (* Amide peaks)

extract of *L. limnetica*. A slight shoulder at ~570 nm is observed in all the four spectra which is characteristic of PE. No striking difference in the λ_{max} of the four spectra was observed (Fig.2.8b).

Table 2.2 Absorption maxima of PB extract in visible region under different nitrate conditions[#].

A. indica	***L. limnetica***	***P. tenue***
620 nm (*4 $\mu Em^{-2}s^{-1}$*)	619 nm (*4 $\mu Em^{-2}s^{-1}$*)	620 nm (*4 $\mu Em^{-2}s^{-1}$*)
619 nm (*10 $\mu Em^{-2}s^{-1}$*)	619 nm (*10 $\mu Em^{-2}s^{-1}$*)	619 nm (*10 $\mu Em^{-2}s^{-1}$*)
618 nm (*16 $\mu Em^{-2}s^{-1}$*)	610 nm (*16 $\mu Em^{-2}s^{-1}$*)	619 nm (*16 $\mu Em^{-2}s^{-1}$*)
619 nm (*white light*)	619 nm (*white light*)	619 nm (*white light*)
620 nm (*red light*)	620 nm (*red light*)	618 nm (*red light*)
619 nm (*blue light*)	620 nm (*blue light*)	619 nm (*blue light*)
615 nm (*green light*)	619 nm (*green light*)	616 nm (*green light*)

 # Light conditions are shown in the bracket

The absorption maxima in *P. tenue* under all the three conditions of light intensity does not seem to be greatly affected. A difference of only 1 nm is observed in the λ_{max} under all the three conditions as seen in (Fig.2.7c). Green light does seem to affect the λ_{max} of PB extract from *P. tenue*. Green light induces a blue shift of ~3 nm. No prominent differences was observed in the λ_{max} in PB extract from the cells grown under white, red and blue light. As in *L. limnetica* a slight shoulder was also observed at ~570 nm (Fig.2.8c)

FT-IR spectra of the different samples showed no major variation. (Fig.2.9)

2.3.3 *Protein Profiling*:

The assembly of phycobiliproteins is dependent on a group of polypeptides named linker polypeptides. The linkers are tightly associated with the biliproteins and their separation has involved gel electrophoresis under strongly denaturing conditions. Certain changes in the relative concentrations of the polypeptides, induced by light are revealed in the banding pattern obtained on electrophoresing the PB extract on a polyacrylamide gel in presence of sodium dodecyl sulphate, a denaturing agent.

Typical electrophoretic patterns, obtained with PB extracts of the three test strains grown under different light intensities and different parts of light spectra are presented in Fig.2.10a and Fig.2.10b, respectively.

Prior to staining, no more than four blue coloured bands corresponding to the α and β phycocyanobilin bearing subunits of both APC and PC were detectable. The subunits were not very clearly resolved on the gels. Core membrane linker (L_{cm}) denotes the largest, multifunctional linker in the PBS which also aids in attachment of the phycobilisome to the thylakoid membrane. According to the banding pattern the L_{cm} in *P. tenue* and *L. limnetica* seems to be of 75 kD and the one in *A. indica* seems to be of 99 kD. Another high molecular peptide is also observed in *A. indica* of around 170 kD.

Cyanobacteria generally increase their cellular contents of antenna proteins and pigments in response to low light intensity (Sidler, 1994) and high light intensity seems to lead to protein degradation affecting PBS as well. Though the " Light intensity samples" have not resolved very well on the gel, yet it can be noted that, many linker peptides especially belonging to group II (30 kD-70 kD) and group III (25 kD-30 kD) seem to disappear in '16 $\mu Em^{-2}s^{-1}$' PB extract. In *A. indica* the number of linkers seem to rise to 16 in green light as compared to white and red light which has 13 clear linkers. In *L. limnetica* the number of linkers is not much affected with all the samples showing ~13 linkers clearly. The electrophoretic pattern for extracts of

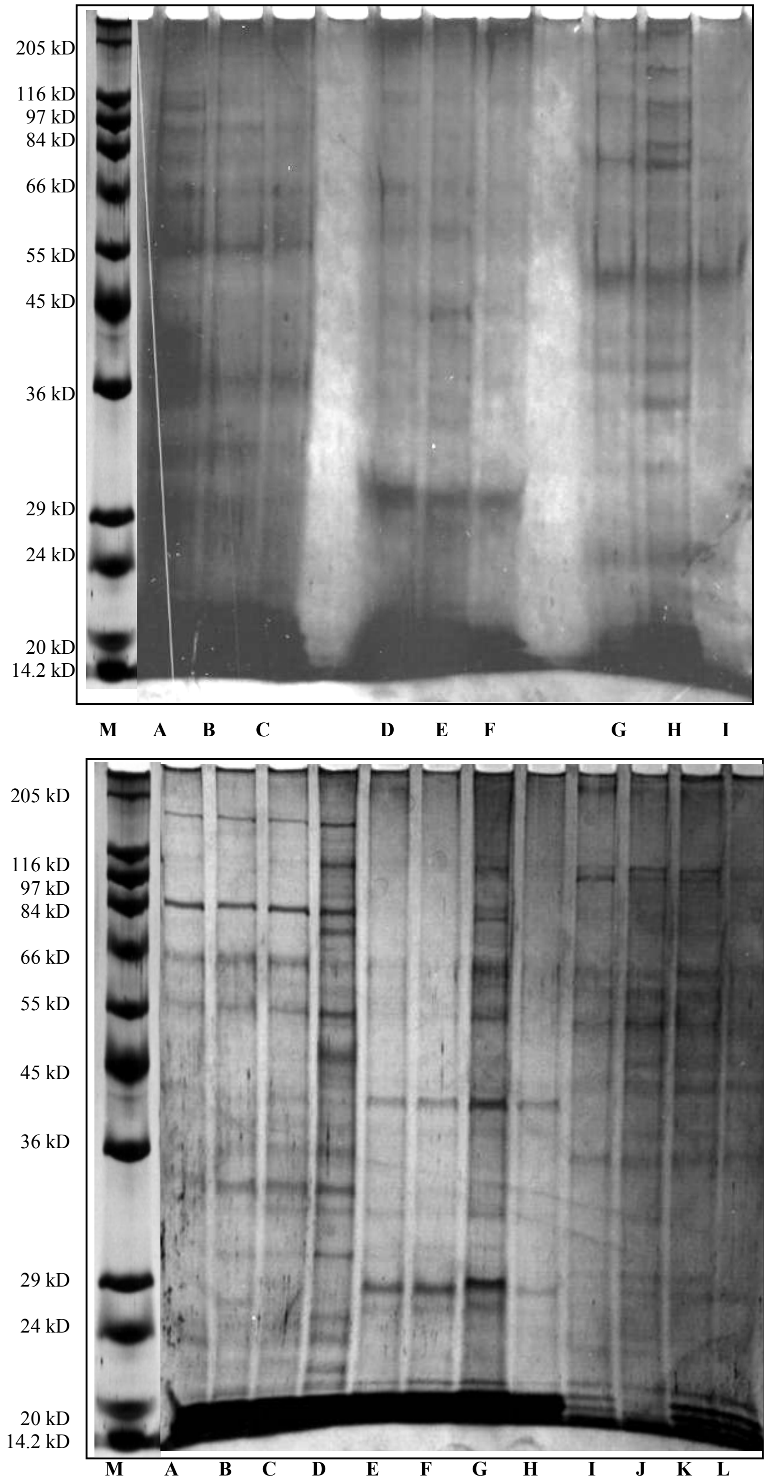

Fig.2.10 Protein profile of PB extract of cultures grown under different light conditions. **(a)** LaneA: *A. indica* (4 μEm^{-2}s^{-1}), LaneB: *A. indica* (10 μEm^{-2}s^{-1}), LaneC: *A. indica* (16 μEm^{-2}s^{-1}), LaneD: *L. limnetica*(4 μEm^{-2}s^{-1}), LaneE: *L. limnetica* (10 μEm^{-2}s^{-1}), LaneF: *L. limnetica* (16 μEm^{-2}s^{-1}), LaneG: *P. tenue* (4 μEm^{-2}s^{-1}),LaneH: *P. tenue* (10 μEm^{-2}s^{-1}), LaneI: *P. tenue* (16 μEm^{-2}s^{-1}), LaneM: Marker. **(b)** LaneA: *A. indica* (white) LaneB: *A. indica* (red) LaneC: *A. indica*(blue) LaneD: *A. indica* (green) LaneE: *L. limnetica* (white) LaneF: *L. limnetica* (red) LaneG: *L. limnetica* (blue) LaneH: *L. limnetica* (green) LaneI: *P. tenue* (white) LaneJ: *P. tenue* (red) LaneK: *P. tenue* (blue) LaneL: *P. tenue* (green) LaneM: Marker

white, blue, green and red light of *P. tenue* are identical except for a few linkers belonging to group II and III which showed slight variation in no. In *P. tenue* 30 kD linker, which is known to be a rod core linker, seems to be absent in “green light” PB extract. Green light shows one linker less as compared to the other samples (30 kD) as mentioned above, an average figure being 14 for all the samples.

2.4 Discussion

The light-harvesting complex of cyanobacteria, mainly composed of chl ‘*a*’ and phycobiliproteins, is actively regulated in accordance with changing environmental conditions, thereby modifying the light-harvesting capabilities of cyanobacteria.

In response to light modulations, cyanobacteria exhibit changes in the pigment content. Low light intensities show a higher pigment content as compared to high light intensities. All the 3 selected strains show higher pigment content in 4 and 10 $\mu Em^{-2}s^{-1}$ as compared to that in 16 $\mu Em^{-2}s^{-1}$. These results comply with observations made by Tandeau de Marsac and Houmard (1988), that there is an inverse correlation between light intensity and pigment content. The less light energy available, the more photosynthetic pigments are synthesized by the cells. High light intensity has a negative effect on the pigment content. All the tested cultures show a decrease in PB content when grown under 16 $\mu Em^{-2}s^{-1}$. A considerable rise in doubling time was also observed which could be due to photoinhibition under high light intensity. It is generally accepted that photoinhibition occurs when the level of light absorbed by the photosynthetic apparatus exceeds the rate by which it is consumed in photosynthetic reactions. It is believed that the primary site of the photoinhibitory response (damages) is located in PS-II and is reflected as a reduction in light limited oxygen evolution or CO_2 uptake rates (quantum efficiency) (Vonshak et al., 1996). This may be the reason for decrease in growth rate and simultaneous increase in doubling time.

PE possessing strains exhibit chromatic adaptation, (Tandeau de marsac and Houmard, 1988), yet PE lacking, *A. indica* exhibits a rise in PC content when grown in red light. *L. limnetica* is a clear example of chromatically adapting strains as PE shows a considerable rise in content when exposed to green light and in PC content when exposed to red light.

No particular part of visible spectra seems to affect APC quantitatively as highest amount of APC is observed in the cultures under white light.

Correlation of high PE content to green light is well studied but low light intensity also may elevate PE levels as observed in *L. limnetica*, which showed high PE content when grown under 4 $\mu Em^{-2}s^{-1}$.

Chromatic adaptation optimizes cellular pigment content for the quality of light in which the organism grows. Three categories of adaptation have been described, Group I : cyanobacteria do not adapt, Group II : organism control PE levels by adjusting the amount of PE-RAP complexes at the free ends of the rod structures, but do not adjust amount of PC Group III : cyanobacteria control both PE and PC in a complementary process, increasing PE and decreasing PC in green light and reversing this response in red light (Anderson., 1983). According to these categories, *L. limnetica* belongs to group III and non-chromatically adapting strains, *A. indica* and *P. tenue* belong to group I.

A sharp peak at 620 nm is eminent in the spectra taken in the range of 250-700 nm. This signifies the dominance of PC pigment in the PB extract. The other two being less in content show either a slight shoulder at their respective absorption maxima or are overlapped by the major pigment peak.

Certain variation in the λ_{max} of the spectra of PB is observed when cultures were exposed to different light quality and intensity. *L. limnetica* is the most affected strain under high light intensity showing a blue shift of ~8 nm, when grown under 16 $\mu Em^{-2}s^{-1}$. Now, according to Sidler (1994), certain linkers, red shifts the spectrum of PC disk to optimize the rod to core energy transfer. A blue shift, therefore, indicates decrease in optimization of rod to core energy transfer and thus negatively affecting the photosynthesis of the organism. *A. indica* and *P. tenue* have higher resistance to light stress as compared to *L. limnetica* as no striking shift was observed in their PB spectra.

But contrary to light intensity, light quality has a prominent effect on the spectra of *A. indica* and *P. tenue*, which exhibit a shift of ~5 nm and ~3 nm under green light, respectively. *L. limnetica* being a chromatically adapting strain, even when it receives a part of the visible spectrum (500-570 nm) and not the whole, the peak showed no shift, indicating that the energy transfer is not much affected.

Linker polypeptides are specifically associated to the phycobiliprotein oligomers, thereby, modulating the spectral properties and maintaining the structure of PBS (Capuano et al., 1991). Polypeptide analyses of PB by SDS-PAGE gives the protein profile of PBS detecting various linkers involved in the assembly of PBS and attachment of PBS to the thylakoid membrane. In many different cyanobacteria studied, the highest molecular weight linker of the PBS, carries the phycocyanobilin (Glazer., 1982) and attaches the core to the membrane (LCM). LCM of *L. limnetica* and *P. tenue* was observed to be 75 kD. In *A. indica* two molecular weight linkers were observed (~ 99 kD & ~170 kD). The reported LCM of most of the cyanobacteria are 75-99 kD (Glazer, 1982), according to which the LCM of *A. indica* would be 99 kD; and the function of 170 kD linker remains to be elucidated. Linker polypeptides belonging to group II and III disappeared in ’16 $\mu Em^{-2}s^{-1}$’ PB samples in all the three cultures, indicating PBS degradation. Linker profile supports the spectral study inference, that high intensity of light leads to sub-optimal energy transfer rates due to PBS degradation.

Another striking observation was absence of 30 kD linker in *P. tenue* grown in green light. The PB were extracted from cultures in mid-log phase, wherein it was observed that PC content is low when cultures are grown in green light. It has been reported in many strains that 30 kD linker is associated with PC assembly and polypeptides of roughly equal molecular weight are associated with the same biliprotein in the PBS of different organisms (Glazer, 1982). Thus the absence of 30 kD linker can be correlated with the decreasing PC content in *P. tenue*, when grown under green light.