

1.1 Introduction

Aquatic as well as terrestrial habitats exhibit a rich diversity of cyanobacteria. There have been many reports of cyanobacteria being promising agents for the production of fine chemicals, pharmaceuticals, nutraceuticals and even for immunodiagnostic purposes. But exploitation of any organism has to be preceded by extensive physiological, biochemical and genetic studies. Axenic cultures are a pre-requisite for most of the above mentioned studies, also cyanobacteria are to be treated according to the rules of the Bacteriological code, so it becomes all the more essential to do the physiological and molecular studies on axenic cultures (Palinska and Krumbein, 1998).

Presently, a small amount of cyanobacteria are in culture, either axenically or with one or more heterotrophic bacteria. This paucity reflects relatively few attempts to isolate cyanobacteria from some habitats and the difficulty in growing a large variety of forms by using present culture methods (Castenholz and Waterbury, 1984). As is well known, cyanobacteria grow under natural and laboratory condition in close relation or even synergism with chemo-organotrophic bacteria. Production of cyanobacterial axenic culture is often time consuming task contrasted with a low rate of success, i.e. some cyanobacterial cultures invariably die when they are completely axenic (Palinska and Krumbein, 1998).

Many different approaches towards obtaining axenic cultures have been reported earlier e.g. micromanipulation, differential filtration (Heaney and Jaworski, 1977). Various other protocols involving use of antibiotics (Vaara et al., 1979), phenols (Carmichel and Gorham, 1974), ultraviolet or gamma irradiation (Kraus, 1966) and even gliding motility and phototaxis of cyanobacteria have been employed (Vaara et al., 1979). Single cell isolation and washing of cyanobacteria is tedious process that

produces few clean viable cells. Moreover, the single cell isolation technique is rendered useless if the contaminating organisms are attached to the cyanobacterial surfaces (Polne-Fuller, 1991). Certain chemical treatments are also found to be very efficient but then the success rate in this case depends on the cyanobacterial sensitivity towards the chemical employed and the effectiveness of the chemical in killing the bacteria. Another problem, encountered while trying to develop axenic culture is the epiphytic bacteria attached to the large mucilaginous sheath possessed by some of the filamentous cyanobacteria.

This chapter describes an improved method employed for the isolation and purification of cyanobacteria involving a modification of capillary method and use of detergents towards obtaining axenic cultures of the three selected filamentous cyanobacteria.

1.2 Materials and Methods

Materials:

All reagents used were of analytical grade available from commercial sources and used without further purification.

Methods:

1.2.1 Axenic Culture:

Isolation

The fresh water strain *Arthrospira indica* was gifted by Dr. N. Jeeji Bai (Scientist, Parry Agro Industries LTD., Chennai). The culture was found to be contaminated with some unicellular cyanobacteria and heterotrophic bacteria. It was inoculated in different freshwater sterilized mediums (at 120 lbs, 15 min) like BG-11 (Castenholz and Waterbury, 1984), Zarrouk's medium (Becker and Venkatraman, 1982) and CHU10 medium (Castenholz and Waterbury, 1984). The growth was checked by

monitoring the OD_{750} every alternate day for 30 days. Zarrouk's medium was found to be the most suited medium for *A. indica*. Marine cyanobacterial cultures were isolated from the mixed cyanobacterial samples collected from north-west coast of India. The marine mixed cyanobacterial samples were inoculated in sterilized ASN-III medium (Rippka et al, 1979). Cycloheximide at the concentration of 0.1 g/L was added to the medium after filter sterilization to eliminate contamination of eukaryotic organisms. A small amount of the mixed culture was taken in a test tube and vortexed for about 1 min to break the cyanobacterial mat and homogenize the culture. Serial dilution of the homogenized mixture was carried out ($10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}$) and samples from all the culture tubes were observed under microscope. A tube in which the desired organism was quantitatively more was chosen and further isolation was carried out by capillary method (Castenholz, 1988). The tubes were maintained at $20 \pm 2^\circ\text{C}$ and $6-12 \mu\text{Em}^{-2}\text{s}^{-1}$ light intensity.

Modified capillary method

In this method, very thin glass capillaries (~1 mm diameter, 6.0-7.0 cm in length) were used to obtain uni-cyanobacterial cultures. A capillary sterilized prior to use, was dipped in the culture containing tube. After the liquid was raised in the capillary, it was broken in small parts and these parts were observed under the microscope. The part, which had just one filament of the desired organism was selected and inoculated in another culture tube containing sterilized ASN-III medium and incubated at $20 \pm 2^\circ\text{C}$ and $6-12 \mu\text{Em}^{-2}\text{s}^{-1}$ light intensity. The filament was allowed to grow and after about 30 days, the culture was inoculated in different marine mediums [ASN-III, Aquil medium (Castenholz and Waterbury, 1984), MN medium (Castenholz and Waterbury, 1984)] to check out the best suited medium. The growth was checked, by

noting the OD₇₅₀ every alternate day for 30 days. ASN-III medium was found to be the most suited medium for both the marine strains.

Purification

Further purification was mandatory to eliminate the contamination by heterotrophic bacteria in the cultures. The culture was homogenized by vortexing for 1 min. A few drops of the culture (~100 µl) was placed on a very low gel strength agar plate (0.6%) of ASN-III medium. It was then spread so as to form a small circle in the centre of the plate. The plates were incubated at 20±2°C and 6-12 µEm⁻²s⁻¹ light intensity. Filaments being motile, started migrating away from the inoculation point. After about 15-20 days (depending on the migration speed) when the filaments reach the edges of the plates, they were picked up from the edges and inoculated into a test tube containing sterilized ASN-III medium. It was then cultured and after considerable growth (15-20 days), was checked for bacterial contamination by streaking it on nutrient agar supplemented with glucose (0.5%, w/v) and incubated in the dark for 48 hrs.

The same procedure of isolation and purification was followed for *A. indica* but it required a further purification as bacterial colonies, though reduced in number, did appear on the nutrient agar plates. The culture was then treated with detergents to reduce the bacterial load. Two different detergents, sodium dodecyl sulphate (SDS) and sodium lauryl sulphate (SLS) of two different concentrations viz. 0.1 mg/ml and 0.05 mg/ml were employed. The detergent treatment was given for 24 hrs and after that 24 hrs the culture was washed with distilled water and transferred to fresh medium. It was then checked for bacterial contamination by streaking it on nutrient agar supplemented with glucose (0.5%, w/v) and incubated in the dark for 48 hrs.

1.2.2 Microscopy and Growth Curves:

Light microscopy and Scanning electron microscopy were carried out to study the morphology and division pattern of the selected cyanobacterial species.

Fresh culture, without any special sample preparation was mounted on the slide and observed under microscope.

For scanning electron microscopy, the below mentioned protocol was followed for sample preparation:

Protocol

A small amount of the culture was placed on a small glass piece covered with the adhesion substance, polylysine, and dried for about an hour till the medium evaporates but the culture was not completely dried. Fixation of the culture was then carried out by treating it with 0.4% glutaraldehyde in 0.1 M Na-Phosphate buffer (pH 7.5) for about 1-2 hrs. The sample was then washed with 0.1 M Na-Phosphate buffer (pH 7.5) at room temperature ($23\pm 1^\circ\text{C}$) for about 1 hr.

Ethanol washing was then carried out in the following sequence:

25% ethanol - 15 min

50% ethanol - 15 min

75% ethanol - 30 min

90% ethanol - 60 min

Absolute ethanol - 30 min

Samples were kept in the refrigerator at 10°C overnight and then critical point drying was carried out in Critical point dryer CPD 030 (BAL-TEC) for a day. Samples were then mounted on the sample holder and gold sputtered in Sputter coater (SCD 005. BAL-TEC) prior to microscopy.

Growth Curve

Growth pattern of the three selected organisms was studied and growth curves were plotted by measuring the total amount of chl 'a' in the culture every alternate day for about 35 days. Doubling time was then calculated according to Vonshak, (1997); a small alteration was made in the original formula, wherein, the biomass was substituted with chlorophyll concentration. The experiment was carried out in duplicates and the graphical data represents the mean values.

Chl 'a' extraction, was carried out by suspending the cell mass 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.

Estimation of Chl 'a'

Chl 'a' concentration was calculated from the absorbance of the methanolic extract at 665 nm, using the following equation (Tandeau de Marsac and J. Houmard, 1988).

$$\text{Chl 'a' } (\mu\text{g/ml}) = \text{O.D}_{665\text{nm}} \times 13.9$$

1.2.3 PB assay:

The fresh cyanobacterial cells of the three selected strains were harvested by centrifugation at 10000 X g for 30 min. The harvested cell mass was washed twice with distilled water and was suspended in Na-phosphate buffer (0.1 M, pH-7.0). The cell mass was then disrupted by sonication for 60 s. Repeated freezing (–195°C) and thawing at room temperature (23±1°C) in dark was followed by centrifugation at 10000 xg for 30 min. at 4°C, yielding a clear supernatant. UV- Vis absorption of PB extracts was recorded on a CARY 500 Scan UV-Vis, NIR spectrophotometer.

1.3 Results

1.3.1 Axenic culture:

For fresh water, *A. indica*, Zarrouk's medium was found to show optimum growth and the marine cultures, *P. tenue* and *L. limnetica* flourished in ASN-III medium. Serial dilution followed by modified capillary method was found to yield uni-cyanobacterial cultures of all the three desired organisms. But for obtaining axenic cultures further purification needed to be done. Plating on low % agar plates facilitated the fast migration of the cyanobacterial filaments towards the edges and leaving behind the non-motile heterotrophic bacteria. Thus, these filaments picked up from the edges yielded axenic culture of *L. limnetica* and *P. tenue*. But *A. indica* still showed bacterial contamination on the nutrient agar plate. Detergent treatment proved to be a successful mode of acquiring axenic culture of *A. indica*. SDS was found to be more toxic, in the concentrations of 0.05 mg/ml and 0.1 mg/ml, to the culture as microscopic examination of the culture revealed fragmentation and disintegration of the filaments. Among the two concentrations of detergents 0.05 mg/ml of SLS was found to be most suitable to avoid bacterial contamination after a treatment of 24 hrs. Higher concentration of SLS (0.1 mg/ml) also did not yield good results as the treated cultures did not survive for more than a week.

1.3.2 Morphology and Growth Studies:

Morphological and cytological data is not only important for the characterization of the organisms but is also useful in the taxonomy of the organism. It also provides an insight into the mode of reproduction in the organism. Light and scanning electron microscopy were employed to elucidate the morphological and cytological characteristics of the cyanobacterial cultures.

The planktonic strain, *A. indica* is a non-heterocystous, filamentous organism with crosswalls separating the cells which are visible even through light microscope. Individual cells of trichome are broader (8 μm) than long (2 μm). The terminal cell appears rounded under light microscopy, but scanning electron microscopy reveals a cap like structure on the terminal cell (Fig.1.1a). The trichomes are motile and helical, the helix being an open spiral. According to Castenholz (1984), members of the Oscillatorian family divide by binary fission in one plane, but it was observed that under laboratory controlled conditions the trichomes of *A. indica* fragment every 12-18 cells with transition cells (necridium cells) dying off and every fragment of the trichome growing into a new helical trichome (Fig.1.1d). The doubling time of the organism was calculated to be ~ 11 hrs. Growth curves show that, the log phase, where the metabolic activities of the cell is at its peak, reached after 10 days and the organism reached stationary phase after 28-30 days (Fig.1.2a).

L. limnetica is a thin filamentous, non-heterocystous cyanobacteria with a prominent sheath, which is also sometimes seen to extend beyond the terminal cell of the filament. The filament is mostly straight as observed under light microscope. Width of a single cell (0.5 μm) is more than its length (2 μm). Fragmentation was observed to occur as a mode of propagation (Fig.1.1e). The doubling time was calculated to be ~ 10 hrs. Growth experiments showed the commencement of the log phase after about 10 days and the culture reached the stationary phase after around 30 days (Fig.1.2b).

P. tenue is also a non-heterocystous, filamentous cyanobacteria. Length of the individual cells (6 μm) is thrice as much as its width (1.5 μm). End cell is slightly conical but no calyptra is observed (Fig.1.1c). A thin sheath is also observed. Cultures exhibited fragmentation as a mode of reproduction. Filaments were motile

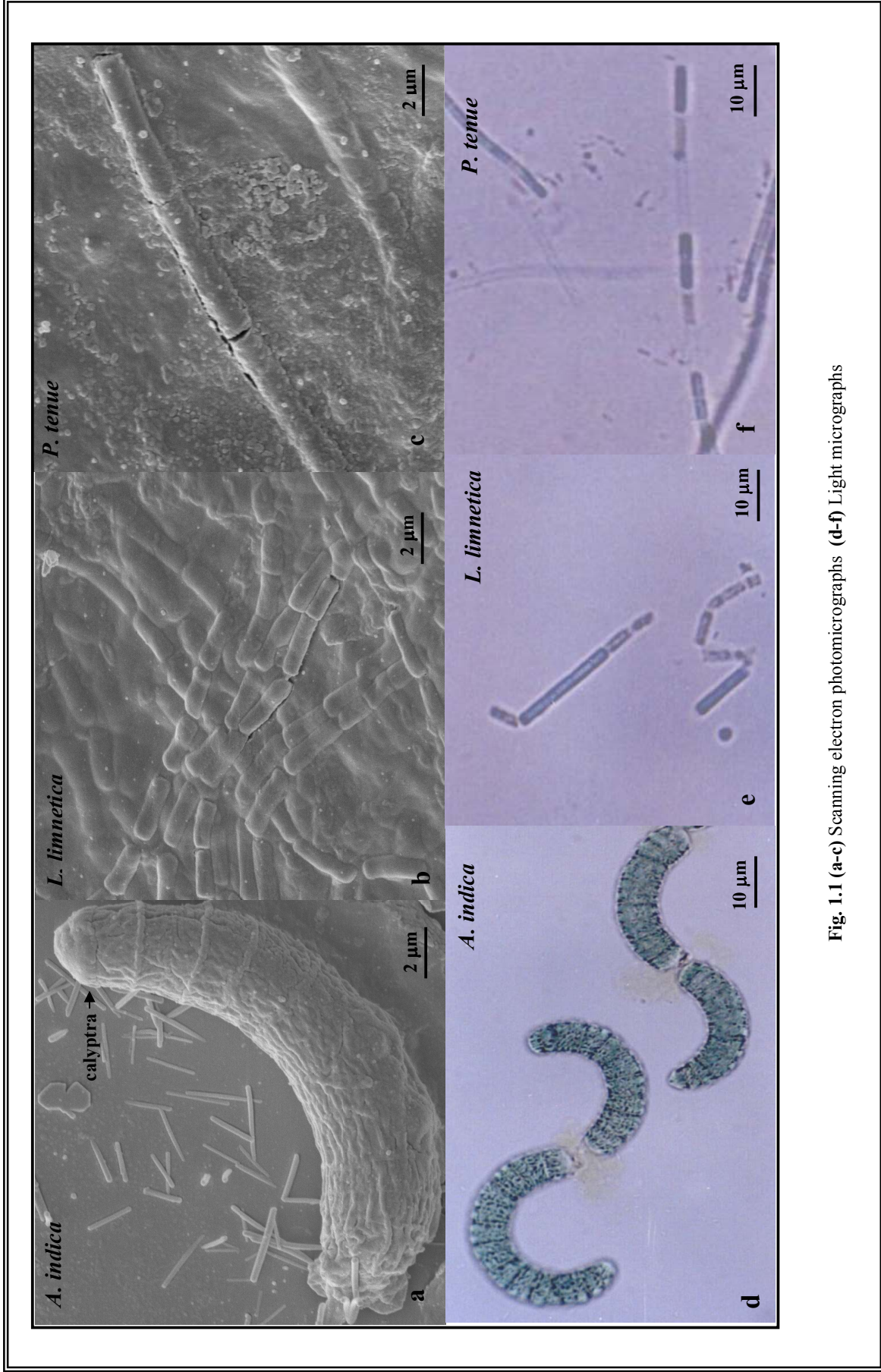


Fig. 1.1 (a-c) Scanning electron photomicrographs (d-f) Light micrographs

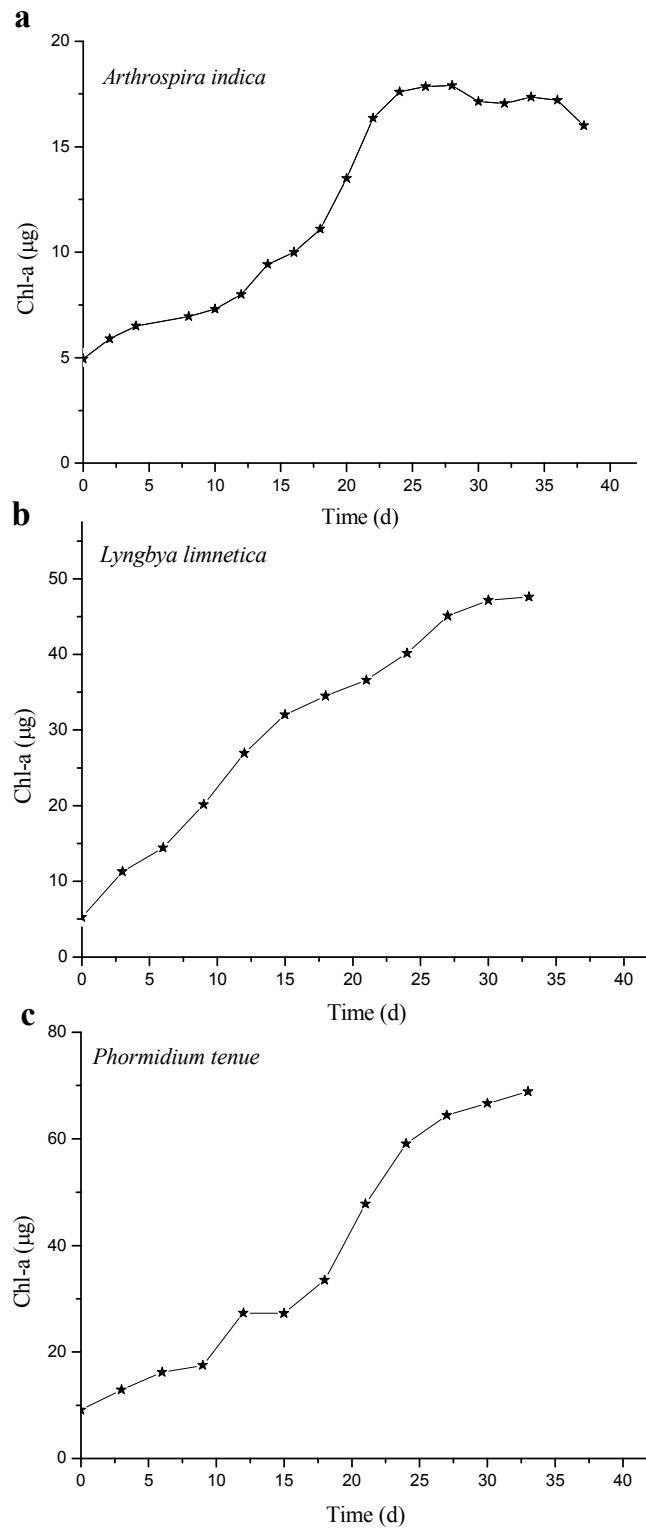


Fig. 1.2 (a-c) Growth curve of cyanobacterial strains

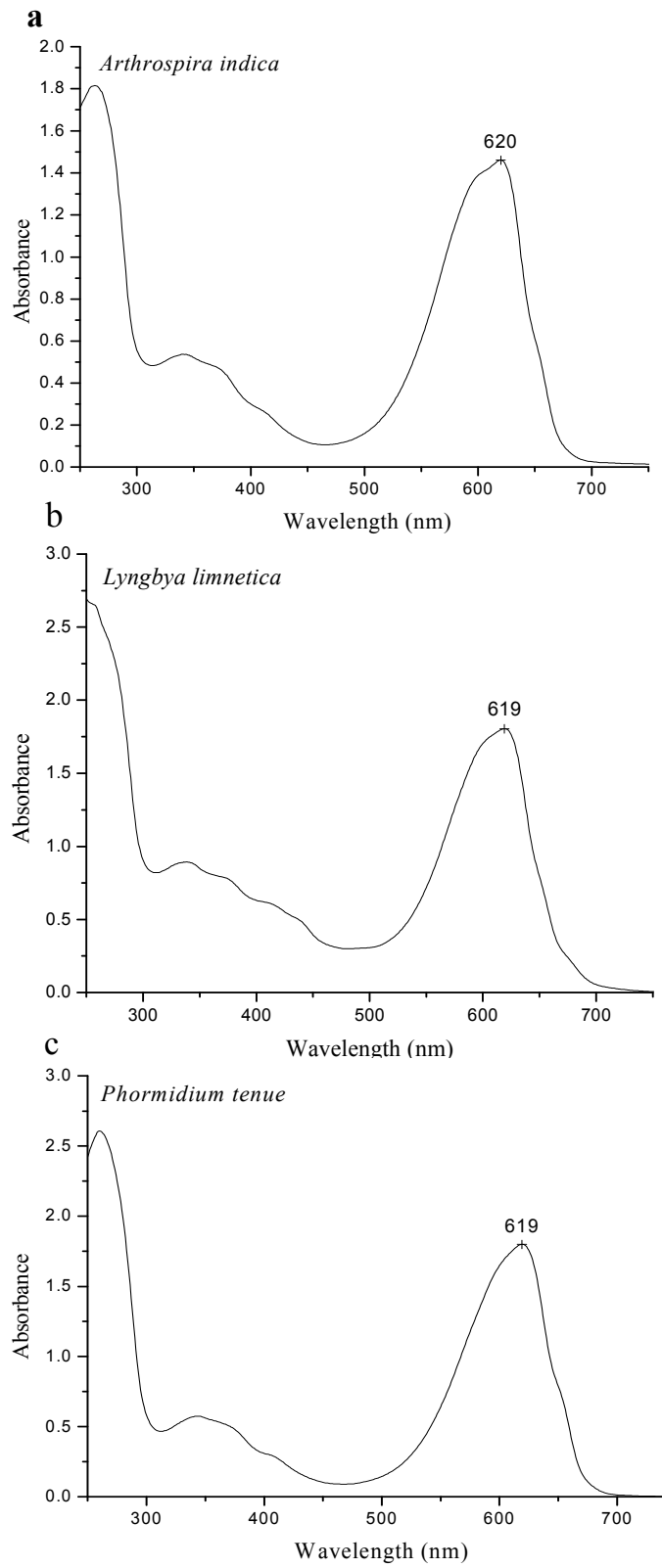


Fig. 1.3 (a-c) Absorption spectra of PB extract

with a straight forward movement. The doubling time (~12 hrs) was found to be longer than *A. indica* and *L. limnetica*. It can be inferred from the growth curve that the organism undergoes a lag phase for the first 10 days and reached a stationary phase after about 30 days.

1.3.3 Qualitative evaluation of PB:

Spectras of PB extract of all the three strains exhibit an intense peak at ~620 nm, due to PC, with relatively weak absorption band at ~350 nm in visible region. Both these bands are due to properties of phycocyanobilin chromophore. In UV region the absorption spectrum showed an intense peak at 278 nm arising from aromatic side chains in proteins. A slight shoulder at ~652 nm representing the presence of APC was also observed in all the three spectra (Fig.1.3).

1.4 Discussion

Various approaches have been undertaken to develop more efficient methods for isolation and purification of cyanobacteria. These include micromanipulation, differential filtration, antibiotic treatment, chemical treatment, UV-gamma rays, gliding motility and phototaxis of cyanobacteria (Palinska and Krumbein, 1998). Yet, till date no universal method is prevalent for obtaining axenic culture. So, a combination of these methods would prove an apt solution. For obtaining axenic culture of the selected filamentous cyanobacterial strains, a combination of serial dilution, isolation by migration on agar plates and detergent treatment proved to be a successful approach for the selected organisms.

Serial dilution is a traditional method which does reduce the number of contaminants and proves efficient in obtaining a uni-cyanobacterial culture, but is not able to completely eliminate heterogenous bacteria which may be attached to the filaments. Gliding motility of these filamentous cyanobacteria allows self isolation method when

used on agar plates. Low % of agar facilitates the faster and easier migration of filaments towards the edges leaving behind the bacteria. Self isolation on agar plate along with serial dilution did yield axenic culture of *L. limnetica* and *P. tenue* but in case of *A. indica* the nutrient agar plate streaked with *A. indica* did show the presence of certain heterotrophic bacteria. Being prokaryotes, cyanobacteria are very sensitive to chemical agents, thus milder agents in low concentration seem to yield better results. Different antibiotic and chemical treatments have been employed to yield axenic cultures (Fogg et al, 1973; Carmichel and Gorham, 1974). But detergents like SLS and SDS seemed to be very successful for acquiring axenic cultures of *A. indica* though concentration of detergents seemed to play an important role. Anionic detergents like SLS and SDS are known to disrupt the association between polypeptide chains. So higher concentration would definitely disrupt the protein organization which in turn may lead to cell death.

Different cell division patterns are observed in the marine and freshwater strains studied. In the marine *L. limnetica* and *P. tenue*, it is observed that LPS “outer membrane” and sheath is continuous and not involved in the division, so may be, only the cytoplasmic membrane and peptidoglycan layer invaginate. But as observed in most unicellular and colonial cyanobacteria, and some filamentous forms, *A. indica* undergoes a binary fission by a constrictive type of division in which all envelop layers (often including sheath) grow inward until cell separation is complete or nearly complete. This firm sheath in *A. indica*, invaginates every 12-18 cells during fragmentation with the transition cells (necridium cells) dying off. So though, both the marine and freshwater strains undergo binary fission yet the pattern differs.

The absorption spectras of the three strains do not show much variation qualitatively. All the three spectras show an intense peak of PC, showing that PC is dominant component in total PB content.

Till date there is no universally accepted taxonomic classification; those put forward by Geitler (1932), Desikachary (1959), Drouet (1968) and Rippka et al., (1979) are widely accepted. Yet, there are lot of discrepancies regarding certain genera, *A. indica* being one of them. There has always been a controversy regarding *A. indica*, whether to put it under the genus of *Spirulina* or to consider it as a separate genus. Some also considered it as a subgenus of *Spirulina* (Geitler 1925, 1932; Fremy, 1930; Elenkin, 1949). Drouet (1968) retained the two genera. The strain considered for the present study *A. indica*, shows a calyptra, though many taxonomists including Desikachary (1959) had earlier claimed that the genus *Arthrospira* does not possess a calyptra. The first confirmatory demonstration of calyptra in the genus *A. indica*, has been reported by Desikachary and Jeeji Bai (Jeeji Bai, 1999). Based on this feature, they have created a new species *A. indica*.

Both the marine strains, *L. limnetica* and *P. tenue* are thin filamentous organisms. Different views regarding taxonomy of these thin filamentous strains have been put forward by different taxonomists. Anagnostidis and Komarek (1988) have put forward a more broadened classification of the Oscillatorian strains. Their classification separates the filaments having a small breadth $<3\mu\text{m}$ from the rest. If this classification is to be considered then *L. limnetica* would be put under the genera *Leptolyngbya*.

In the present study the taxonomic classifications of Geitler (1932), Desikachary (1959) and Rippka et al., (1979) have been referred and accordingly the strains have been identified as *Arthrospira indica*, *Lyngbya limnetica*, *Phormidium tenue*.