CHAPTER 1

General Introduction
1.1 Introduction

Cyanobacteria are the prominent constituents of marine biosphere that account for a significant percentage of oceanic primary productivity, and are among the oldest life forms on the Earth capable of doing oxygenic photosynthesis about 3.5 billion years ago (Schopf, 1993) which is similar to the process found in higher plants (Gralnick et al., 2000; Ting et al., 2002; Guan et al., 2007). The main reason for the evolutionary hardiness of cyanobacteria is their successful combination of metabolic pathways. They are among the few groups that can perform oxygenic photosynthesis and respiration simultaneously in the same compartment, and many cyanobacterial species are also able to fix nitrogen. Therefore, they can survive and prosper under a wide range of environmental conditions (Vermaas, 2001).

Cyanobacteria, also known as blue-green algae occupied an anomalous position in the biological world. They were treated as a division (or class) of algae by botanists because they are autotrophs that use water as an electron-donor and contain two photopigments (chlorophyll $a$ and $\beta$-carotene) that are chemical hallmarks of plant photosynthesis. However, since the advent of the use of the electron microscopy in biology, they have known to have a prokaryotic cell structure (Fig. 1.1).

![Schematic diagram of cyanobacterial cell constituents.](image)

**Fig. 1.1:** Schematic diagram of cyanobacterial cell constituents.
The designation blue-green algae is therefore misleading, although this common name is now so firmly established that its use can probably never be eradicated. These organisms are not algae; their taxonomic association with eukaryotic algal groups is an anachronism, formally equivalent to classifying the bacteria as a constituent group of the Fungi or the Protozoa. Thus, the cyanobacteria are algae from an ecological perspective, but are bacteria and part of kingdom Monera from a cell structure viewpoint. Their cell-wall structure clearly puts them in with Gram-negative bacteria.

The Cyanophyta contains about 150 genera and 2000 species. They are found in most diverse habitats: in fresh water and in the sea, on damp soil, and even in extreme and inhospitable places as glaciers, deserts and hot springs. Most, however, live in fresh water. Part of the success of these blue-green algae can be ascribed to their ability to use low-light intensities effectively, so that they can thrive below the surface, deep in the epilimnion. They can control their buoyancy via gas vacuoles and hence their position in the water-column. Akinetes play an important role in the survival of these organisms in the adverse conditions like drought, cold, nutrient deficiency, etc. Some cyanobacteria possess not only gas vacuoles but also heterocysts: differentiated cells with colourless interior and thick walls, which can fix atmospheric nitrogen. Cyanobacteria with heterocysts can satisfy their nitrogen requirements and grow even in waters where nitrate and ammonium levels are low but where the phosphate levels are high because of pollution with detergent containing polyphosphates.

The majority of cyanobacteria are aerobic photoautotrophs - their life processes require only oxygen, light and inorganic substances. However, there are species of cyanobacteria (Oscillatoria) that are found in mud at the bottom of the Thames which are able to live anaerobically. Cyanobacteria can even live in extreme of temperatures -60°C to 85°C. Cyanobacteria can grow in full sunlight and complete darkness. They are often the first ones to colonize bare areas of rock and soil. In many environments, cyanobacteria are primary producers at the base of the food web of the ecosystem viz. marine waters, hypersaline, brackish waters, soda lakes, fresh water, paddy fields, soils, deserts (Dor and Danin, 1996), mountain streams, cave walls, hot springs (Casenholtz, 1973), polar regions (Skulberg, 1996), snow and ice (Laamanen, 1996) and other extreme environments (Lakshmi, 2007). Cyanobacteria are symbionts of a
variety of other organisms, viz. the marine diatom *Rhizosolenia*, leaves of *Azolla* and the roots of cycas (Thajuddin and Subramanian, 2005).

### 1.2 Relationship of cyanobacteria with plants

Chloroplasts, the sites of photosynthesis within plant cells, comprise a prominent and well known class of plastids with diverse and specialist functions in plant and algal cells. Mereschkowsky is widely recognized as having written the first clear exposition of the hypothesis that plastids are derived from endosymbiotic cyanobacteria or blue-green algae (Raven and Allen, 2003) (Fig. 1.2). Mereschkowsky’s 1905 hypothesis gained support from electron microscopical and biochemical studies which showed that plastids contain DNA, RNA and ribosomes, supplying a structural and biochemical basis for cytoplasmic inheritance of plastid related characters. Molecular phylogenetic studies now make it abundantly clear that the closest bacterial homologs of plastids are indeed cyanobacteria (Raven and Douglas, 2003). Only cyanobacteria and chloroplasts have two photosystems and split water, to make oxygen, as a source of reducing power. It has long been clear that many of the proteins needed for plastid functions, including photosynthesis, are now encoded in the nuclear genome and arrived there during evolution by the wholesale uptake of cyanobacteria, including their genomes, followed by gene transfer into the nucleus (Doolittle et al., 2003).

![Fig. 1.2: Endosymbiotic origin of plastids.](image)

Long before the era of genome sequencing, Weeden (1981) had proposed that plant nuclei must possess large numbers of genes derived from cyanobacteria. Weeden’s insight was based on the fact that plastid genomes are small and extremely limited on their coding potential and cannot possibly encode all the proteins necessary for plastid function. As predicted by Weeden, plant nuclear genomes harbour thousands of
cyanobacterial genes whose proteins are synthesized in the cytoplasm and targeted back to the plastid (McFadden, 1999; Bruce, 2000; Soll and Schleiff, 2004). The reality is even more complex than Weeden would have imagined. It is now known that in addition to producing proteins that service their compartment of origin, nuclear-encoded cyanobacterial genes have the potential to acquire all manner of functions in plant and algal cells. Genomic studies have been important in showing the evolutionary fate of the cyanobacterial genes that originated from the endosymbiotic pre-plastids. The genes in pre-plastids were retained, lost, or transferred to the nucleus. The process of transfer of genes to the nucleus would have involved duplication of each plastid gene, and a nuclear copy of the gene becoming able to produce a functional product in the cytosol or with, appropriate targeting sequences, in other compartments (Raven and Allen, 2003).

1.3 Photosynthesis

Oxygenic photosynthesis is arguably the most important biological processes on Earth. Approximately 2.3 billion years ago (Bekker et al., 2004; Anbar et al., 2007; Kaufman et al., 2007), the energy transduction pathway transformed Earth’s atmosphere and upper ocean ultimately facilitating the development of complex life forms that depend on aerobic metabolism (Blankenship and Hartman, 1998; Falkowski et al., 2005; Raymond and Segre, 2006). Cyanobacteria are widely accepted as the progenitor of oxygenic photosynthesis, and the clade has evolved into one of the largest and most diverse groups of bacteria on this planet (Whitton and Potts, 2000). Cyanobacteria contribute significantly to global primary productivity (Waterbury et al., 1979) and are diazotrophic taxa central to global nitrogen cycle (Capone et al., 1997; Zehr et al., 2001; Karl et al., 2002). No other prokaryotic group has had a greater impact on the biogeochemistry and evolutionary trajectory of Earth, yet its own evolutionary history is poorly understood.

The photosynthetic electron transport chain in cyanobacteria is essentially identical to that in plants, even though some of the polypeptides that are a part of electron transporting enzymes appear to be of different evolutionary origin in the two systems. One important difference between cyanobacteria and plants is that the stoichiometry of PS-I and PS-II in at least some species of cyanobacteria is much larger than 1,
whereas in higher plants an equal amounts of PS-I and PS-II is the rule (Vermaas, 2001). PS-II uses light energy to split water and to reduce the PQ pool. Electrons are transported from the PQ pool to the cytochrome b_{6f} complex and from there to a soluble electron carrier (plastocyanin or cytochrome C_{553}, depending upon the species and on the availability of copper) on the luminal side of the thylakoid membrane. Either of the soluble one-electron carriers can reduce the oxidised PS-I reaction centre chlorophyll, P700⁺. This oxidised form of the reaction centre chlorophyll is formed by a light-induced transfer of an electron from PS-I to ferrodoxin (Fd) and eventually to NADP. Reduced NADP can be used for CO₂ fixation. Photosynthetic electron transfer leads to a proton gradient across the thylakoid membrane. In PS-II, protons are released into the lumen upon water splitting, and protons formed upon plastoquinol oxidation by the cytochrome b_{6f} complex are released into the lumen as well. The proton gradient across the thylakoid membrane is used for ATP synthesis by ATP synthase in the thylakoid; this ATP may be applied for CO₂ fixation and other cell processes.

1.4 Light-harvesting complex ‘phycobilisomes’

Cyanobacteria harvest light energy for photosynthesis with macromolecular antennae complexes, termed ‘phycobilisomes (PBS)’, which are peripherally attached to the photosynthetic membrane (Grossman et al., 1993; Jung et al., 1995; Kahn et al., 1995; Kahn and Schaefer, 1995; Kalla et al., 1988; Ong and Glazer, 1991; Raps, 1990; Sidler, 1994) (Fig. 1.3). These complexes absorb visible light in the range of 500-680 nm and efficiently transfer the captured light energy to chlorophylls of the photosynthetic apparatus. Phycobilisomes consists of two structural domains: a core which connects with the photosynthetic membrane and rods, generally six in number that radiate from the core (Kahn et al., 1997). Each domain contains pigmented and non-pigmented linker polypeptides (Grossman et al., 2001). The composition of PBS varies from organism to organism, and individual organisms have PBS that are changed by the environment in diverse ways (MacColl, 1998).

The study of PBS started in 1965-66 when Gantt and Conti, using red algae, performed a series of experiments that resulted in the isolation of extrinsic granules from the outer or stromal surface of the chlorophyll ‘a’ containing thylakoid
membrane. Following this lead, many other researchers showed that cyanobacteria also contain PBS (Edwards et al., 1968; Gantt and Conti, 1969; Edwards and Gantt, 1971; Wildman and Bowen, 1974). There are different structural types of phycobilisomes like hemidiscoidal, hemiellipsoidal, bundle-shaped, block-shaped, and hemiellidiscoidal (Wehrmeyer, 1983; Wehrmeyer et al., 1988; Ducret et al., 1998). Hemidiscoidal PBS, having a tricylindrical core and six rods, are extensively found in cyanobacteria. All PBS have the chromoproteins (phycobiliproteins), categorized into 3 types by bilin energy: those of high energy (phycoerythrin or phycoerythroyanin), intermediate energy (phycocyanin) and low energy (allophycocyanin). Energy flows from the highest to the lowest energy pigments and that is how PBS are organised (Fig. 1.3).

1.4.1 Phycobiliproteins, bilins and linkers
Phycobiliproteins (PBP) are obtained as dissociation products of PBS. Two general types of proteins make up the PBS: small, acidic, pigmented proteins called phycobiliproteins with covalently attached tetrapyrrole chromophores, and larger, basic, generally non-pigmented proteins known as linker polypeptides (Tandeau de Marsac and Cohen-Bazire, 1977; Glazer, 1982). When the cyanobacterial cells are broken and the cellular contents escape into the low-ionic strength aqueous medium, the PBS dissociate into the various biliproteins, either with or without attached linkers.

![Fig. 1.3: Typical structure of a phycobilisome.](image-url)
Phycobiliproteins, comprising 60% of the soluble protein in cyanobacterial cells (Apt et al., 1995), can be divided into 3 major groups based on their spectral properties: phycoerythrin (PE, $\lambda_{\text{max}}$ - 540-570 nm), phycocyanin (PC, $\lambda_{\text{max}}$ - 610-620 nm) and allophycocyanin (APC, $\lambda_{\text{max}}$ - 650-655 nm) (Bernard et al., 1992) (Fig. 1.4). All the PBPs are generally composed of two non-identical polypeptide subunits (α and β), which contain one or more covalently linked open-chain tetrapyrrole chromophores. The chromophores, known as phycobilins, are covalently attached to the phycobiliproteins by either one or occasionally two cysteinyl thioether linkages (Apt et al., 1995). There are several structurally distinct phycobilin chromophores (Fig. 1.5) with different spectroscopic properties. When covalently attached to the phycobiliproteins, the chromophores phycourobilin (PUB), phycovoilobilin (PXB), phycoerythrobilin (PEB) and phycocyanobilin (PCB) have absorbance maxima at approximately 498 nm, 568 nm, 535 to 567 nm and 620-660 nm, respectively (Apt et al., 1995).

![Fig. 1.4: Purified phycobiliproteins: (1) Allophycocyanin, (2) Phycocyanin, (3) Phycoerythrin.](image)

The number and type of bilins carried by various phycobiliproteins may vary (Glazer, 1989). The colour of a given PBP is not determined solely by the chemical nature of its bilins. The spectroscopic properties of each bilin within a PBP are strongly influenced by the conformation and environment imposed on the bilin by the native protein. Conservation of sites of attachment of the bilins is seen in all of the PBPs. The important finding is that the location of the bilin residue in the primary structure
is fixed whereas the chemical nature of the bilin at a given site differs from one PBP to another (Glazer, 1989).

![Chemical structure of phycocyanobilin and phycoerythrobilin]

**Fig. 1.5:** Phycobiliprotein chromophore groups: (a) phycocyanobilin; (b) phycoerythrobilin.

The PBPs can form trimer or hexamer complexes, which assemble into higher-order structures in the presence of specific linker proteins. PBS linkers (Fig. 1.6) transfer energy of PBPs to favour unidirectional flow of excitation energy from the peripheral rod of PBS to PBS core and from PBS core to the photosynthetic reaction centre (MacColl, 1998). Linker proteins function to stabilize PBS structure and determine positions of the PBPs within PBS structure. At the same time, linkers also interact indirectly or directly with the chromophores to cause PBS structure changes that can modulate different PBP subassemblies and optimize absorbance characteristics (Gottschalk et al., 1991; Gottschalk et al., 1993; Reuter et al., 1999). The structural function of the PBS linkers in PBPs has allowed cyanobacteria to colonize environment and show a great diversity in terms of light quality and quantity.
Grossman et al., 1993; Allen and Matthijs, 1997). The importance of linker polypeptide for the assembly of defined complexes and their roles for tuning spectral characteristics of the complexes has been well understood (Man et al., 2003; Steglich et al., 2005).

**Fig. 1.6:** Typical phycobilisome structure depicting all the type of linker proteins.

### 1.4.1.1 Phycoerythrin

The intensely red phycoerythrins, having $\lambda_{\text{max}}$ = 540-570 nm, serve as major light-harvesting proteins in numerous cyanobacteria (Schoenleber et al., 1984). PE extends the light-harvesting capability of the PBS into the green region of the visible light spectrum, and its location at the PBS periphery reflects the position of PE in the sequential energy transfer pathway (Anderson and Grossman, 1990). High phycoerythrin content is a distinguishing adaptive characteristic of marine unicellular cyanobacteria (Waterbury et al., 1979; Wilbanks et al., 1991). In their natural habitat, these strict photoautotrophs are exposed primarily to blue-green light with maximum intensity near 500 nm which is strongly absorbed by phycoerythrins (Waterbury et al., 1986). The marine strains contain two different kinds of phycoerythrins, whereas all other cyanobacteria contain a single type of phycoerythrin. The phycoerythrins of the marine cyanobacteria also vary in relative proportion of the phycoerythrobilin (PEB) and phycourobilin (PUB) prosthetic groups they carry (Ong and Glazer, 1991; Swanson et al., 1991). The number of bilins per monomer differs, generally 5 or 6. Those with 5 bilins have three on the $\beta$ polypeptide and two on the $\alpha$, and those with 6...
have an additional PUB on α-subunit. Such bilin diversification signifies light-harvesting as an important aspect of the ecology for these organisms (MacColl, 1998).

1.4.1.2 Phycocyanin
Intermediate energy possessing phycocyanin, having $\lambda_{\text{max}}$ - 610-620 nm, is a universal biliprotein present in almost all cyanobacteria. C-phycocyanin is found as a complex of $\alpha_3\beta_3$ or $\alpha_6\beta_6$ or other oligomers. The hexamers ($\alpha_6\beta_6$) are disc-shaped formed by face-to-face assembly of trimers. Rods are formed by face-to-face assembly of these discs. C-phycocyanin has phycocyanobilins as their chromophore groups - two on α chain and three on β chain.

1.4.1.3 Allophycocyanin
Allophycocyanin (APC), having $\lambda_{\text{max}}$ - 650-655 nm, is the most efficient biliprotein for energy transfer (Lemasson et al., 1973; Gantt et al., 1976; Gysi and Zuber, 1978) and is the key pigment in funnelling the excitation energy from the other biliproteins into the chlorophyll ‘a’ of the PS-II. APC accounts on a weight basis for 10% or less of the total cellular phycobiliproteins. The allophycocyanins, present in the core, are of two functional types: allophycocyanins, having 650 nm maximum and two lower energy allophycocyanins, the Lcm polypeptide and the $\alpha^B$ polypeptide, which transfer energy to chlorophyll ‘a’ (Gantt, 1975; Bryant et al., 1979; Sidler, 1994; Bald et al., 1996). APC is generally found as a trimer near neutral pH having three α and three β polypeptides; each having one chromophore (PCB). Trimers ($\alpha_3\beta_3$) are ring-like assemblies of three monomers (αβ) having three-fold symmetry. The difference between the absorption spectra of monomers and trimers of APC is striking. Homogenous monomers were found to have an absorption maximum at 614 nm, while trimers had a sharp maximum at 650 nm and a prominent shoulder at 610-620 nm (MacColl, 1998).

1.5 Energy transfer
The energy migration in phycobilisomes occurs via ‘resonance energy transfer’ mechanism. It requires the spectral overlap of donor fluorescence and acceptor absorbance and is exquisitely sensitive to distance and relative geometry of the donor/acceptor chromophore pair (Glazer, 1985). The energy levels of the bilins in the
phycobiliproteins are not equivalent. Though all of the bilins in a given PBP absorb excitation energy, the fluorescence of the protein originates from the bilins with the longest wavelength absorption bands. Bilins which absorb light energy but transfer the excitation energy to the other bilins are called ‘donors’. Those bilins that absorb excitation energy and fluoresce are called ‘acceptors’. The intramolecular energy transfer within the PBP trimers and hexamers is very fast, and in consequence the steady state fluorescence emission originates almost exclusively from the acceptors.

In 1970s, it became clear that light energy collected by the phycobiliproteins within the PBS was transferred mainly to PS-II, with APC acting as a link between the phycocyanin and membrane bound chlorophyll \( a \) (Halldal, 1970; Gantt and Lipschultz, 1973; Lemasson et al., 1973). However, the detailed description of the energy transfer within the phycobilisome awaited the work of Glazer and collaborators for about 10 years (Glazer, 1989; Sidler, 1994). Glazer, in 1976, postulated that allophycocyanin was the evolutionary ancestor of phycocyanin, which in turn preceded phycoerythrin (Tandeau de Marsac, 2003). The properties of the native biliprotein chromophores are profoundly influenced by non-covalent interactions with the protein (Scheer, 1982). Both intense visible absorption and fluorescence of the phycobiliproteins, which are crucial for their functions, are caused by these interactions, and are absent in the denatured pigments and in the free chromophores.

Energy transfer from biliproteins to chlorophylls has also been demonstrated in solution (Frackowiak et al., 1979). Phycobilisomes collect light efficiently in the wavelength range between 480-650 nm, but their fluorescence is emitted almost exclusively (≥ 95%) by a small fraction of a minor constituent, allophycocyanin. The morphological ordering from PE to PC to APC when going from periphery to the centre of the PBS corresponds perfectly to the decrease in excitation energy among the three pigments, which can form an energy transfer chain in which fluorescence of any preceding one overlaps reasonably well with the absorption of the next one. Teale and Dale (1970) suggested that part of an energy transfer chain is already present within each individual biliprotein. The absorption of PCs and PEs can be resolved into components of slightly different energies. These are assigned to individual
chromophores of often the same molecular structure, but with different absorption energies due to different interactions with the proteins. The ones absorbing at shorter wavelengths act as sensitizers to the ones absorbing and fluorescing at longer wavelengths, which provide an efficient fine-tuning of the energy transfer.

A large fraction of energy transfer in phycobiliproteins proceeds via a Forster type process. This has been deduced from the efficiency of the energy transfer on one hand, and the low occurrence of strong couplings on the other. Grabowski and Gantt (1978) have critically investigated a series of biliproteins and calculated Forster’s critical distances, which are mostly in the range of 40 Å – 60 Å, well beyond the diameter of phycobiliprotein subunits (approximately 30 Å). The energy transfer from PE to PC appears to be faster than that from PC to APC core (Suter et al., 1984). In isolated biliproteins, the transfer times increase with decreasing aggregation i.e. time taken by a trimer is less than that by a monomer.

1.6 Structural assembly and stability of phycobiliproteins

The 3D structure of the phycobiliproteins (Schirmer et al., 1985, 1986, 1987; Duerring et al., 1990, 1991; Ficner et al., 1992; Ficner and Huber, 1993) reveal similar tertiary structures for the α and β subunits. The rigorous structural constraints have limited evolutionary divergence among the biliprotein family, and the PBS is a protein scaffold for bilin chromophores that is predominantly (approximately 85%) composed of variations on a single subunit structural motif (Anderson and Toole, 1998). It has long been recognized that there are numerous similarities among the different phycobiliprotein subunits, leading to the proposal that different subunit belong to a protein family that has evolved from a single ancestral protein (Glazer et al., 1976; Glazer, 1980).

Biliprotein subunit structure consists of nine α-helices arranged in two domains. At N-terminus, helices X and Y comprise a platform that extends out from the globular domain, which contains helices A-B-E-F-F'-G-H arranged in a globin fold (Schirmer et al., 1985). Regardless of the structural similarity across the biliprotein classes, oligomerization in the PBS assembly is selective, and the primary assembly interactions occurs between the α and β subunits of the same class of biliprotein,
where the XY platform of one subunit binds to the globin domain of its assembly partner in a symmetric interaction that yields an αβ heterodimer (Anderson and Toole, 1998). The heterodimer is defined as the biliprotein ‘monomer’, a traditional term that reflects the extreme stability of the αβ complex \textit{in vitro} relative to other biliprotein aggregation forms, notably trimer (α3β3) or hexamer (α6β6) (Glazer, 1976). Linker polypeptides are believed to bind in the central cavity of the trimers and hexamers.

Phycobiliproteins (trimers or hexamers), assembled with the aid of linkers, are organized into two structural domains – the core and the rods. The core forms the physical connection with the outer surface of thylakoid membranes. Radiating from the core are the series of rods that are composed of stacked phycobiliprotein discs. Disc proximal to the core are of PC, while those distil to the core are of PE or PEC. Measurements of the energy have shown that light energy absorbed by PE is sequentially transferred to PC, then APC and finally is passed primarily through terminal energy acceptor to chlorophyll \textit{a}. This transfer of energy occurs with an efficiency approaching 100% \textit{in vivo} (Glazer, 1989).

1.7 Influence of environment on cyanobacteria

In photosynthetic organisms, the pigment content is modulated by many environmental factors such as nutrient availability, temperature and light (Mazel et al., 1986). Environmental factors can strongly influence protein stability. Cyanobacteria deprived of essential macronutrient such as N, S, C or P exhibit approx 6-fold increase in the basal rate of proteolysis (Collier and Grossman, 1994). The increased rate of proteolysis may affect most cellular proteins or a specific subset of them. Our understanding of the regulation and specificity of proteolysis \textit{in vivo} is limited. Properly folded proteins are stable because proteolysis-sensitive domains are hidden whereas damaged, misfolded or unassembled proteins are rapidly degraded (Gottesman and Maurizi, 1992; Hill et al., 1993). Cyanobacteria exhibit a suite of responses during nutrient limited growth that are either specific responses triggered by the depletion of a single nutrient or general responses that occur in medium that lacks any one of a number of different nutrients (Allen and Smith, 1969; Allen, 1984; Simon, 1987; Reithman et al., 1988). General responses to nutrient limitation include changes in both cellular morphology and physiology. A dramatic response exhibited
by cyanobacteria when they are limited for S, N, P, C or Fe is a decrease in the abundance of pigment molecule on the cells (Allen and Smith, 1969). During N and S starvation, there is rapid and near complete degradation of the PBS. Degradation of PBS could provide cells with amino acids useful for synthesis of proteins important for the acclimation process. These amino acids can be converted into carbon skeletons and used to produce other cellular constituents. The use of phycobiliproteins as amino acid storage molecules may be important for marine cyanobacteria, since nitrogen is frequently limiting in marine environments (Wyman et al., 1985). The degradation of PBS during N or S limited growth is an orderly process (Fig. 1.7). However, on addition of the limiting nutrient back to deprived cultures, new phycobilisomes are rapidly synthesized. Studies concerning environmentally regulated degradation of the PBS will provide insights into processes involved in the targeting of macromolecular complexes for degradation and the machinery that implements this degradation.

Fig. 1.7: Orderly process for phycobilisome degradation.

The acclimation of photosynthetic organisms to changes in light colour is ubiquitous and may be best illustrated by the colourful process of complementary chromatic adaptation (CCA). During CCA, cyanobacterial cells change from brick red to bright blue-green, depending on their light colour environment (Kehoe and Gutu, 2006) (Fig. 1.8). Cyanobacteria often live in fresh water and marine environments. Because water preferentially absorbs longer wavelength (more red) light, cells at the surface of the water column experience more red-enriched light than at moderate depth, where green and blue light predominate, or even deeper, where only blue light penetrates. As a result many cyanobacterial species experience dramatic differences in light-colour ratios in their natural environments.
Cyanobacteria use various bilins as the primary pigments, which allow photon capture between the blue and red regions of the spectrum that are not efficiently trapped by chlorophyll. These bilins can confer dramatic colour phenotypes. However, because each bilin absorbs relatively specific light colour, the ability to control the production of a variety of bilin-containing phycobilisomes, each with an absorption profile that closely matches the ambient light colour, can provide a fitness advantage (Stomp et al., 2008). Chromatic adaptation requires cyanobacteria to precisely measure ratios of specific light colours in the environment. CCA exists in species producing both red-absorbing PC and green-absorbing PE (Tandeau de Marsac, 1977; Palenik, 2001). More than 70% of the PE containing cyanobacteria examined chromatically adapts (Tandeau de Marsac, 1977). CCA is likely an important contributor to global primary productivity. Tandeau de Marsac (1977) grouped chromatically adapting species based on their growth in red and green lights. Group I species have unaltered PC and PE levels during growth in these two light conditions. Group II species have PE levels high in green light and low in red light, whereas PC levels are not affected by light colour. Group III species are most complex. They also have PE levels that are high in green light/low in red light, like in group II, but in addition, they regulate their PC
content in response to light colour i.e. PC levels are high in red light/low in green light, just opposite of the way that PE is regulated. A more recently discovered a fourth type (Palenik, 2001) is the one where PC and PE do not change; instead a green-light-absorbing bilin is added to PE in green light and a blue-light-absorbing bilin is added to PE in blue light (Everroad et al., 2006).

1.8 Developments in purification and structural characterization of phycoerythrin

Phycoerythrin, a highly fluorescent water-soluble pigment, is the primary light-harvesting pigment of many marine cyanobacteria, a globally important group of marine phytoplankton. This pigment is typically part of organized phycobilisomes that provide nearly all light energy to PSII (Glazer, 1999). Thus, the additional wavelengths of light that can be used for photosynthesis are determined by the spectral forms of PE the organisms synthesize. This, in turn depends on the relative concentration of two different chromophores (PEB and PUB) that can be incorporated into the PE heterodimer. PEB provides for sufficient utilization of green wavelengths of light; as the relative abundance of PUB increases, the spectral signature of the PE becomes more complex (i.e. additional peaks and shoulders in the fluorescence excitation and absorption spectrum). The ecological effect of increasing the PUB:PEB ratio is that the cell’s ability to use shorter wavelengths that penetrate more transparent seawater greatly increases. Being an important pigment from the ecological point of view, several studies have been done on various aspects of phycoerythrin stating from purification till its structural analyses.

Bermejo et al. (2001) described a fast two-step chromatographic method for the purification of PE from *Porphyridium cruentum* – expended-bed adsorption chromatography using Streamline DEAE followed by conventional ion-exchange chromatography (DEAE cellulose). The same authors then did the baseline separation of its α, β and γ-subunits by a reverse-phase HPLC gradient semipreparative method. Circular dichroism and spectroscopic characterization was done for PE (Bermejo et al., 2003). Rossano et al. (2003) extracted and purified R-PE from Mediterranean red algae *Corallina elongata* Ellis & Solander, using hydroxyapatite column. The purity index for PE obtained in this study was above 6. Isailovic et al. (2004) isolated and
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characterized R-PE subunits and its enzymatic digests. Their results show efficient absorption and fluorescence of the R-PE subunits and digest peptides, originating from the incorporation of PEB and PUB chromophore groups in them. In addition, HPLC-ESI-MS and SDS-PAGE were used to determine the molecular masses of phycobiliprotein subunits and the chromophore-containing peptides, as well as the amino-acid sequences of the latter. Tripathi et al. (2007) extracted and purified an unusual phycoerythrin from a terrestrial and desiccation-tolerant cyanobacterium *Lyngbya arboricola*. PE was purified using acetone precipitation, gel filtration and ion-exchange chromatography resulting in PE of purity index 5.

Several gene-related studies have also been done for phycoerythrin. Anderson and Grossman (1990) studied structure and light-regulated expression of phycoerythrin genes in wild-type and phycobilisome assembly mutants of *Synechocystis* sp. strain PCC 6701. They found that the control of PE levels in *Synechocystis* sp. strain PCC 6701 is complex and may involve posttranscriptional processes. Bernard et al. (1992) characterized genes encoding PE in the red alga *Rhodella violacea*. The molecular events affecting the genes, such as *rpeB* intron, could give a clue to elucidate some aspects of the molecular processes involved in the evolution of plastid genes. Federspiel and Scott (1992) characterized light-regulated gene encoding a phycoerythrin-associated linker protein from the cyanobacterium *Fremyella diplosiphon*. Kahn et al. (1997) studied the role of *cpeYZ* in cyanobacterial PE biosynthesis. Their results showed that the *cpeYZ* gene products function in PE synthesis, possibly as a lyase involved in the attachment of PEB to the α or β subunit. Six et al. (2005) characterized two novel PE-associated linker proteins in the marine cyanobacterium *Synechococcus* sp. strain WH8102. Zhang et al. (2006) cloned, expressed and characterized PE gene from *Ceramium boydenn*. They found that there are several sequence regions which might reflect functional or structural requirements of the PE organization, and several residues known for their functional importance are conserved in almost all the sequences.

Only few phycoerythrin crystal structures are available in PDB of which only two are from cyanobacteria one of which is truncated phycoerythrin from *Phormidium tenue* (Soni et al., 2010) and the remaining ones are from red algae. Duerring et al. (1990)
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determined the three-dimensional structure of phycoerythrocyanin from the cyanobacterium *Mastigocladus laminosus* at 2.7Å resolution by X-ray crystallography. The three (αβ)-subunits are arranged around a 3-fold symmetry axis, as in C-phycocyanin. The two chromophores of the β-chain are phycocyanobilin, whereas that of α-chain is a chemically different phycobilivoilin. Chang et al. (1996) determined the crystal structure of R-PE from *Polysiphonia urceolata* at 2.8Å resolution (Fig. 1.9). The structure was solved by the multiple isomorphous replacement method. There are 5 chromophore groups – four PEB and one PUB attached to an (αβ) unit covalently bound to the cysteine residues. They basically discussed the energy transfer and the relationship between cysteine residues and chromophores.

![Image of crystal structure](image)

**Fig. 1.9:** Crystal structure of R-phycoerythrin from *Polysiphonia urceolata* at 2.8 Å resolution (ILIA).

Jiang et al. (1999) described the structure and function of chromophores in R-PE from *Polysiphonia urceolata* at 1.9Å resolution. They have proposed that aromatic residues in linker proteins not only influence the conformation of chromophore, but may also bridge chromophores to improve the energy transfer efficiency. Wilk et al. (1999)
determined the crystal structure of a cryptophytes phycoerythrin at 1.63Å resolution. One of the key components of this cryptophytes system is water-soluble PE-545 whose expression is enhanced by low light levels. The structure of PE-545 demonstrates the evolution of a functional protein complex based on the rearrangement of related elements and the addition of new subunits that are unlikely to fold in isolation. The structure helps to understand how the cryptophytes light-harvesting system operates. Ritter et al. (1999) determined the crystal structure of phycourorubinin-containing PE from red alga Griffithsia monilis at 1.9Å resolution. The model consists of (αβ)2 dimer with an internal noncrystallographic dyad and a fragment of the γ-polypeptide. The α chain has two PEB chromophore groups and the β chain has two PEB and one PUB chromophore groups. They have briefly discussed possible energy transfer pathways. Contreras-Martel et al. (2000) determined the crystal structure of R-PE from Gracilaria chilensis at 2.2Å resolution. The molecule had structural features similar to those described for R-PE in Polysiphonia urceolata and Griffithsia monilis. However, they proposed the presence of a helical segment of linker protein in the central channel of R-PE because of interpretation of weak electron densities.

1.9 Economic importance of cyanobacteria and phycobiliproteins
Cyanobacteria have the appeal of being a raw unprocessed food, rich in carotenoids, chlorophyll, phycobiliproteins, amino acids, minerals, exopolysaccharides and many other bioactive components. Cyanobacterial phycobiliproteins, because of their unique colour, fluorescence and antioxidant properties possess a wide range of promising applications in the food and cosmetics replacing synthetic colorant, biomedical and therapeutic industries as fluorescent markers of cells and macromolecules. Cyanobacterial extracellular polymeric substances (EPSs) (polysaccharidic in nature) possess unique biochemical properties that make them interesting from the biotechnological point of view. Cyanobacteria produce complex exopolysaccharides composed of at least 10 different monosaccharides and are characterized by the presence of pentoses, which are usually absent in other polysaccharides of prokaryotic origin, and by their anionic nature, which is due to the presence of acidic sugars (glucuronic and/or galacturonic acids) and anionic organic
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(acetyl, pyruvil) and inorganic (phosphate and sulphate) substituents. Besides the standard applications of EPSs as food coating, emulsifying and gelling agents, flocculants and hydrating agents, the anionic nature of cyanobacterial polysaccharides makes them interesting for biomedical applications also. Cyanobacteria such as *Microcystis*, *Anabaena*, *Nostoc* and *Oscillatoria* produce a great variety of secondary metabolites (Singh et al., 2005). Cyanobacteria produce a wide variety of toxins and other bioactive compounds having activities such as cytotoxic, anticancer (Gantar and Svircev, 2008), antiviral, antibiotic and others such as antimalarial, antimycotic, multi-drug resistance reversers, antifeedant, herbicides and immunosuppressive (Burja et al., 2001). The cyanobacterial composition depends on the nutrient contents of location and environment in which they are grown. The environment includes altitude, temperature, sunlight, water and other such factors which can greatly affect the lipid and pigment content in cyanobacteria. Consequently, cyanobacteria in itself or its products/constituents have found their applications in food and cosmetics, fluorescent markers, therapeutics, bioremediation, biofuels and many other such commercially and socially important fields.

Cyanobacteria have good potential as a food. Dried *Anthrospira* (*Spirulina*) is sold in the market as a health food with annual sales estimated at 40 million US $ (Pulz and Gross, 2004; Gantar and Svircev, 2008). Due to the toxic effect of several synthetic dyes, there is an increasing preference to use natural colours for various end uses in food, pharmaceuticals, cosmetics, textiles and as printing dyes. However, their utility is limited to only few of these since the natural dyes have low tinctorial values and persistence. Cyanobacterial phycobiliproteins are stable at low temperature for long duration, with addition of preservatives like citric acid and so they can be used in food industries as food colorant (chewing gum, jellies), health drink and colouring agent in sweet confectionary and cosmetics (Patel et al., 2004; Eriksen et al., 2008; Mishra et al., 2008). Cyanobacterial biopigments are also used in fluorescent labeling of antibodies that are applied in diagnostic kits in immunology, cell biology and biomedical research (Sekar and Chandramohan, 2008).

Cyanobacteria improve metabolism and also have a cholesterol-lowering effect in animals and humans. Cyanobacteria contain significant amounts of carotenoids (β-
carotene, lycopene, and lutein) having antioxidant properties (Soni et al., 2008). By the quenching action on the reactive oxygen species, these carotenoids also have anti-inflammatory activities. Omega 3-fatty acids like eicosapentanoic acid (EPA) and docosahexaenoic acid from microalgae have therapeutic importance (Pulz and Gross, 2004; Gantar and Svircev, 2008). EPA is used in the treatment of heart and inflammatory diseases. The protection of human lymphoblastoid T cells from the cytopathic effect of HIV infection with the extract of cyanobacteria *Lyngbya lagerheimeii* has been reported (Gustafson et al., 1989). A new class of HIV inhibitors called sulfonic acid, containing glycolipid, was isolated. Cyanobacterial extract containing lipopeptides and compounds were found to be active against the HIV virus. Cyanoviridin-N, isolated from cyanobacteria, inactivates the strains of HIV virus and inhibits cell to cell and virus to cell fusion (Yang et al., 1997).

Phycobiliproteins are chromo-proteins having anti-inflammatory, hepato-protective and antioxidant properties, which could play a crucial role in photodynamic action during tumor and leukemia treatment (Vadiraja et al., 1998; Khan et al., 2005; Patel et al., 2006; Paul et al., 2006; Kulshreshtha et al., 2008). Phycoerythrin is beneficial for human health, having antioxidant, radical scavenging, anti-inflammatory and anticancer properties (Li and Qi, 1997; Gonzalez et al., 1999; Olvera-Ramirez et al., 2000; Bhat and Madhyastha, 2000; Estrada et al., 2001; Tseng, 2001; Soni et al., 2008).

In the field of bioremediation, cyanobacteria have been known to be involved in dye degradation and moreover cyanobacterial exopolysaccharides are used to remove toxic metals from polluted waters (Otero and Vincenzini, 2003). Biofuel-bioenergy production has generated intensive interest due to increased concern regarding limited petroleum-based fuel supplies and their contribution to atmospheric CO₂ levels. Biofuel research is not just a matter of finding the right type of biomass and converting it to fuel, but it must also be economically sustainable on large-scale. Several aspects of cyanobacteria and microalgae such as oxygenic photosynthesis, high per-acre productivity, non-food based feedstock, growth on non-productive and non-arable land, utilization of wide variety of water sources (fresh, brackish, seawater and wastewater) and production of valuable co-products along with biofuels have
combined to capture the interest of researchers and entrepreneurs. Currently, worldwide biofuels mainly in focus include biohydrogen, bioethanol, biodiesel and biogas. Consequently we can observe that though cyanobacteria are one of the most primitive organism, yet have a significant value commercially and socially even in the twenty first century.

1.10 Objectives of the study

The objectives of the research presented in this thesis are as follows:

- Isolation of intact phycobilisomes from different cyanobacterial cultures.
- Purification and characterization of allophycocyanin from Geitlerinema sp. A28DM.
- Purification and characterization of intact phycoerythrin and its cleaved 14 kDa functional subunit from marine cyanobacterium Phormidium sp. A27DM.
- Purification, characterization and comparison of phycoerythrins from three different cultures namely, Phormidium sp. A27DM, Lyngbya sp. A09DM and Halomicronema sp. A32DM.
- Influence of light on phycobiliprotein composition in the same three marine cultures namely, Phormidium sp. A27DM, Lyngbya sp. A09DM and Halomicronema sp. A32DM.
- Structure determination of phycoerythrins from Phormidium sp. A27DM and Lyngbya sp. A09DM.
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