ABSTRACT

This thesis describes the research on C-phycocyanins (C-PC) from three cyanobacteria and an alkaline thermoactive xylanase (ATBXYL-C) from a Bacillus species. The first chapter involves a general introduction to the proteins under study, based on extensive review of available reports, publications and communications. The second chapter incorporates all the materials and methods employed in the course of the work. Next two chapters (3 & 4) record the results of various experiments on cyanobacterial phycocyanins, their analysis and comparison to existing knowledge. Similarly, last two chapters (5 & 6) involve documentation of results and relevant discussion about the xylanase.

The major light-harvesting capacity of prokaryotic cyanobacteria and eukaryotic red algae is associated with large antennae complexes called phycobilisomes located on the surface of the photosynthetic thylakoid membranes. The phycobilisomes are composed of rods and a core, consisting of various phycobiliproteins and linker polypeptides. The phycobiliproteins are divided into three major classes: phycoerythrins, phycocyanins and allophycocyanins. The rods in phycobilisome normally have phycocyanin and the core has allophycocyanins. C-phycocyanins are composed of α and β subunits exhibiting high mutual affinity to form (αβ) monomers, which in turn aggregate into (αβ)₃ trimers and (αβ)₆ hexamers. Phycocyanins not only absorb light energy but also transfer the absorbed energy from phycoerythrins to allophycocyanins in the core and finally to the photosynthetic reaction center. In this thesis, we report the purification, crystallization and crystal structure analysis of C-PCs from the Indian cyanobacteria Phormidium and Lyngbya spp. of marine habitat and Spirulina sp. of freshwater habitat. The crystal structure analysis of C-PCs has thrown light upon how the organization of C-PC units as seen in crystals helps in energy transfer.

Xylanases produced by extremophiles are important due to their biotechnological applications and as model systems in structure-function studies. The paper and pulp industries use xylan-degrading enzymes for the pretreatment of paper pulp to enhance the bleaching effects. By using xylanases the quantity of bleaching chemicals can be reduced,
thereby reducing the amount of toxic by-products and results in cost benefits also. Many studies on xylanases belonging to family G/11 have been reported. However, information, especially structural characterization, about very few alkaline xylanases has been reported. The active site characterization, fluorescence studies and determination of three-dimensional structure are of great interest, since they can lead to a better understanding of the catalytic mechanism and structural stability which in turn helps in the rational design of enzyme with selected specificity and enhanced stability. In the second part of the thesis detailed structural and active site characterization and fluorescence studies on an alkaline thermoactive xylanase from an alkalophilic Bacillus sp (NCL 87-6-10) is presented.

Chapter 1
General introduction

This chapter deals with general introduction, history, classification, role and application of C-phycocyanins (C-PCs) and xylanases. A detailed survey of literature on different algal C-phycocyanins and bacterial family G/11 xylanases is presented in this chapter.

C-phycocyanins

Phycobiliproteins are major photosynthetic accessory pigments, found primarily in prokaryotic cyanobacteria, and two groups of eukaryotic algae, the red algae and cryptomonads. The red algae and cyanobacteria also contain chlorophyll-a, and the cryptomonads contain both chlorophyll-a and c. The chlorophylls are located in the thylakoid membranes, whereas the phycobiliproteins are found on the exterior of these membranes. The phycobiliproteins have capacity to harvest light energy at wavelengths at which chlorophyll-a shows less absorption. This property of phycobiliproteins is especially useful in underwater habitats, where the light is both attenuated and altered in spectral distribution by passage through water. In particular, light tends to become blue green as it proceeds down the water column, and this light is better absorbed by phycobiliproteins than by chlorophyll-a. The rods in phycobilisome normally include phycocyanin; they sometimes also contain phycoerythrin or phycoerythrocyanin at the tips of the rods in some species. The phycobilisome core is composed of
allophycocyanins and linker polypeptides. The hexameric CPCs, the major component of the rods, not only absorb light energy but also transfer the absorbed energy from phycoerythrins to allophycocyanins in the core. The energy is finally transferred to the photosynthetic reaction center.

**Xylanases**

The increased interest in the study of extremophilic microorganisms can be attributed mainly to their importance as biotechnological resources and their specific properties are expected to find applications in novel industrial processes. Useful insights gained from the study of enzyme systems in extremophiles can extend the understanding of protein chemistry along with expanding the horizon of application of biocatalysts. Xylan is the major component of hemicellulose, which accounts for 30% of the dry weight of some plant tissues. Microbial xylanases are the preferred catalysts for xylan hydrolysis due to their high specificity, mild reaction conditions, and negligible substrate loss by the efficient generation of product. Cellulase-free xylanases have attracted much interest, especially in paper and pulp industry. Xylanases play an important role in the development of eco-friendly processes by reducing the use of hazardous chlorine chemicals in bleaching processes. They are also important from the point of view of fundamental research, since multiple xylanases produced by an organism have diverse structure-function correlation. The commercial and academic importance of extremophilic microorganisms, prompted us to carry out biochemical and structural studies of a xylanase from an alkalophilic *Bacillus* sp.

**Chapter 2**

**Materials and methods**

This chapter describes the details of materials and methods used for the preparation of C-phycocyanin from *Spirulina sp.*, *Phormidium sp.*, *Lyngbya sp.* and alkaline thermoactive xylanase from alkalophilic *Bacillus* sp., their fermentation, purification, crystallization, X-ray diffraction data collection, data processing, structure determination, structure refinement, analysis of the refined structure and biochemical/biophysical characterizations.
The proteins were purified by ion-exchange chromatography using DEAE-sepharose and CM-sepharose and by Gel-filtration using G-100. Hanging-drop vapour-diffusion method was used for crystallization. The X-ray data were collected using Raxis IV++ detector mounted on a rotating anode at NCL, Pune, India. The X-ray images were processed using DENZO and SCALEPACK program in HKL suite. The crystal structures were determined using molecular replacement technique implemented in AMoRe and PHASER. The REFMAC5 program was used for structure refinement in cycles along with the program QUANTA for display and model fitting. The programmes of CCP4 suit were used for most of the calculations.

Chapter 3
Purification, crystallization and preliminary X-ray characterization of C-phycocyanins from Phormidium and Lyngbya spp. (marine) and Spirulina sp. (fresh water).

C-phycocyanins from three cyanobacterial cultures of Phormidium, Lyngbya spp. (marine) and Spirulina sp. (freshwater) were purified to homogeneity and crystallized by hanging-drop vapour-diffusion method. Blue-coloured crystals in different crystal forms, monoclinic for all three species and hexagonal for two, were obtained. The crystals took 1–12 weeks to grow to full size using polyethylene glycols of different molecular weights as precipitants.

The biochemical and biophysical properties, i.e. molecular weight, stability etc. of C-PCs from fresh water and marine forms showed differences. The C-PCs were crystallized to investigate their structural differences in marine and fresh water forms. All of them crystallized in monoclinic space group P2_1 with unit-cell parameters a = 107.33, b = 115.64, c = 183.26 Å, β= 90.03° for C-PC from Spirulina sp. and had similar parameters for CPC crystals from Phormidium and Lyngbya spp. also. Crystals belonging to the hexagonal space group P6_3, with unit-cell parameters a = b = 154.97, c = 40.35 Å and a = b = 151.96, c = 39.06 Å, were also obtained for C-PCs from Spirulina and Lyngbya spp. X-ray diffraction data was collected at resolutions of 3.0 Å for the monoclinic and 3.2 and 3.6 Å for the hexagonal forms. The estimated solvent content was
around 50% for the monoclinic crystals assuming the presence of two hexamers, \((\alpha\beta)_6\), per asymmetric unit. By assuming the presence of one \((\alpha\beta)\) monomer per asymmetric unit the solvent content estimated for the hexagonal crystals was 66.5 and 64.1% respectively for *Spirulina* and *Lyngbya* spp..

**Chapter 4**

**Crystal structure analysis of C-phycocyanins from *Phormidium, Lyngbia* and *Spirulina* spp.**

The crystal structures of C-phycocyanins from cyanobacteria (blue-green algae) *Phormidium*, *Lyngbya* and *Spirulina* spp. have been determined using molecular replacement technique. After refinement the final crystallographic \(R_{factor}\) and \(R_{free}\) were 19.9 and 24.0 (*Spirulina* sp. C-PC), 19.68 and 24.6 (*Phormidium* sp. C-PC) and 21.5 and 25.7 (*Lyngbya* sp C-PC), respectively, for monoclinic crystals of data at 3.0 Å resolution. The asymmetric unit contained two \((\alpha\beta)_6\) hexamers, each hexamer may be considered the functional unit in the native antenna rod of cyanobacteria.

The molecular structure resembles that of other reported C-PCs. The aggregation of two \((\alpha\beta)_6\)-hexamers in the asymmetric unit suggests pathways of lateral energy transfer between adjacent hexamers involving phycocyanobilin chromophores. The chromophores involved, distance between two adjacent hexamers in the asymmetric units and conformation were different in C-PC crystals of marine (*Phormidium* and *Lyngbia* spp.) and freshwater (*Spirulina* sp) forms. In the asymmetric units monoclinic crystals of *Spirulina* C-PC the chromophores are closer to each other. Whereas the chromospheres between two hexamers in the asymmetric units of the crystals of *Phormidium* and *Lyngbya* C-PCs are not close. Conformation and closeness of chromophores between two hexamers in the asymmetric units of C-PCs from *Spirulina* sp. can facilitate efficient energy transfer. The growth of *Spirulina* sp. is faster compared to *Phormidium* and *Lyngbya* spp.. However, a careful analysis it is found that the difference in asymmetric
units, are a results of the choices of origin in the asymmetric unit. Thus both type of interactions are found in all C-PC crystals.

Chapter 5

Crystallization, active site characterization and stability studies of xylanase from alkalophilic Bacillus sp.

This chapter discusses the crystallization, chemical modification of active site residues and fluorometric studies on the stability of xylanase from Bacillus sp.

Initial screening provided conditions that gave thin crystals which were subsequently improved to get better crystals and higher resolutions of diffraction data. Enzyme inactivation using the residue or group specific reagents such as N-bromosuccinimide, Woodward’s reagent K and Phenyl glyoxal combined with substrate protection studies identified Trp and Arg in substrate binding while carboxyl group in catalysis. Two population of tryptophans, one in polar and the other in hydrophobic environment, were detected by the intrinsic fluorescence spectrum of native xylanase. Thermal behaviour showed an increase in activity of the enzyme till 60°C and then a sudden drop at 70°C, associated with 2 to 3 times increase in fluorescence ascertaining role for Trp in activity.

1-Anilino-8-sulfonic acid (ANS) binding data showed presence of exposed hydrophobic patches at extreme acidic pH of the enzyme. Although, enzyme is inactivated by 1M guanidium hydrochloride (Gdn-HCl), substantial increase in fluorescence occurred only at 3M, further increase in Gdn-HCl resulted in a red shift in $\lambda_{\text{max}}$ which could be an indication of unfolding. Fluorescence quenching studies using acrylamide, potassium iodide (KI) and cesium chloride (CsCl) showed maximum quenching due to acrylamide and no quenching due to CsCl. Quenching of fluorescence by KI is interpreted as indication of positively charged Trp environment. Maximum intrinsic fluorescence quenching by simple xylo-oligosaccharides such as xylobiose (X2)
and xylotriose (X3) were only 4%, 9%, respectively, indicating that the affinity of xylanase for latter is higher. All these observations have been correlated with the structure analysis in the next chapter.

Chapter 6

The three-dimensional crystal structure analysis of xylanase in two crystal forms, orthorhombic and tetragonal.

This chapter presents the complete three-dimensional structure analysis of the alkaline thermoactive xylanase from alkalophilic Bacillus sp. The structure solution was obtained by molecular replacement methods using PDB code 1H4G as model. The structures are refined at resolutions 2.8 (space group P2₁₂₁₂₁) and 2.4 Å (space group P4₃2₁2) to $R_{\text{factor}}$, $R_{\text{free}}$ 16.9, 23.0 and 21.0, 24.0, respectively. Coordinates of space group P2₁₂₁₂₁ and P4₃2₁2 were deposited in PDB with PDB ID 2F6B and 2NQY respectively. Comparison between the crystal structures of monoclinic and tetragonal forms and with other reported structures of family G/11 xylanases are discussed.