CHAPTER I

INTRODUCTION

Recently , there has been considerable interest worldwide in the development of food colorants from natural sources. Although, natural food coloring has a fairly long history , one indication of this is the number and distribution of food colorant patents issued in the years 1969 through 1984. In addition , natural colorants are attracting more interest than synthetic colorants. Since certain synthetic pigments are not only harmful to man but may also be carcinogenic , many countries have limited their uses in the food industry. There were 356 patents on natural sources compared to 71 synthetics (Francis , 1987).

The algal biliproteins, or phycobiliproteins, function as important light harvesting component for driving the photosynthetic reactions in organism of the prokaryotic blue-green algae (Cyanophyta) , the eukaryotic red algae (Rhodophyta) and of the eukaryotic cryptomonad (Cryptophyceae). They are composed of algal bile pigment , or phycobilins, covalently linked to a specific protein. The absorption spectra of the major biliproteins lead to the classification of these proteins into three groups:

phycoerythrins , red biliproteins with the phycoerythrobilin chromophores having absorption maxima at 570 nm; phycocyanin , blue biliproteins with the phycocyanobilin chromophores having a major absorption band at approximately 620 nm; and allophycocyanin, the blue biliprotein with a sharp absorption maximum at 650 nm (Emerson and Lewis,1942). The mechanism of the energy transfer process was examined by Arnold and Oppenheimer (1950). They concluded that only mechanism of importance for the transfer of energy from phycocyanin to chlobophyll was the resonance transfer of energy from one oscillator to another in resonance with it.

The characteristics of phycobiliprotein is conferred by covalently linked linear tetrapyrrole chormophores (bilins). Crespi and Smith (1970) proposed that phycocyanobilin was doubly linked to the peptide chain , one bond being an ester involving the β-carboxyl group of an aspartyl residue and the hydroxyl group of the enol form of ring A of the bilin and the other bond a thioether derived from a cysteine side-chain to the methine carbon of the ethylidene group at position 2 of ring A of phycocyanobilin (Figure 1).

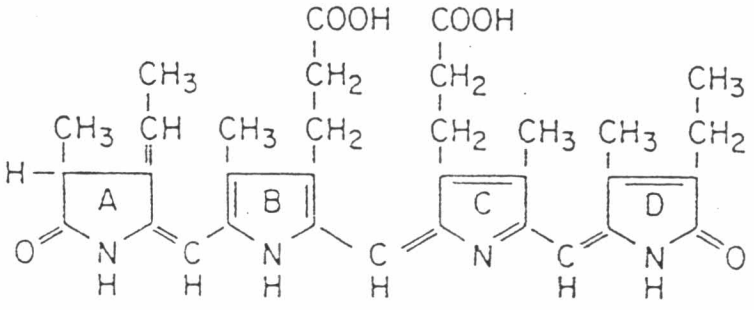

Figure 1 Chemical structure of phycocyanobilin (Glazer, 1984)

Phycobiliproteins are intensely colored. Thus , phycocyanin and phycoerythrin could be utilized as natural pigments for food, drug and cosmetics industries to replace the currently used synthetic pigments that are suspected of being carcinogens. Pigment preparations soluble in either water or alcohol can be prepared and are suggested for use in chewing gum, frozen confections, sherbets confectioneries, candied ices (Francis,1987) and mix in liquor (Dainippon Ink and Chemicals Inc,1980). Ten patents were applied in the year 1979 through 1987, of three deal with *Spirulina* (Francis,1987). Phycocyanin from *Spirulina* has already been commercialized by Dainippon Ink & Chemicals of Japan under the name of "Linablue". The product is an odorless nontoxic blue powder with a slight sweetness and has brilliant blue with a faint reddish fluorescence in water. In another patent, Dainippon Ink describes the

buffer extract of *Spirulina*, the blue pigment obtained being used for eye shadow, eye liner and lipstick. For other applications, the phycobiliproteins have been widely applied as fluorescent tags in a variety of analytical and diagnostic procedures particularly in multiparameter fluorescence activated cell analyses (Glazer,1988).

The phycobiliproteins are organized into granules , or phycobilisome localized on the outer surface of the thylakoid membranes (Gantt,1975). The thylakoid membranes of phycobilisome, uncommon with those of higher plants, contain photosystem I (PS I) and Photosystem II(PS II). There are some noteworthy differences in the composition and organization of photosynthetic structure in these organisms and in green plants. Higher plants contain both chlorophyll a and chlorophyll b. The latter is a prominent component of the antenna of PS II (Glazer,1983). Cyanobacteria and red algae contain only chlorophyll a. However, additional light harvesting capacity is provided by a family of brightly colored proteins (biliprotein) present in large amounts. The grana regions, formed of appressed thylakoids, so characteristic of higher plant chloroplasts, are absent in cyanobacteria and red algae. Instead, the outer surface of their thylakoid membranes is studded with regularly spaced phycobilisomes multiprotein complexes of which biliproteins make up over 85% (Tandeau de Marsac and Cohen-Bazire , 1977 ; Yamanaka,

Glazer and Williams, 1978). The strong absorption bands of the major biliproteins lie in the region of 470-650 nm, whereas those of the chlorophyll a complexs are at approximately 430-440 nm and 670 nm. This separation of the major absorption bands permits analysis of the relative contributions of the biliproteins and of chlorophyll a to the action spectra of PS I and PS II (Glazer,1984). It is found that in cyanobacteria, P-700, the reaction center of PS I , is associated with an antenna of some 140 chlorophyll a molacules. The reaction center of PS II is associated with only approximately 20-50 chlorophyll a molecules, but receives most, if not all , of energy harvested by phycobilisomes (Glazer,1984).Membrane-bound pigment-protein complexes that function in harvesting or absorbing radiant energy and tranferring it to the photosynthetic reaction center are found in all photosynthetic organisms. In prokaryotic cyanobacteria and eukaryotic red algae , the major light-harvesting complex is the phycobilisome, a water-soluble,supermolecular structure attached to the stromal surfaces of the thylakoid membranes (Conley, Lemaux and Grossman,1988). The phycobilisome is far more efficient than any man-made device for the transduction of solar energy. Depending on their organismal origin, phycobilisomes contain between 300-800 tetrapyrrole chromophores which absorb light over much of the visible spectrum. The design of the

phycobilisome is such that excitation energy is delivered from any one of these many chromophores to a reaction center in the photosynthetic membrane with an efficiency approaching 100 % (Glazer, 1984).

Phycobilisomes have unusual stability properties. They are stable in concentrated solutions of certain salts (for example , 0.65-1.0 M sodium potassium phosphate or 0.8 M Na_2SO_4) at pH 7-8 , but dissociate into a mixture of their constituent complexes upon dilution of the salt. Either partial or complete dissociation into water-soluble subcomplexes can be achieved by appropriate manipulation of conditions. The final and perhaps most important feature is that the open-chain tetrapyrrole chromophores (bilins) of the biliproteins are all covalently linked to the polypeptide chains through stable thioether bonds (Glazer,1984).

In primary structure all of the major biliproteins are oligomers of an $\alpha\beta$ monomer , where α and β are dissimilar polypeptide chains of approximately 160-180 residues (Glazer,1984). The intensely colored phycobiliproteins allophycocyanin , phycocyanin and phycoerythrin have two dissimilar polypeptide chains (α and β) that vary in molecular weight from 17 to 22 kDa. The number of chromophores varies for the different phycobiliprotein subunits (Conley et.al.,1988). As shown in Figure 2, for example, R-phycocyanin , $(\alpha\beta)_3$, contains six phycocya-

nobilin (PCB) and three phycoerythrobilin (PEB) groups per trimer ; each α subunit carries one PCB, whereas each β subunit carries one PCB and one PEB. The absorption and fluorescence emission maxima are given for the higher aggregate of each of the biliproteins,i.e., in the case of R-phycocyanin,the values refer to the $(\alpha\beta)_3$ aggregate. The precise values differ slightly with the organismal source of the protein (Glazer,1984).

The basic building block of all the biliproteins is a monomer , αβ made up of two different polypeptide chains. As documented later , in phycobilisomes the biliproteins of the rod (phycoerythrins,phycoerythrocyanins, phycocyanins) are present as hexameric, $(\alpha\beta)_6$, complexes with linker polypeptides and the complicated allophycocyanin-containing complexes of the core can be broadly described as trimeric (Glazer,1984).

The biliproteins are isolated in any one of a number of aggregation states , which include αβ, $(\alpha\beta)_2$, $(\alpha\beta)_3$, $(\alpha\beta)_4$ and $(\alpha\beta)_6$ depends on the organism from which the protein is isolated , the pH , ionic strength , composition of the solvent , protein concentration and temperature (Glazer, 1984). Native phycocyanin is unstable at pH values below 4.0 and above 9.0 (Glazer,1976).

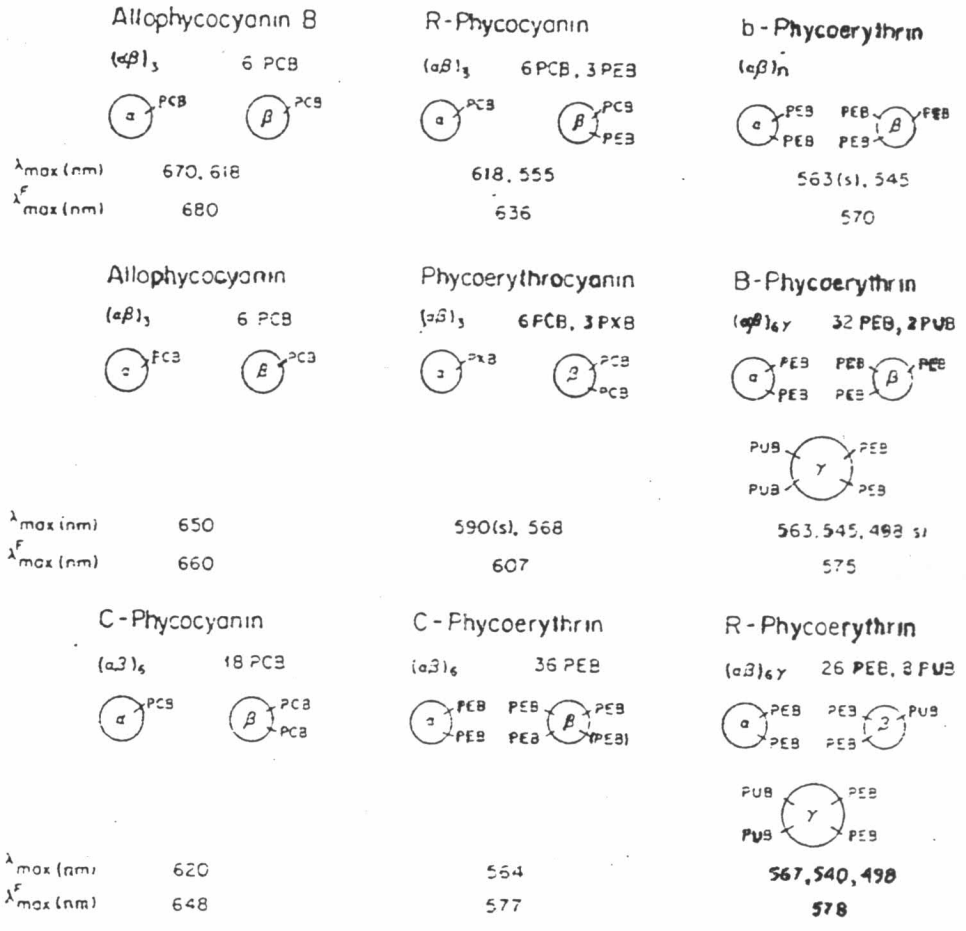

Figure 2 The nature and distribution of bilin prosthetic groups among the subunits of the various biliproteins and the numbers present in higher aggregates of each protein (Glazer, 1984)

Since phycobiliproteins are the major proteins in cyanobacteria , a lot of work have been carried out to study these proteins. Reunjitchachawaly et. al., (1988) studied optimal condition to produce pigment from *Spirulina* sp. and found that concentration of NaCl less than 3.0 % did not affect growth of cells. However at 3.0 % NaCl chlorophyll a and phycocyanin contents were decreased to 33% and 13 % respectively.

Allen and Smith (1969) were the first to point out the possibility that phycocyanin may be a nitrogen storage compound in the cell and nitrogen deficiency reduce phycocyanin in *Anacystis* sp. . Later on Boussiba and Richmond (1980) showed that C-phycocyanin serves as a nitrogen source in *Spirulina platensis* during nitrogen starvation. De Loura (1987) reported that nitrogen deficiency during growth causes a change in pigment composition but no significant changes in whole cell lipid and fatty acid composition of the two cyanobacteria, *Pseudanabaena* sp. and *Oscillatoria splendida*. Nitrogen deficiency does not affect the cellular content in chlorophyll a , but it causes a selective loss in phycobiliproteins ; carotenoid content increases with phycocyanin depletion (De Loura , Dubacq and Thomas , 1978).

Myers and Kratz (1955) reported the effect of light intensities and temperature on photosynthetic characteristics and pigment content in *Anacystis nidulan*. They found that the high temperature (40°C) and increasing light intensities resulted in the increase in photosynthesis. However , the pigment content was decreased with increasing light intensities.

Engelmann and Gaidukov (1902) reported that the pigmentation of certain cyanobacteria can be modified by light quality. The growth of such organisms behind a series of different colors filters caused increased light absorption by the cell in the specific spectral region to which they have been exposed , a phenomenon which these authors termed " complementary chromatic adaptation ". However, the existence of such a chromatic response was questioned by several investigators, who were unable to repeat the observations of Engelmann and Gaidukov on other strains of cyanobacteria. The controversy was resolved by Boresch (1922) who was the first to comfirm the results of Engelmann and Gaidukov. He showed that the chromatically induced change in the color of the cells is largely attributable to a change in phycoerythrin-phycocyanin ratio. Phycocyanin predominates after growth in red light and phycoerythrin predominates after growth in green light , the transition occurring at a wavelength of approximately 590 nm. Bogorad (1975) also

studied chromatic adaptation in two filamentous cyanobacteria, *Tolypothrix tenuis* and *Fremyella diplosiphon*. Two main conclusions have emerged from this work. The chromatically induced modification of the phycoerythrin-phycocyanin ratio involves de novo protein synthesis (Emerson and Lewis,1942). There is an evidence, both indirect and direct, which suggests that the relative rates of phycoerythrin and phycocyanin synthesis are controlled by a regulatory pigment analogous to , but not identical with , phytochrome. This regulatory pigment appears to exist in two forms , interconvertible by irradiation with specific wavelengths of light (Tandeau De Marsac, 1977).

Tandeau De marsac and Cohen - Bazire (1977) isolated phycobilisomes from eight different species of cyanobacteria and found that light quality affected differentially the rate of phycoerythrin and phycocyanin synthesis ; phycobilisomes prepared from algae after growth in white, red and green light differed markedly in their phycobiliprotein composition. "Green-light" phycobilisomes had a high PE:PC ratio, "white-light" phycobilisomes had a somewhat lower PE:PC ratio , and "red-light" phycobilisomes were virtually devoid of phycoerythrin. These light induced modifications of the major phycobilisomal light-harvesting proteins were accompanied by marked changes in the relative concentration of the

colorless group II polypeptides (Bennett and Bogorad, 1971).

In this study , the unicellular cyanobacterium , *Aphanothece halophytica* was chosen as a source of biliprotein. This alga is classified into Chroococcales order , chroococcacean cyanobacteria subgroup. Sexual reproduction is absent in *A. halophytica.* The cell reproduces by binary fission.

Objectives

The objectives of the research are:

1. To select the method for phycocyanin extraction from *Aphanothece halophytica.*

2. To study the effect of environmental factors on *Aphanothece halophytica* cultivation for high phycocyanin production.

3. To mutagenize *Aphanothece halophytica* by irradiation with UV-Light and treatment by chemical mutagen (NTG) and compare the mutated cells with the normal cells for phycocyanin content.

4. To partially purify phycocyanin.