CHAPTER IV

DISCUSSION

Methods for Phycocyanin Extraction

Several methods are available and selection of a particular procedure is based on the ease of cell breakage. The algal biliproteins can somtimes be extracted by placing the algae in water and allowing the proteins to leach out for several days (Svedberg and Katsurai, 1929). A general procedure which has proven useful for extracting many algae is repeated freezing and thawing in the presence of phosphate buffer with neutral pH. Some blue-green algae can be disrupted with lysozyme (Crespi et. al.,1962) which is simply achieved on a small scale by adding uncooked egg white to the algae. The extraction process may have to be repeated several times for a quantitative yield. The extracted biliproteins are separated from the algae by filtration or centrifugation.

Many preliminary experiments were done in order to select the best method of extracting phycocyanin from *Aphanothece halophytica*. The method of freeze thaw did not work because this method was not able to completely break the cell wall of *Aphanothece halophytica*. Thus, all of phycocyanin could not be released from the cells. Another important reason that we did not use this method

because it took a long time which was probably due to unsuitable conditions used in the experiment.The methods of sonication was also used. But the conditions for this method was so strong that after centrifugation the color of the supernatant became dark green (instead of blue supernatant) because chlorophyll came out together with phycocyanin. Before we could measure the absorbance of phycocyanin at 620 nm we had to remove chlorophyll by using acetone extraction. Thus, it was not a convinient method. However, Lysozyme digestion method was chosen because of its ability to completely digest the cell wall of *A. halophytica*. So all of phycocyanin could be released from the cells with a relatively short incubation time of 1 hr. This was confirmed by the appearance of the cell debris which was devoid of the blue colour. In addition , lysozyme digestion method has many advantages ; reproducible , time saving and suitable for quantitative experiments.

Effect of Environmental Factors on *Aphanothece halophytica* Cultivation for High Phycocyanin Production

The complexity of the trophy terminology of algae stems largely from overlapping of the various nutritional modes due to the ability of many algal species to change their nutritional patterns in response to environmental circumstances. In general , two major forms of nutrition exist in algae : autotrophy and heterotrophy

and both modes are employed in algaculture. Autotrophs (or lithotrophs) obtain all the elements they need from inorganic compounds and the energy for their metabolism from light or the oxidation of inorganic compounds or ions. Heterotrophic algae obtain their material and energy needs from organic compounds synthesized by other organisms (Richmond,1986). Much research has been conducted to determine the optimum mineral composition of growth media for various algal species. Most formula for culture media differ greatly from what the algae would have in the natural environments because the concentrations of all the required nutrient element usually far exceed their natural levels.

In response to salinity , the algae as a group exhibit an extremely wide range of tolerance to salts in their surrounding. Some species can tolerate only milimolar amounts of salt, while others survive in saturated brine. As for the adaptation to salinity, algae may be roughly divided into halotolerant and halophilic , the latter requiring salt for optimum growth and the former having response mechanisms that permit their existence in saline medium (Richmond, 1986). The observation that chloride is essential for production of oxygen by isolated chloroplasts has led to the view that chloride acts as an electron-tranporting agent in photophosphorylation. In addition , high-salt algae have

a higher chlorophyll content per cell and are able to absorb more light per cell. They grow better under high light intensities and exhibit a higher chlorophyll-a/b ratio. Also , high-salt algae perform a higher rate of photosynthesis and need more light (Gimmler et. al., 1981). *Aphanothece halophytica* can grow under a wide range of NaCl contration (0.1-3 M) (Reed et. al., 1984). Hence, the photosynthetic apparatus must adapt to different degree of salinity in the habitat by means of either osmoregulation or modification of enzyme structure. Plant and algal cells regulate the intracellular osmoticum against high external salt by accumulation of glycerol , mannitol , proline and other low molecular weight substances (Tel-Or and Melamed-Harel, 1981). The study of the effect of NaCl concentration of growth and phycocyanin production indicated that there were slightly differences on growth of *A. halophytica* when grown in the range of NaCl concentration between 0.25-1.5 M after day 9. The growth was delayed at either too low salinity (0.125 M NaCl) or too high salinity (2.0 M NaCl) (Figure 9). The highest phycocyanin content at about 100 mg/g dry weight was obtained at day 9 when cells were grown under 0.25 M or 0.5 M NaCl (Figure 10). Since at day 9 the number of *A. halophytica* cells when grown under 0.5 M NaCl was higher than that under 0.25 M NaCl, it appeared total phycocyanin content was the highest when cells were grown under 0.5 M NaCl and

total phycocyanin content was decreased when grown at above or below this concentration. Moreover, we chose NaCl concentration at 0.5 M to be the optimal condition for later experiments. The result indicated that salt stress reduced the growth and phycocyanin production. Ben-amotz et. al., (1982) found that salt stress injured the chloroplast. This is reflected in the reduction of growth rate.

Nitrogen is quatitatively the most important element contributing to the dry matter of algae cells. Nitrogen requirements and metabolism in relation to alga culture were summarized by Kaplan et.al., (1986). The proportion of nitrogen as the percent of dry weight may vary from 1 to 10 % and in exponentially growing cells of nondiatomaceous microalgae, nitrogen accounts for about 7 to 10 % of dry matter and carbon for about 50% (Vaccaro, 1965). Role of nitrogen in the plant is its presence in the structure of the protein molecule. In addition, nitrogen is found in such important molecules as purines , pyrimidines , porphyrins and coenzymes. Purines and pyrimidines are found in the nucleic acids essential for protein synthesis. The porphyrin structure is found in such metabolically important compound as the chlorophyll pigment and the cytochromes. Coenzymes are essential to many enzymes (Devlin & Witham, 1983). The study of the effect of N-source on growth and phycocyanin production of *A. halophytica* indicated that the cell growth was

gradually increased when grown under urea as a N-source. However, the cell growth was the highest when grown under sodium nitrate as a N-source. On the other hand the cell growth was sharply decreased when grown under ammonium acetate and stable when grown under ammonium nitrate as a N-source (Figure 11). Phycocyanin content was the highest when grown under sodium nitrate as a N-source and decreased when grown under urea , ammonium nitrate and ammonium acetate as a N-source,respectively (Figure 12). The results indicated that N-source affected growth and phycocyanin production of *A. halophytica*. N-source such as ammonium acetate and ammonium nitrate were composed of ammonium ion and urea could be converted to NH_4^+ by the cells. The NH_4^+ has been recognized as toxic substance for cells. *A. halophytica* was not able to grow under ammonium acetate or ammonium nitrate or urea as a N-source. Phycocyanin content was also decreased when grown under such N-sources. To sum up, sodium nitrate in the culture medium was the best N-source for growth and phycocyanin production of *A. halophytica*.

Inorganic nitrogen source , $NaNO_3$, was utilized for complete growth of *A. halophytica* in Turk Island Salt Solution + modified BG_{11} medium. It was indicated that $NaNO_3$ was an essential chemical for growth. Growth of *A. halophytica* cultured in Turk Island Salt Solution + modified BG_{11} medium without $NaNO_3$ was slightly

increased in the first 3 days and then obviously decreased until day 15. In addition, the cells ceased to grow after 6 days of cultivation at 0.1 g/l of $NaNO_3$. We assumed , from the fact that nitrogen is an essential constituent of protein , that nitrogen deficiency must cause a decrease in protein synthesis, which subsequently causes a decrease in cell size and especially cell division. So the reduction of growth was brought about primarily by the lack of sufficient proteins and nucleic acids for continued production of new cells. Not surprisingly, nitrogen deficiency limited growth more severely than deficiency of other elements.Algae completely deprived of nitrogen are frequently unable to grow larger and regenerate (Greulach ,1973). From Figure 14, phycocyanin content was the lowest in the medium without $NaNO_3$ and highest at 1.5 g/l of $NaNO_3$.As nitrogen deficiency develops, the amount of chlorophyll in the cells decreases faster than the total nitrogen content. Nothing is known of the changes in enzymatic balance which may occur (Lewn, 1962). The most easily observed characteristic of nitrogen deficiency was the yellowing or chlorosis which probably resulted not only because of inadequate nitrogen for chlorophyll synthesis but also because of reduced chloroplast protein production. From the results of the effect of $NaNO_3$ content on phycocyanin content , we summarized that $NaNO_3$ at 1.5 g/l was the optimal concentration to

produce the higest phycocyanin content in *A. halophytica*. However, phycocyanin content decreased when nitrate content dropped to 0 and 0.1 g/l. Similar results were reported in *Anacystis nidulan* by Allen and Smith (1969). They found that phycocyanin may be a nitrogen storage compound in the cell and nitrogen deficiency reduce phycocyanin. The study of effect of nitrate starvation on growth and phycocyanin production indicated that the cell growth was slightly increased when grown without $NaNO_3$ for 68 hr and clearly increased in the first day after the addition of $NaNO_3$ until final concentration was 1.5 g/l (Figure 15). The phycocyanin content was sharply decreased when grown under $NaNO_3$-free medium for 68 hr and sharply increased in the first day after the addition of $NaNO_3$ until final concentration was 1.5 g/l (Figure 16). Under the condition of nitrogen deficiency , the contents of photosynthetic pigment decrease and the rate of photosynthesis is reduced (Fogg, 1966). The accessory pigment phycocyanin is rapidly and specifically degraded in nitrogen-limited cell and reappears rapidly when nitrogen becomes available (Lau , Mackenzic and Dootittle, 1976; Foulds and Carr, 1977). In *Anabaena cylindrica* (Wood and Hasselkorn, 1976) protease can degrade phycocyanin and appears to be activated or preferentially synthesized during nitrogen starvation.

Light initiates the process of photosynthesis through photosynthetic apparatus, equipped with an intricate

array of membranes and pigments. Algal and higher plant photosynthesis requires the presence of light as an energy source. The light intensities in our experiment were 1,500 , 3,000 , 5,000 and 8,000 lux. There were no differences on growth of *Aphanothece halophytica* when the light intensities was increased but phycocyanin content became clearly decreased (Figure 17 ,18). Because high light intensity was usually accompanied by the higher temperature.This might be attributed to the excessive heat generated by high intensity of light causing the destruction of phycocyanin. In conclusion,light intensity at 1,500 lux was appropriate to produce highest phycocyanin content in *A. halophytica*. The quantitative data on the growth of *A. halophytica* showed that the growth in white light was higher than that in either red light or green light (Figure 20). However, the quantitative data on cellular pigment content of phycocyanin of *A. halophytica* showed that phycocyanin in red light was higher than that in either green light or white light (Figure 21). Our results indicated that the quality of light greatly affected phycocyanin content in *A. halophytica*. Similar results were reported by Engelmann and Gaidukov (1902). They found that the pigmentation of certain cyanobacteria can be modified by light quality : phycocyanin predominates after growth in red light and phycoerythrin predominates after growth in green light.

Initial chlorophyll concentration is an important factor for algal growth and phycocyanin production of *A. halophytica*. From Figures 22 and 23, the highest growth rate occurred at minimum initial chlorophyll concentration of 50 μg/ml. However, phycocyanin content was the highest when initial chlorophyll concentration was 100 μg/ml. Growth was slow at high initial chlorophyll concentration due to the overshadowing during the course of the experiments. Venkataraman (1985) suggested that the culture should be diluted several times when overshadowing occurs.

Temperature is one of the important factors for algal growth influencing the composition of cell structures, metabolic regulatory mechanisms and specificity of enzyme reactions. In addition, temperature affects cell reaction rates. Therefore, *A. halophytica* cultivation in optimal temperature is essential for high yield. From the results in Figure 24 and 25, the lowest growth and lowest phycocyanin occurred at 25 °C cultivation. However, there were no differences in growth between 30 and 40 °C of cultivation but phycocyanin content was the highest at 30 °C cultivation. So we chose 30°C as the temperature for *A. halophytica* cultivation since it gave the highest phycocyanin content and cultivation temperature in nature and in our laboratory for stock culture was 30°C.

Effect of Mutagenesis on *Aphanothece halophytica* Cultivation for High Phycocyanin Production

Cyanobacteria appear to be ideal organisms for the genetic study. They perform , within a noncompartmentalized prokaryotic cell , photosynthesis similar to that of higher plants. (Astier et. al.,1984). The studies on mutagenesis and genetics of these organisms were started only in the early 1960s. The present knowledge of the genetics of blue-green algae is far less than that of bacterial genetics because of several practical and technical problems that have hampered progress. A major problem was the isolation of mutants of cyanobacteria since it was the lack of specific agents suitable for enriching the mutant population (Ladha and Kumar, 1978). The incidence of mutation in the unicellular species *Synechocystis* PCC6714 and *Synechocystis* PCC6803 as measured by the frequency of colonies resisted to *p*-fluorophenylalanine or fructose,respectively. (Thiel and Leone, 1986). However, Guikema and Sherman (1980) have developed a protocol for the isolation of temperature-sensitive photosynthesis mutant of *Synechococcus cedrorum* using the redox-active drug metronidazole (2-methyl-5-nitroimidazole-1-ethanol). In this study , we performed mutagenesis on *A. halophytica* by classical methods including use of UV irradiation and chemical mutagen (NTG). Cycloserine was employed as a selective agent for mutants.

growth of *A. halophytica* mutants when grown under Turk Island Salt Solution + modified BG_{11} medium was not different from the normal *A. halophytica* (Figure 29,34). However , phycocyanin content of *A. halophytica* mutants were lower than the normal *A. halophytica* (Figure 30,35). Van Baalen (1968) studied the effects of UV-Light on survival and photosynthetic rates of the coccoid *Agmenellum quadruplicatum.* He found a direct correlation between loss of photosynthesis and survival after exposure to UV-light. This UV-induced loss of photosynthetic activity could be recovered by exposure to photoreactivating conditions. Therefore, he suggusted that UV primarily damage the photosynthetic apparatus and that photorecovery occurred by photorepair of the photosynthetic system. As the blue-green algae are photosynthetic, it is somwhat difficult to determine whether the killing by UV irradiation results from the DNA damage or is caused by some injury to the photosynthetic machinery. The results in the present study indicated that mutagenesis on *A. halophytica* by classical method (non-specific mutant) may reduce photosynthetic rates and destroy enzymes for phycocyanin synthesis. Therefore, mutagenesis on *A. halophytica* cultivation for high phycocyanin production should be specifically targeted at the genes coding for enzymes synthesizing phycocyanin.

Partial Purification of Phycocyanin

Partial purification was based on the use of two DEAE-cellulose columns. The first DEAE-cellulose column facilitated the removal of the contaminating chlorophyll in the crude extract since the green pigment was trapped on the top of the column. Kufer and Scheer (1979) previously reported that after loading the crude phycocyanin, the column was developed, first to remove unbound protein and some yellow pigment then linear gradient should be performed. It should be noted that crude extract containing biliprotein could be free from chlorophyll by ultracentrifugation (1 hr , 78,000 g , A_{620}/A_{280}=1.4).

After purification by two columns of DEAE-cellulose the result from the native gel electrophoresis showed only one band (Figure 38) and the result from SDS-PAGE also showed one band. Compared with the pattern of Linablue, a marker for phycocyanin the band was likely to be phycocyanin. From absorption spectrum of Linablue, a single peak with maximum absorption at about 620 nm was observed (see Appendix 3). So we could use this method for partial purification of phycocyanin with high efficiency.

Purified phycocyanin should be stored in the dark at -4°C (Bennett and Bogorad, 1970) or lyophilized and kept at -20°C (Kufer and Scheer, 1979). Addition of antioxidant such as L-ascorbic acid or sodium erythonate increase its stability (Kawasaki and Kaneko, 1978).

Treatment by a proteinase will also increase color stability (Yamanaka et.al., 1978).