CHAPTER IV

Discussion

I) Isolation

Spirulina is commonly found in various environmental habitats. However,it is present in small number. In nature various kinds of microorganisms including algae coexist with *Spirulina*. Isolation of *Spirulina* from natural water is very difficult since *Spirulina* in nature is so sensitive to the change of environments that it will cease growing and eventually die upon encountering with new environment. It is therefor necessary to maintain *Spirulina* in such condition in Zarrouk medium prior to their isolation via single cell isolation technique. The reason why we could find large amount of *Spirulina* in Makkasan Pond and Wat Benjamaborpit Pond was due to the blooming season of *Spirulina* at that time in which *Spirulina* was predominant species in that water.In order to know the real condition of natural water that *Spirulina* was grown it is generally suggested that the chemical analysis of water sample

should be done such as ; measuring pH,salinity, dissolved oxygen (D.O.), temperature. Also the water sample should be analyzed for its alkalinity, its contents of bicarbonate, nitrate, ammonia, nitrogen, sodium, phosphate and hardness by standard method for the analysis of water quality. In the present experiment we only measured pH of the water as well as observing their characteristics in the water without doing any chemical analysis.

In 2.1 Spirulina in 50 ml flask containing Zarrouk medium pH 8.85 could be grown although a 5 weeks period was required. This is because in both Makkasan and Wat Benjamaborpit ponds water was polluted with wastes and oil drainage. There were many factors contributing to the difficulty for growing Spirulina with air pump. For example, the evaporation of the water resulting in the reduced volume of medium hence concentrations of chemicals were increased. Another factor was bubble generated by air pump might be too strong and could cause cell breakage. Inoculum should be of suitable size and correspond to light intensity (Venkataraman, 1983). If cell concentration is too high a phenomenon of cell shading will occur resulting in a low growth due to an uneven exposure of individual cells to light. On the other hand if cell concentration is too low a bleaching of cells will result. Temperature is also

an important factor. Spirulina can grow well between 28-34°C. If the temperature is lower than 20°C and higher than 37°C Spirulina will not grow and will eventually die (Venkataraman, 1985).

Single cell isolation technique can be done in many different ways depending on type of algae . We modified this technique in order to make it easier to isolate Spirulina with high efficiency.

This technique is difficult and needs high skill because it is very hard to control capillary pipette due to the capillary force. The size of capillary depends on the cell type and size. Algal cells less than 10 Mm in diameter are difficult to see and to isolate with capillary pipette.

Many preliminary experiments were done in order to select the best method of extracting phycocyanin from Spirulina . The method of lysozyme digestion did not work even when high concentration of lysozyme was used. The lysozyme was not able to completely digest the cell wall of Spirulina which was probably due to the unsuitable conditions used in the experiment. Another important reason that we didn't use this method because lysozyme is very expensive. The methods of Sonication and French press were also used. However, the conditions for these

two methods were 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. Breaking cell by homogenization was not a convenient method .It took a long time and was not reproducible. However, freeze thaw method was chosen because of its many advantages ; reproducible , time saving and suitable for quantitative experiments. Although the method could not break the whole cell (partial breakage) all of phycocyanin could be released from the leakage of the cell because we gave enough incubation time and in the experiment we resuspended it twice until the cell debris became devoid of blue color.

In this experiment we detected the population of Spirulina by measuring the major parameters to follow the overall turbidity and dry weight . Thus, measurement of turbidity is the most rapid way to determine changes in the population density. However, measurement of turbidity or dry weight gives only a quantitative indication of the variations in biomass that take place in the culture. It should be noted that these measurements should always be accompanied by a detailed microscopic evaluation because environmental change may

cause morphological modification in some species which should be recorded and analyzed. The most important reason for microscopic examination is to monitor the development of foreign algal species as well as protozoa or fungi that may threaten to take over the cultivated species or cause severe damage to the culture. When the invading organisms are detected early, they should be easier to control, but no less important, the mere appearance of significant populations of other organisms in the culture should be regarded as a warning signal that the cultured species have become under some pressure. It may be that the temperature decreases or increases too much, giving a relative advantage to another species. It may also signal that the concentration of a particular nutrient has declined below the concentration threshold for the culture species. Indeed, any weakness of the culture species provides an opportunity for foreign organisms to rapidly increase their population (Richmond , 1986).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Three strains of Spirulina used in this experiment could not precipitate into pellet after centrifugation because of the presence of gas vacuoles in the cell. The density of the cells becomes less and hence pellet down becomes difficult. It can be harvested by using nylon sieve or even by filtration. This is a well established practice. But in this experiment, for

a quantitative analysis we still used centrifugation method. Some of Spirulina cell floating on the surface of the tube could be recovered by using a Pasteur pipette.

II) Optimization

The complexity in the trophy terminology of algae stems largely from overlapping and intergrading 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 formulas for culture media differ greatly from what the algae would have in the natural environments because the concentrations of all the required nutrient elements usually far exceed their natural levels .

Spirulina has been cultured with Zarrouk medium (see appendix) internationally. This medium is very expensive and contains several chemicals. For larger scale cultivation it is necessary to establish an optimal nutrient standard since the prices are high and contribute considerably to the production costs. Zarrouk medium has its own advantage to prevent contamination of other organisms and other kinds of algae because the pH of medium is high about 8-9 and will increase up to 10 when *Spirulina* is growing.

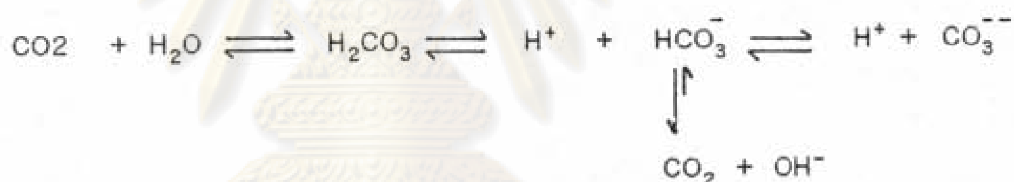
One of the major obstacles in cultivation of *Spirulina* in artificial systems is the high bicarbonate requirement of this alga. The amount of bicarbonate in the standard medium for *Spirulina* is about 16.8 g/l. It was found that the amount of bicarbonate in medium could be reduced significantly by slowly adapting the alga to lower concentrations without affecting growth (Venkataraman, 1985).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

The observation that high concentration of $NaHCO_3$ at 16.8 and 25.2 g/l caused the reduction of phycocyanin content (Figure 15) could be partly explained in relation to the nitrogen requirement . As growth at high $NaHCO_3$ content could be sustained, it was likely that nitrogen reserves in the cells were also utilized by the cells in conjunction with $NaHCO_3$. The resulting depletion of nitrogen reserves was possibly

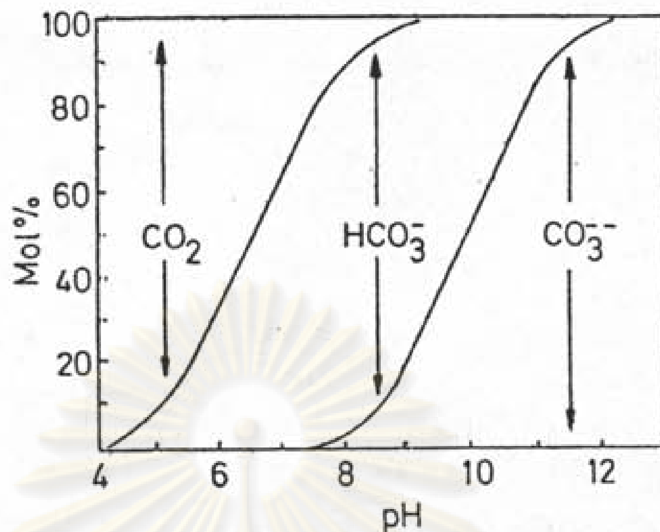
compensated by the degradation of phycocyanin in the cells.

In most cases, the growth of algae is limited by the supply of carbon. In a poorly buffer system like nutrient medium, the assimilation of carbon dioxide and bicarbonate by rapidly growing algae caused the equilibrium to shift, resulting in increased pH, due to a release of OH^- ions.



At pH values above 9, the carbonate may precipitate in form of salts which results in the removal of nutrients. As mentioned above , the change of pH is a function of the photosynthetic growth of the algae and the pH rises as carbon and nitrate are assimilated by the cells. The addition of carbon dioxide in the form of pure gas from cylinders helps to stabilize the pH, and also acts as a carbon source for autotrophic growths (Venkataraman, 1985).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย



After carbon ,nitrogen is quantitatively the most important element contributing to the dry matter of algae cells.In standard Zarrouk medium for Spirulina it contains 2.5 g/l of nitrate. Nitrogen requirements and metabolism in relation to alga culture, were summarized by Kaplan, Richmond, Dubinsky and Aaronson (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 the dry matter and carbon for about 50 % (Vaccaro,1965). Under condition for nitrogen deficiency, the contents of photosynthetic pigments 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, Mackenzie and Doolittle,1976; Foulds and

Carr, 1977). In *Anabaena cylindrica* (Wood and Haselkorn, 1976) and *Spirulina platensis* (Boussiba and Richmond, 1980) protease can degrade phycocyanin and appears to be activated or preferentially synthesized during nitrogen starvation. In the present study NaNO3 in the medium did not show much effect on growth of *Spirulina* but could affect phycocyanin production. The culture containing 0 g/l NaNO3 could grow because there was some $NaNO_3$ carried over by the inoculum. *Spirulina* could use this nitrate as nitrogen source for growth.

Phosphorus requirements for algaculture and its metabolism were summarized by Kaplan, Richmond, Dubinsky and Aaronson (1986). Phosphate is one of the major nutrient elements required for normal growth of algae. It plays a major role in most cellular processes, particularly those involved in energy transfer and in nucleic acid synthesis. The major form in which microalgae cells acquire phosphorus is as inorganic phosphate ($H_2PO_4^-$ + HPO_4^{2-}). The phosphate requirements for optimum growth differ considerably from species to species even if no other external factor is limiting. The uptake of phosphorus from the surrounding medium by algal is generally stimulated by light. It is an energy-dependent reaction as shown by its sensitivity to uncoupler (Healey,1973). The uptake rate is also influenced by the phosphate concentration in the medium

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

the pH, and in several algae species, on the availability of Na^+, K^+ or Mg^+ (Kuhl, 1974 ; Mohleji and Verhoff ,1980). Some of the symptoms of phosphorus deficiency are similar to those observed in nitrogen deficiency. The contents of protein, chlorophyll-a, RNA and DNA tend to decrease (Healey,1982).

In response to salinity, the algae as a group exhibit an extremely wide range of tolerance to salts in their surroundings. Some species can tolerate only millimolar 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). Indeed, high-salt algae have a higher chlorophyll content per cell and are thus 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 and a higher bicarbonate concentration for half-saturation of CO_2-assimilation (Gimmler, Wiedemann and Moller, 1981). Chiu (1980) found that the optimum concentration of sodium chloride for growth of Spirulina platensis was between 0-0.5 g/l and Tel-Or (1980) found that Spirulina can grown in sodium chloride up to 20-30

g/l.

Effect of light intensities on phycocyanin in Figure 23 showed that at 6500 and 8000 lux phycocyanin was much lower than that at 5000 lux. This might be attributed to the excessive heat generated by high intensity of light causing the destruction of phycocyanin. It is suggested that the cool white lamp should be used in order to overcome the problem of overheat.

It has been reported that the efficiency of light utilization by algae can be increased by exposing the cell to alternate light and dark periods. The maximum duration of the light phase, giving the highest photosynthetic efficiency, will be negatively correlated with light intensities equal or grater than one, above which the efficiency begins to decrease under constant illumination (Venkataraman ,1985). Standardization of the saturation curve is often delineated in terms of only two parameters : the initial slope (alpha) and the chlorophyll-a specific carbon production rate at saturating light (P max) often referred to as the assimilation number (Jassby and Platt,1976). Light saturation of photosynthesis is nevertheless influenced by several other factors such as nutrients and chemical composition and particularly, by the temperature

(Verity,1981). An important aspect of the interaction of light and temperature was that the optimum temperature for photosynthesis increased with increasing light intensities. At low light intensities (42 and 99 $\mu E/m^2/sec$), the photosynthetic rate decreased with increasing temperature, whereas at night temperature (40°C) the photosynthetic rate increased with increasing intensities (Verity,1981). The optimum light intensity that effect growth of Spirulina was related with temperature (Venkataraman, 1985). Spirulina in outdoor scale have the optimum growth between 20000-30000 Lux. In laboratory conditions where temperature was lower than in out door scale the optimum light intensity for growth was between 8000-10000 Lux (Ciferri,1983).

The transmission spectra of the two filters used are shown in Figure 26. Growth of Spirulina in green and red light experiments was determined turbidimetrically by the measurement of optical density at 750 nm with a spectrophotometer. In Figure 27 and 28, growth in white light was higher than in either red light or green light. However, it should be pointed out that the high turbidity occurring in the sample grown in white light might partly resulted from the dead cells as we noticed that the culture turned pale green during the first days after inoculation. In this respectivable count should be done in order to check the real amount of living cells

especially when Spirulina was grown for a long time in order to prevent a change in growth rate as a result of overshadowing during the course of the experiments. Venkataraman (1985) suggested that the culture should be diluted several times when overshadowing occurs.

The result in quantitative data on the cellular pigment content of phycocyanin of Spirulina showed that in both red light and green light phycocyanin was higher than that in white light (Figure 29). Furthermore, it was noted that in green light phycocyanin was higher than that in red light. Our results indicated that the quality of light greatly affected phycocyanin content in Spirulina .

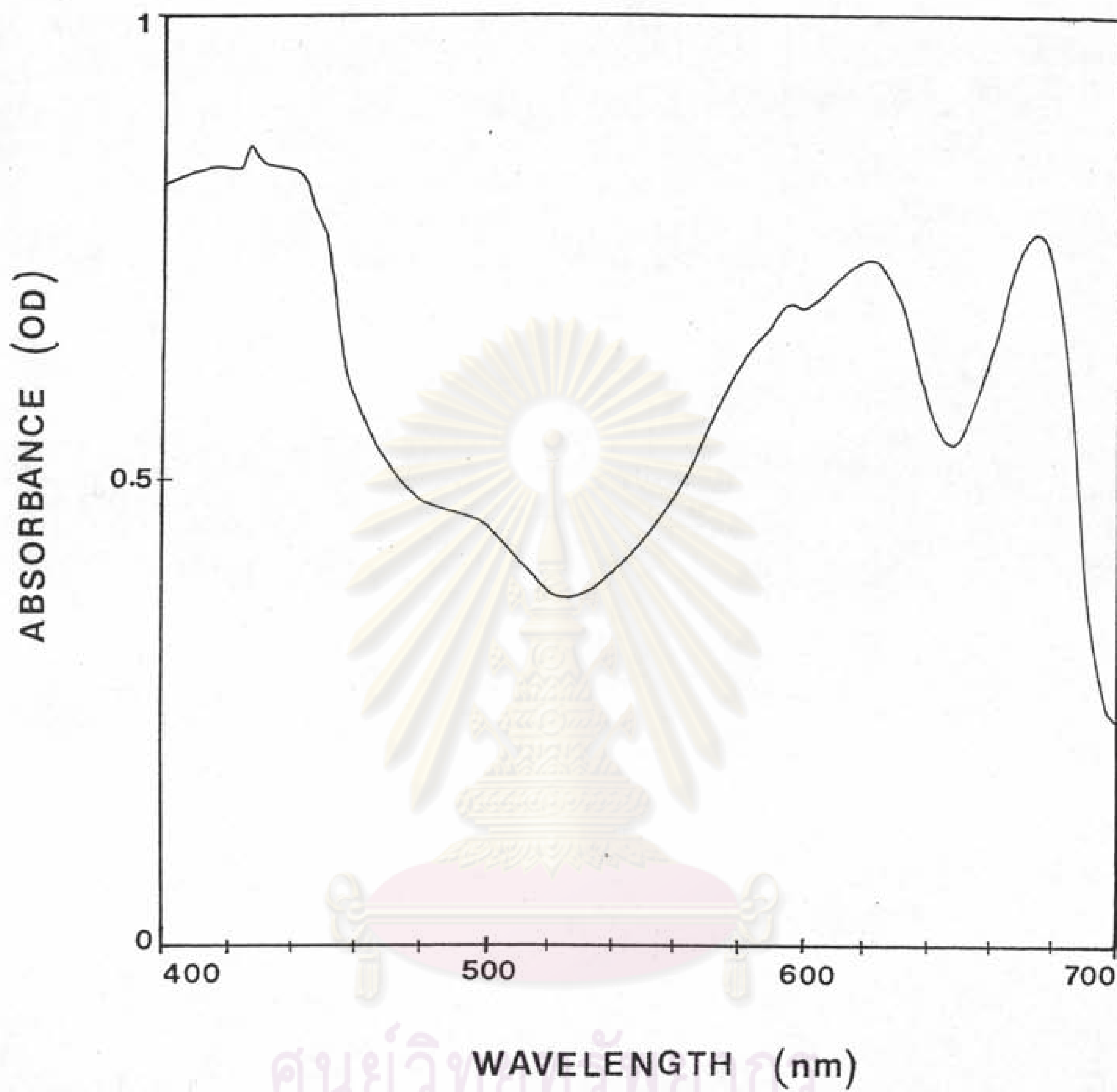
Since nitrate can affect phycocyanin contents in the cell (Boussiba and Richmond, 1980 ; De Loura, Dubacq and Thomas, 1978) we thought it would be worthy of testing the effect of sudden changes of nitrate in the medium on the phycocyanin content during a short time period. Phycocyanin content was found to markedly increase when nitrate in the medium was changed from 2.5 g/l to 10 g/l (Figures 33,34 and 35). It was therefore , concluded that c-phycocyanin was produced in large excess in the algal cell due to the increase of nitrogen supply in the medium. This is also supported by the fact that c-phycocyanin is a nitrogen storage compound in the

cell (Boussiba and Richmond, 1980). It may be worth noting that in the cyanobacteria, c-phycocyanin differs from other known storage materials in that it has a dual role in the cell and that its synthesis occurs largely during the logarithmic phase of growth and practically ceases at the stationary phase. This is in contrast to other storage products which are usually accumulated at the end of the growth phase or in the final stage of the life cycle (Boussiba and Richmond,1980). The advantage of c-phycocyanin as a storage form of reduced nitrogen skeletons may be speculated : When cells which grow logarithmically with an abundant nitrogen source are exposed to a temporary shortage of nitrogen, they would be able to grow unhindered for up to another generation before their growth ceases. Also, when cells of these algae so multiply as to cause dense blooms, a portion of c-phycocyanin becomes potentially a storage material, which facilitates immediate resumption of accentuated growth when cells from such blooms are dispersed and light ceases to be limiting to growth (Boussiba and Richmond,1980).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

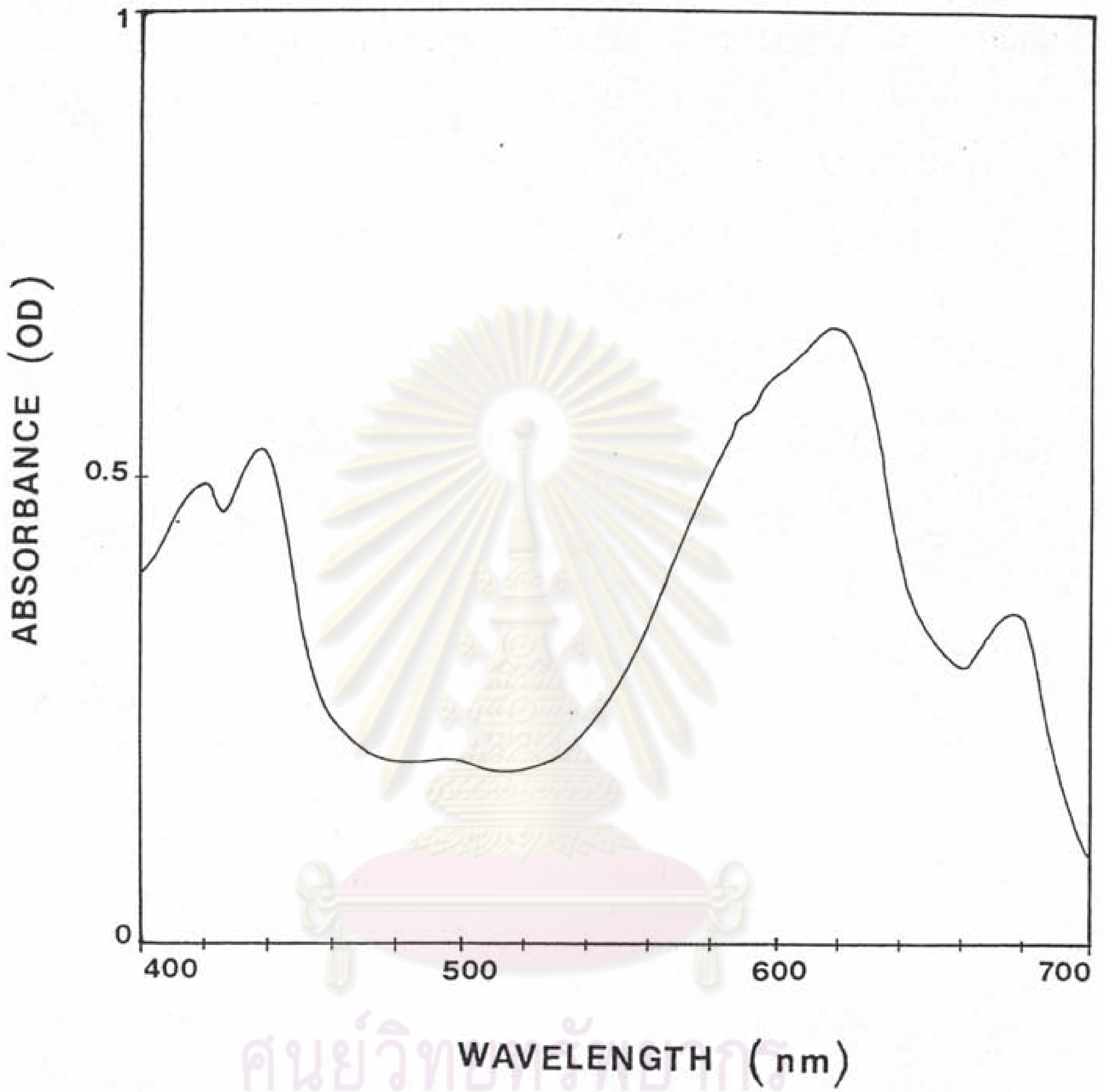
III) <u>Partial purification</u>

The crude extract containing phycocyanin was contaminated with chlorophyll because when loading on the first DEAE-cellulose column , a lot of green pigments were trapped on the top of the column causing poor



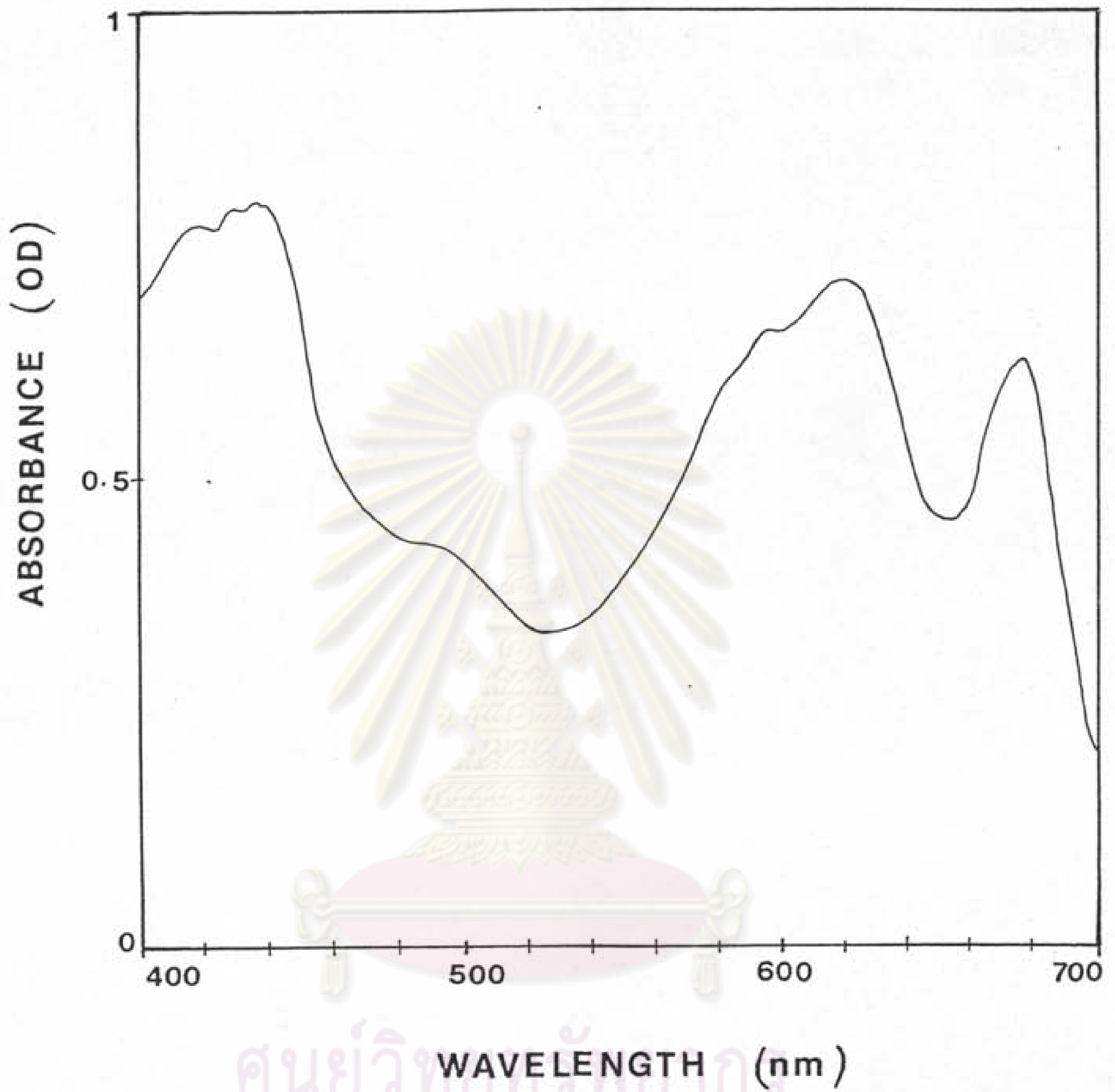
ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Figure 65 Absorption spectra of 65 % $(NH_4)_2SO_4$ fraction of Spirulina (BP)



ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Figure 66 Absorption spectra of 65 % $(NH_4)_2SO_4$ fraction of Spirulina (NIFI)



ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Figure 67 Absorption spectra of 65 % $(NH_4)_2SO_4$ fraction of Spirulina (MP)

resolution. Another evidence was seen in the absorption spectra of crude phycocyanin in Figures 65, 66 and 67 showing peaks of chlorophyll as well as carotenoids. After passing through the second column of DEAE-cellulose no green pigments were trapped on the top of the column. It should be noted that crude extract containing biliprotein should be free from chlorophyll by ultracentrifugation (1 hr,78,000 x g , A_{620} / A_{280} = 1.4) (Kufer and Scheer,1979). After loading the crude phycocyanin, the column was developed, first to remove unbind protien and some yellow pigments then linear gradient should be performed (Kufer and Scheer,1979). The yellow pigments that came out first is mainly carotenoids. But there are other yellow pigments present in algal also.

After purifying by three columns the results from the native gel electrophoresis showed that there seemed to exist more than one band. The results from SDS-PAGE showed two bands with high and low intensity of band. Compared with Linablue, the upper band with high intensity was likely to be phycocyanin. From absorption spectra of Linablue a single peak with maximum absorption at about 620 nm was observed (Figure 57). We then tried to prove whether the low intensity band might be allophycocyanin contaminant or not by passing the peak fraction of the third column (Sephadex G-150) on

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Hydroxylapatite column which was shown to separate phycocyanin from allophycocyanin (Boussiba and Richmond,1979). The elution profile in Figures 58,59 and 60 showed that in three strains of Spirulina phycocyanin (OD 620) and allophycocyanin (OD 650) could be well separated. We then confirmed this by running absorption spectra of the peak fraction of these 2 pigments. As shown in Figures 62,63 and 64 there was one main peak at 620 nm for phycocyanin sample and one peak at 650 nm with a slight shoulder at 620 nm for allophycocyanin sample indicating that the allophycocyanin sample was slightly contaminated with phycocyanin which was further confirmed by the analysis by SDS-PAGE (Figure 61). The algal biliproteins remain colored after treatment with SDS and the separation can thus be followed visually. The same holds true for electrophoresis of native biliprotein on polyacrylamide gels (Glazer and Cohen-Bazire, 1971).

Purified phycocyanin should be stored in the dark at 4°C (Bennett and Bogorad, 1971) 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 with a proteinase will also increase color stability (Yamanaka,Chiba,Morinaga, 1978).