CHAPTER III

Results

1.Collection of sample from natural water sources

Approximately 100 water samples were collected during May and June 1988. Spirulina cells could be detected in 20 samples taken from various locations and Spirulina was found relatively abundant in Makkasan pond and Wat Benjamaborpit pond. The samples from these two ponds also contained the cells of Oscillatoria. In other locations, for example Srinakarintaravirot University at Bang Saen, Pimarnmake Palace, the cells could be detected but the appearances of the cells were not in good shape, i.e., some were cracking or bleaching.

2.Isolation and purification of Spirulina as unialgal culture.

All 20 samples containing Spirulina cells were isolated and purified as described in section 3. Only 2 samples from Makkasan Pond and Wat Benjamaborpit pond could be isolated and purified as unialgal culture. This

was probably due to high concentration of cells existing in the sample initially (Table 1, items 3 and 4). Another factor contributing to the success of the isolation and purification of those 2 samples was because of the basic pH of the sample. Figures 8, 9 and 10 showed that Spirulina appeared as filaments composed of cylindrical cells arranged in unbranched helicoidal trichomes.

3.Optimization studies for three strains of Spirulina

3.1 Effect of $NaHCO_3$ on growth and phycocyanin

Figures 11, 12 and 13 showed that 12 day culture gave the highest yield of phycocyanin for all 3 strains of Spirulina. Consequently in later optimization experiments we followed the results after 12 day cultivation. Growth of 3 strains of Spirulina was compared during 15 days cultivation under 5 concentrations of $NaHCO_3$ .It was found that in 9 days there was no difference in terms of growth. After 9 days at 2.1 g/l slightly less growth was observed in all three strains than at other 4 concentrations of $NaHCO_3$ (Figure 14). Figure 15 showed that at day 12 all three strains of Spirulina gave the highest yield of phycocyanin when cultured with 8.4 g/l $NaHCO_3$. Furthermore, at this concentration of $NaHCO_3$ phycocyanin

Table 1 Locations and Characteristics of Water Samples

Locations	Characteristics of Water	pH	Concentration of Spirulina (unit / ml)	Characteristics of Spirulina	Other Living Organisms
1. Fishing Pond at Bang-Pli, Samutprakarn	green water	about 7	$5 \times 10^5 - 7.5 \times 10^5$	Spiral cell	Daphnia, Diatom, Oscillatoria, Spherical algae
2. Fishing pond at Bang-Poo, Samutprakarn	green water	about 7	2.5×10^5	Spiral cell	Daphnia, Diatom, green algae
3. Turtle Pond at Wat Benjamaborbit, Bangkok	green water see green dust of Spirulina	7.80	$7.5 \times 10^5 - 1.0 \times 10^6$	Spiral cell, large cell, predominant species	Diatom, Oscillatoria
4. Makkasan Pond (Under express way), Bangkok	green water see green dust of Spirulina	8.10 about	$2.5 \times 10^5 - 5 \times 10^5$	Spiral cell, large cell, predominant species	Daphnia, many living organism
5. Dusit Zoo Surrounding Canal, Bangkok	Clear, no color	7	very low	Spiral cell, bleaching cell	Diatom, Daphnia, Oscillatoria
6. Alanton Canal at Srinakarin Road, Bangkok	Clear, no color	0	very low	short cell, bleachig cell	Diatom is predominant species

Locations	Characteristics of Water	pH	Concentration of Spirulina (unit / ml)	Characteristics of Spirulina	Other Living Organisms
7. Onnoj Canal, Bangkok	Clear, no color	about 7	very low	Spiral, short cell	Daphnia, Diatom
8. Onnoj Fish Farm, Bangkok	green water	about 7	very low	Spiral, short cell	Daphnia, Diatom
9. Fish Farm at Bang-Pli, Samutprakarn	green water	8.50	2.5×10^5	Spiral, long cell	Spherical algae
10. Fish Farm at Bang-Pli, (around people area), Samutprakarn	green water	8.13	very low	Spiral, short cell	Green algae, Diatom
11. Fish Farm in Front of Bugn-Yai, Bangkok	green water	8.57	very low	Spiral, short cell	Daphnia, algae, Diatom
12. Canal around Udom-Suk Road, around Prem-Rutai Technical School, Bangkok	dark green water	7.49	very low	Spiral, short cell	Oscillatoria is predominant species
13. Canal behind Klong-Chan, Bangkok	green water	8.22	2.5×10^5	Spiral	Oscillatoria
14. Srinakarintaravirot Bang-San, Chon-buri	green water	8.10	$1 \times 10^5 - 2.5 \times 10^5$	Spiral, large cell	Oscillatoria is predominant species

Locations	Characteristics of Water	pH	Concentration of Spirulina (unit / ml)	Characteristics of Spirulina	Other Living Organisms
15. Fish Pond behind Tab Buiding, Chulalongkorn University	Clean, no color, have fountain	7.80	very low	Spiral, large cell, cell aggregate	Spherical algae, Diatom, Daphnia, Oscillatoria
16. Fish Pond at Dusit Palace, Bangkok	green water	about 7	very low	Spiral, short cell	Daphnia, Oscillatoria is predominant species
17. Klong-Pram Prison, Laksaee, Bangkok	green water	about 7	2.5×10^5	Spiral, short cell, bleaching cell	many living organism
18. Pond in Front of Thaitextile Company, Laksaee, Bangkok	brown water	about 7	very low	Short cell, bleaching cell	non
19. Pond in Soan-Siam, Bangkok	green water	about 7	2.5×10^5	Spiral, large cell	many living organism
20. Pond in Pimarn-Make Palace, Bangkok	green water	about 7	$5 \times 10^5 - 7.5 \times 10^5$	Spiral, large cell	Oscillatoria

Figure 8 Spirulina from Wat Benjamaborpit pond

(x 50)

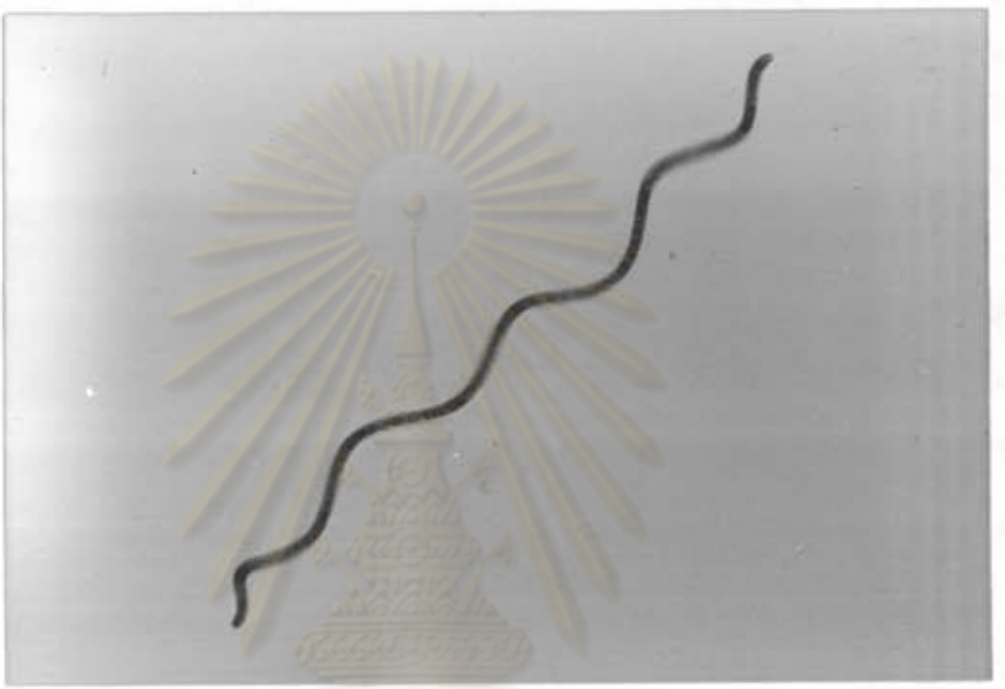


Figure 9 Spirulina from the National Inland Fisheries Institute (x50)

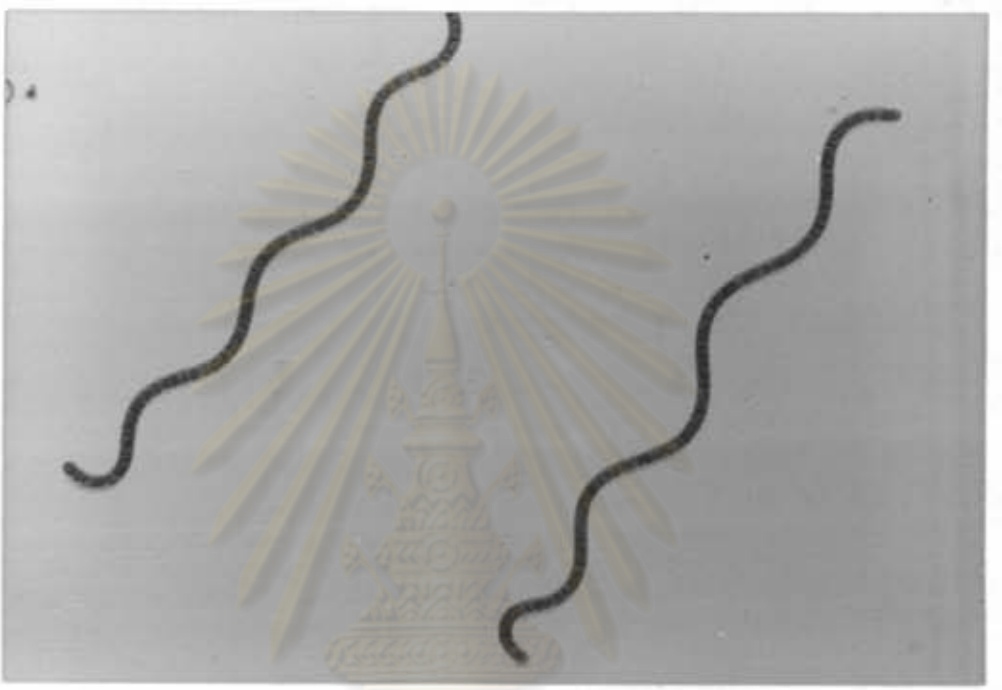


Figure 10 Spirulina from Makkasan pond

(x50)

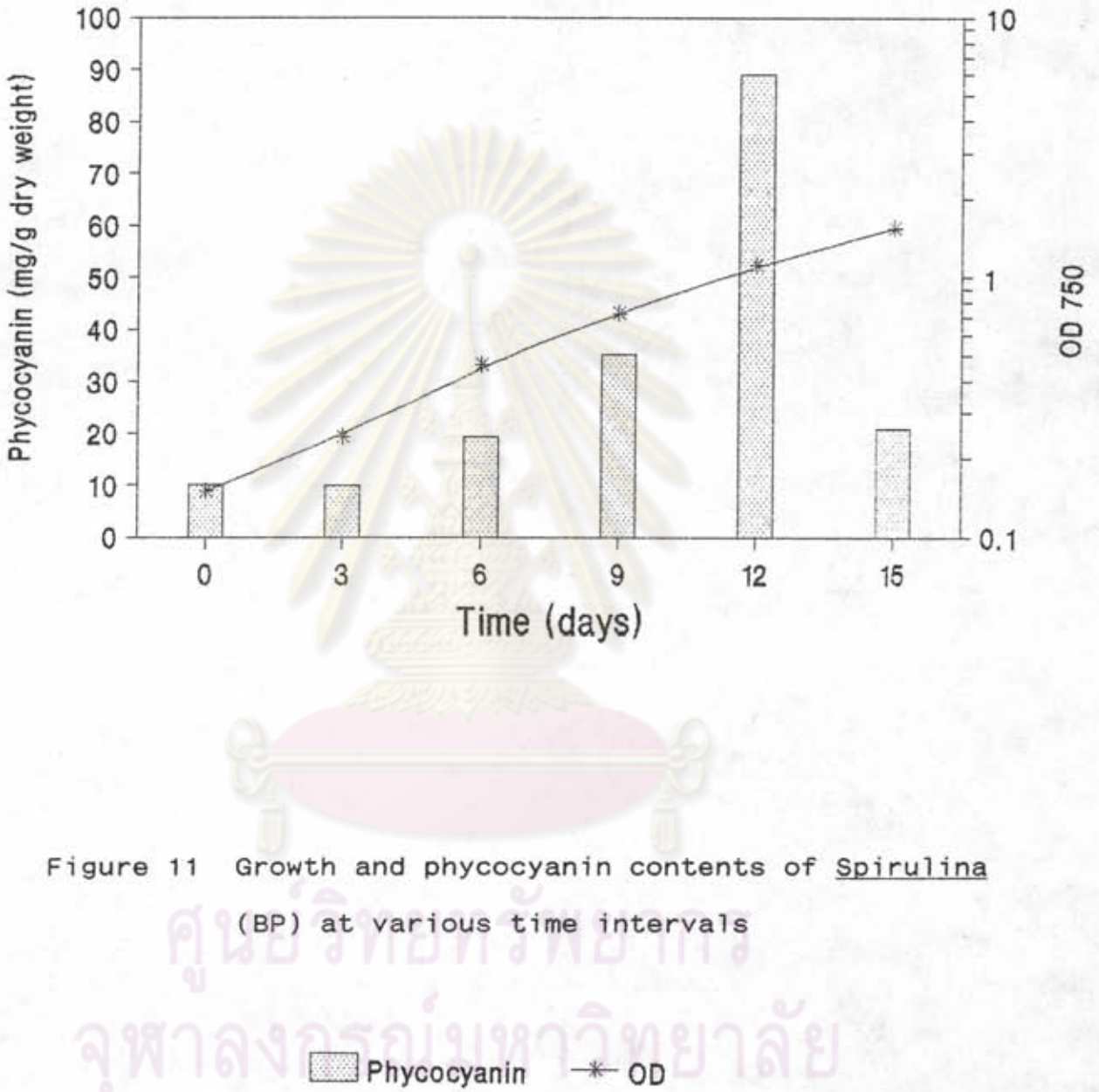


Figure 11 Growth and phycocyanin contents of Spirulina (BP) at various time intervals

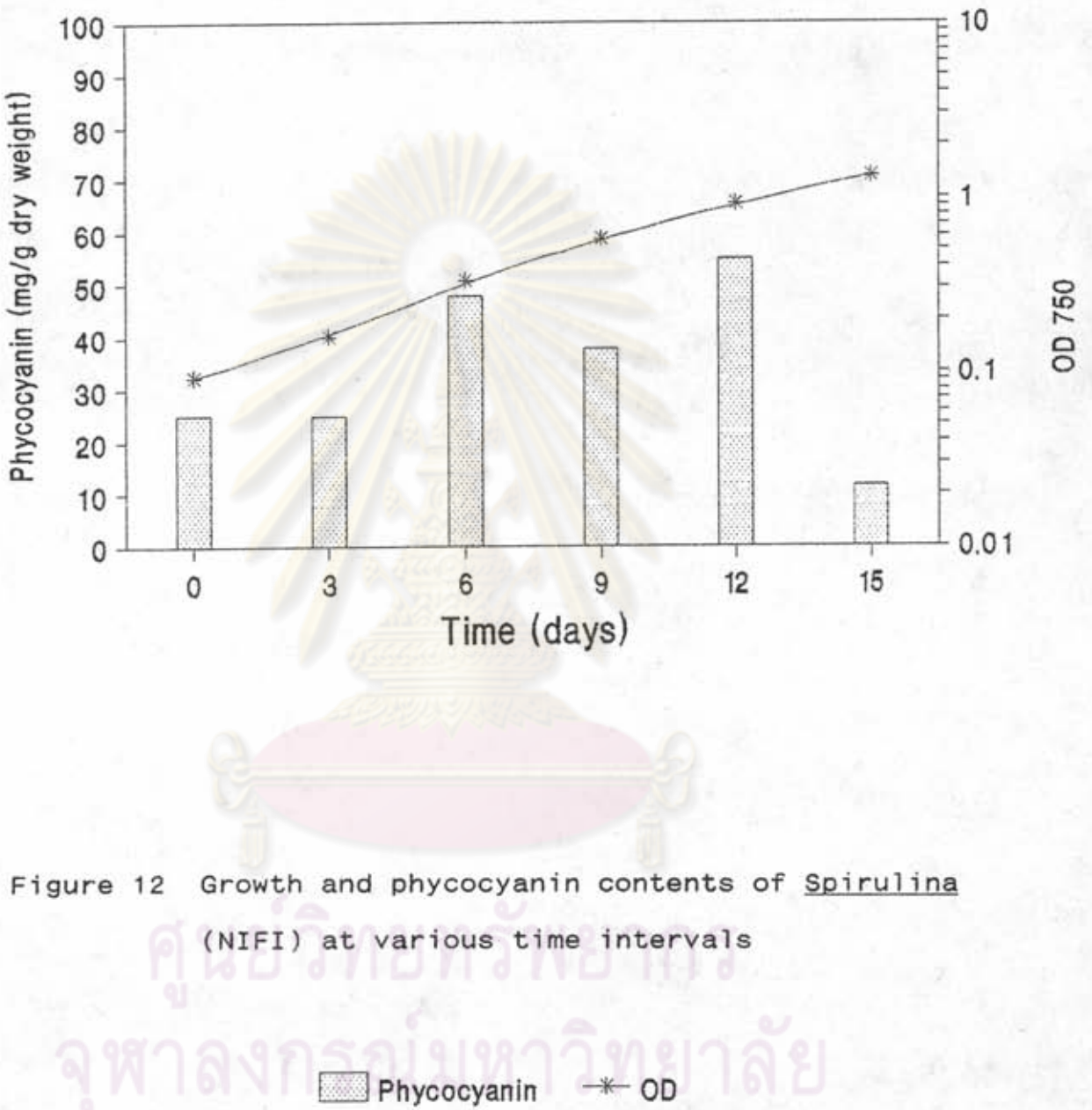


Figure 12 Growth and phycocyanin contents of Spirulina (NIFI) at various time intervals

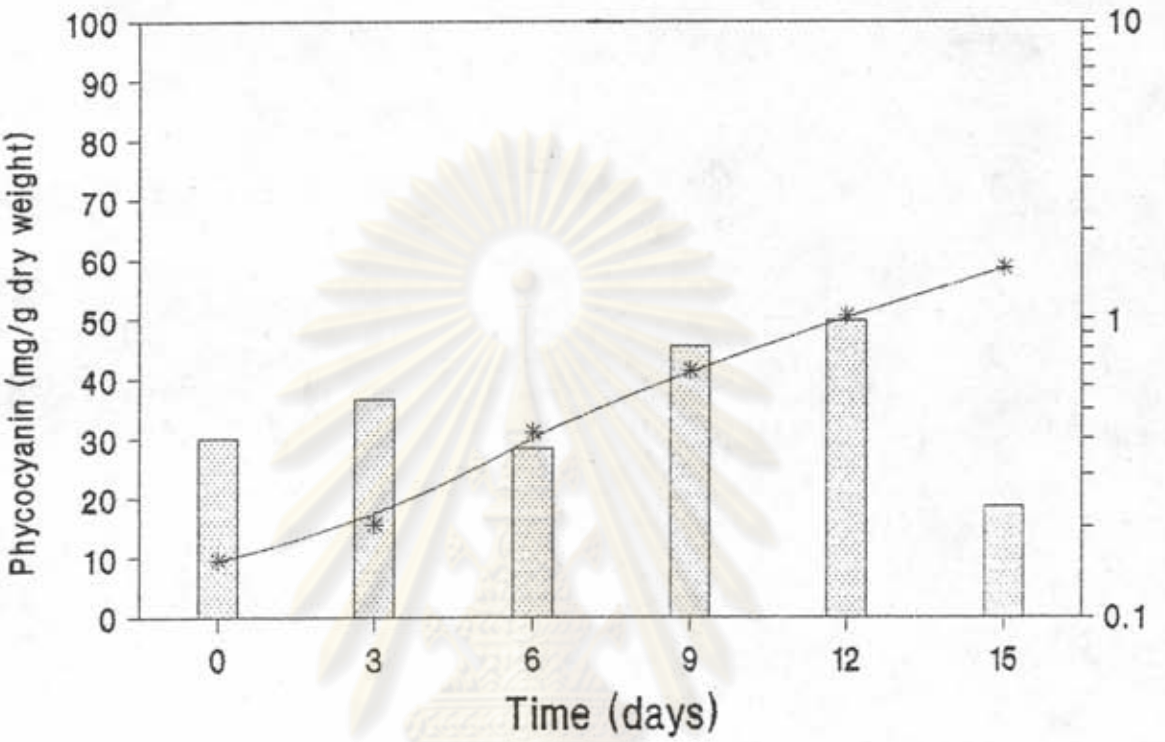


Figure 13 Growth and phycocyanin contents of Spirulina (MP) at various time intervals

Figure 14 Growth of Spirulina in Zarrouk medium containing various $NaHCO_3$ concentrations

(—•—) $NaHCO_3$ 2.1 g/l

(—+—) $NaHCO_3$ 4.2 g/l

(—*—) NaHCO3 8.4 g/l

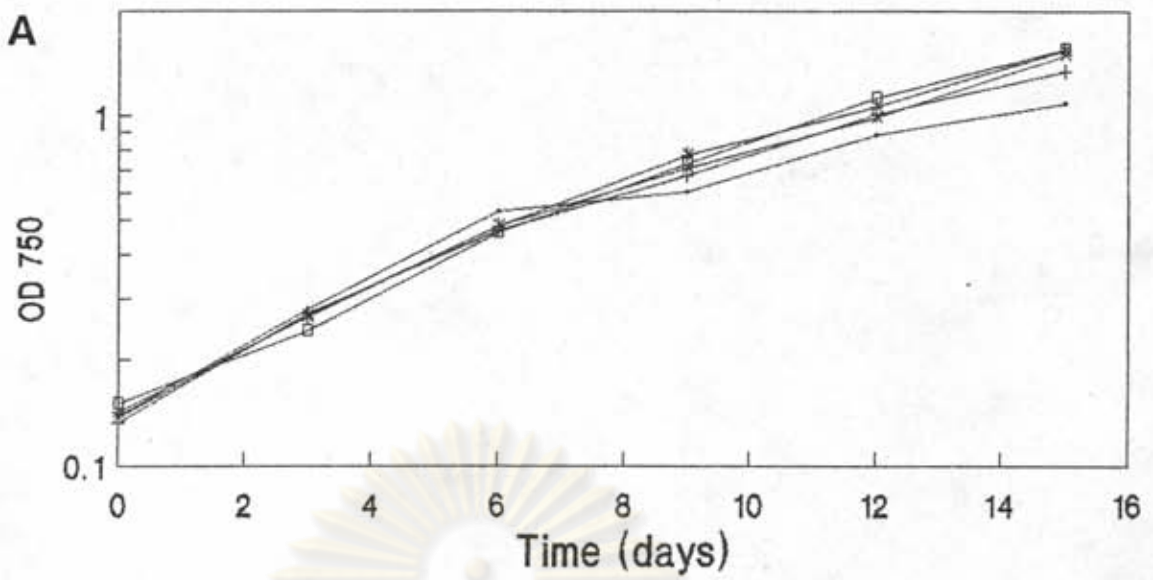
(—□—) $NaHCO_3$ 16.8 g/l

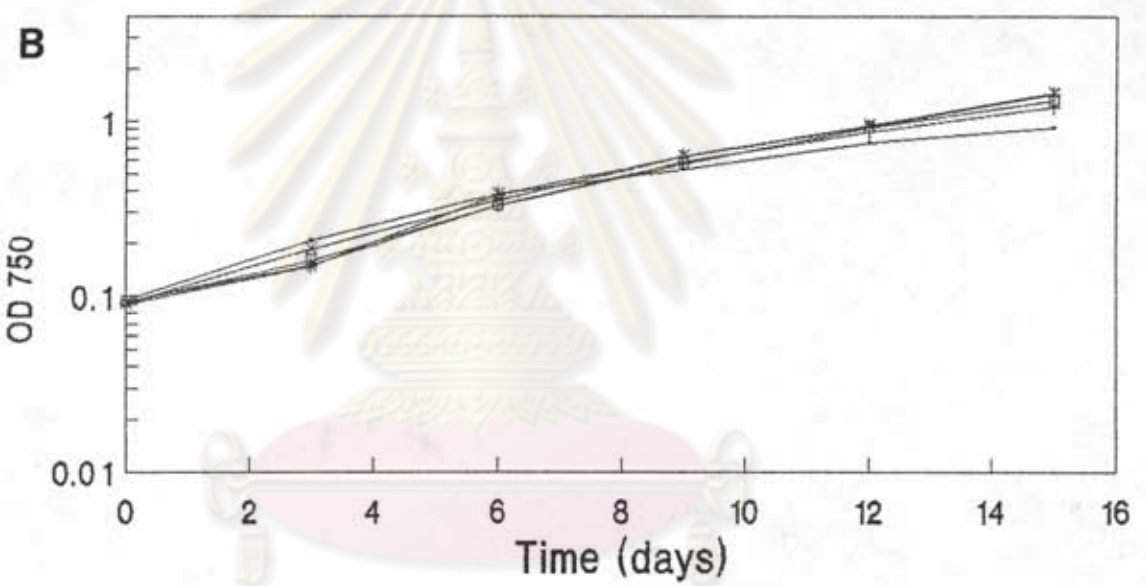
(—×—) $NaHCO_3$ 25.2 g/l

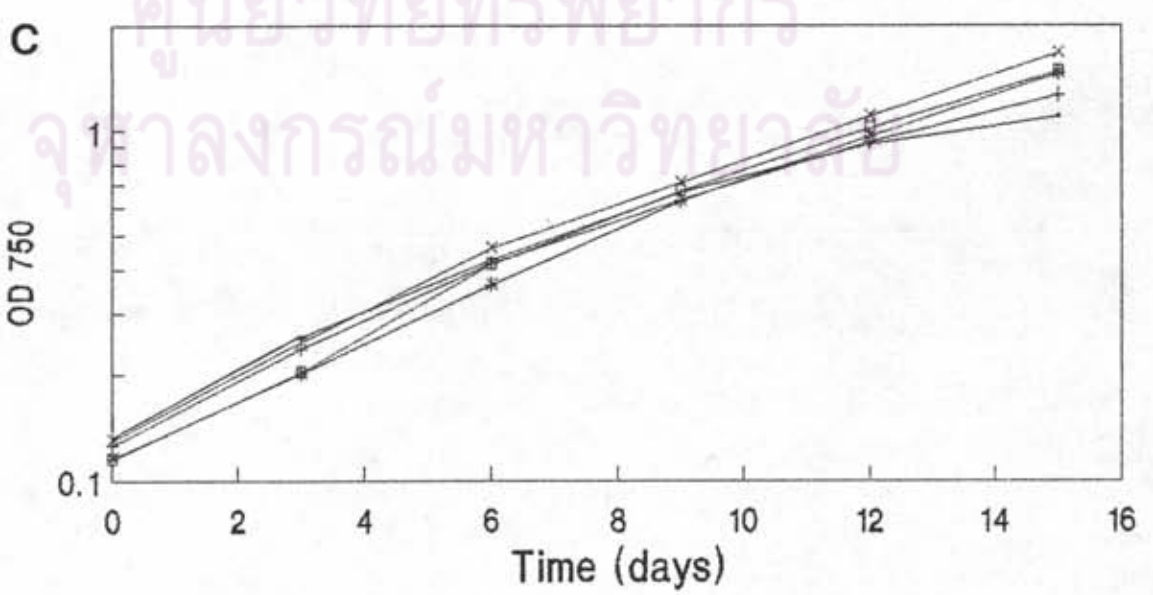
A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)







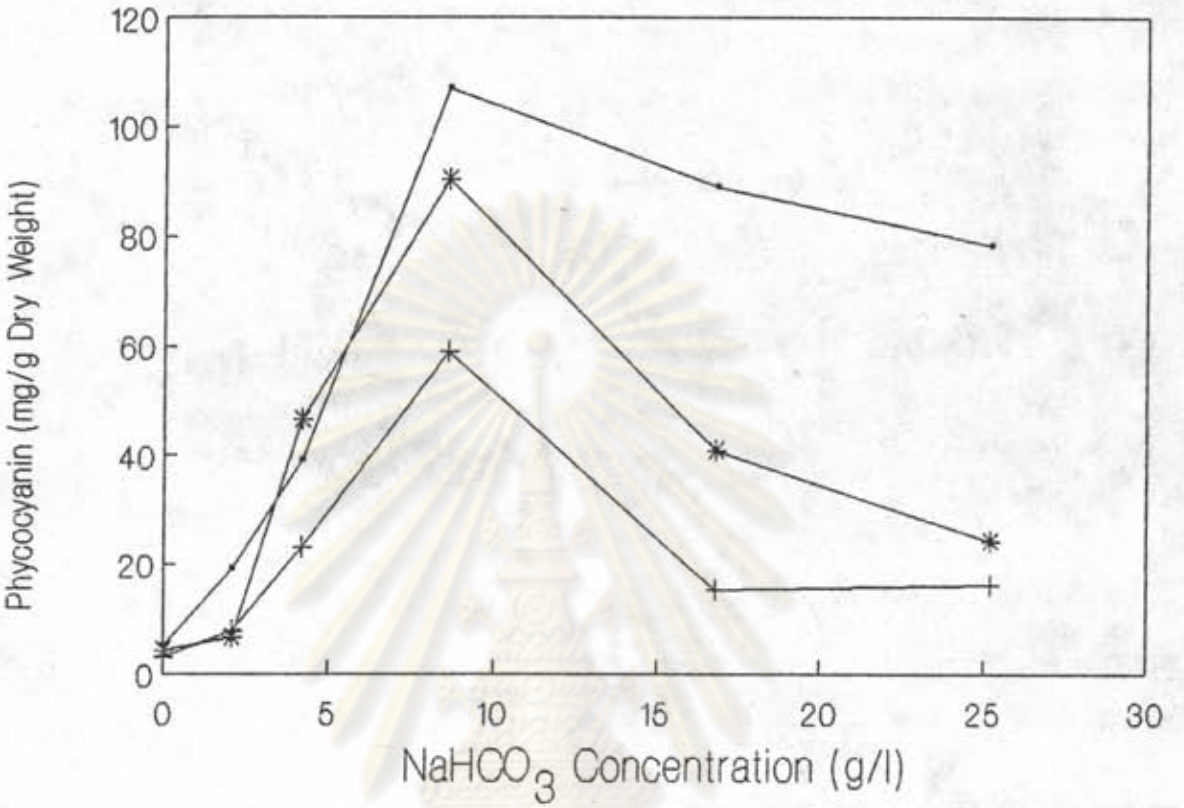


Figure 15 Effect of $NaHCO_3$ concentration on phycocyanin at day 12 of three strains of Spirulina

(—•—) Spirulina (BP)

(—+—) Spirulina (NIFI)

(—*—) Spirulina (MP)

of Spirulina (BP) was highest followed by that of (MP) and (NIFI) respectively. In later experiments $NaHCO_3$ concentration was fixed at 8.4 g/l.

3.2 Effect of $NaNO_3$ on growth and phycocyanin

Concentrations of sodium nitrate did not appear to cause changes in growth of 3 strains of Spirulina (Figure 16) . In Spirulina (BP),there was slightly reduced growth after 9 days when grown under 1.25 or 5.0 g/l $NaNO_3$. Figure 17 showed that the minimum value that gave high yield of phycocyanin for Spirulina (BP) was 2.5 g/l NaNO3.For Spirulina (NIFI) and Spirulina (MP) the phycocyanin content was not affected by $NaNO_3$ concentrations ranging from 1.25 to 5.0 g/l . In later experiments , the content of $NaNO_3$ was fixed at 2.5 g/l.

3.3 Effect of K_2HPO_4

Spirulina (BP) and Spirulina (MP) had high growth in 0.370 g/l K_2HPO_4 whereas Spirulina (NIFI) had high growth at 0.093 g/l K_2HPO_4 (Figure 18). Figure 19 showed that no difference in yield of phycocyanin under various concentrations of K_2HPO4 was observed. However, we chose K_2HPO_4 concentration at 0.185 g/l to be the optimum condition for three strains of Spirulina. In later experiments K_2HPO_4 concentration was fixed at 0.185 g/l.

Figure 16 Growth of Spirulina in Zarrouk medium containing various $NaNO_3$ concentrations

(—•—) $NaNO_3$ 0 g/l

(—+—) $NaNO_3$ 1.25 g/l

(—*—) $NaNO_3$ 2.50 g/l

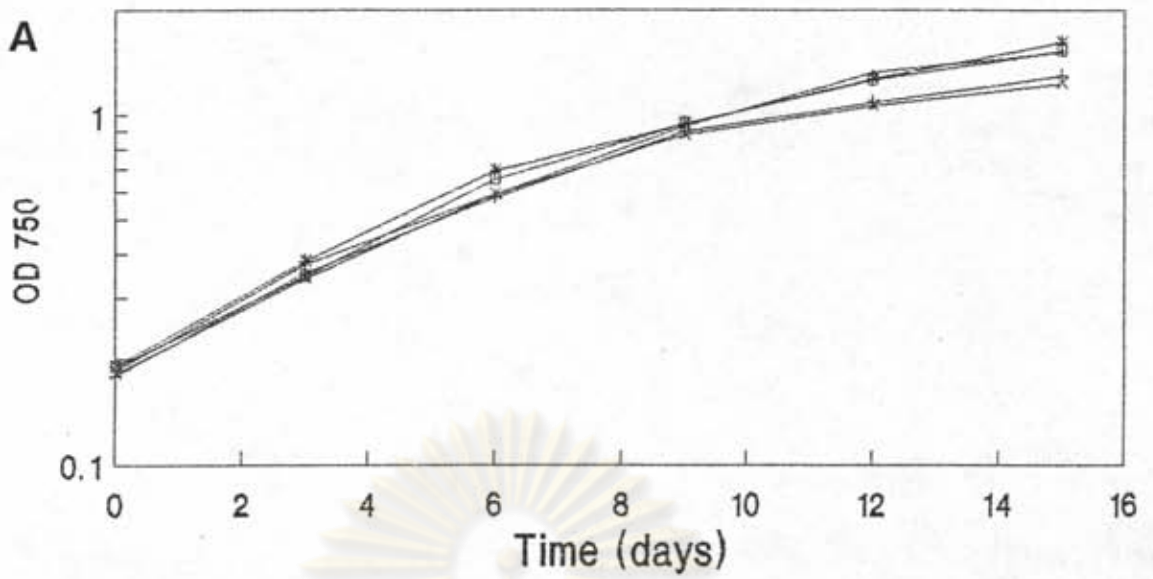
(—□—) $NaNO_3$ 3.75 g/l

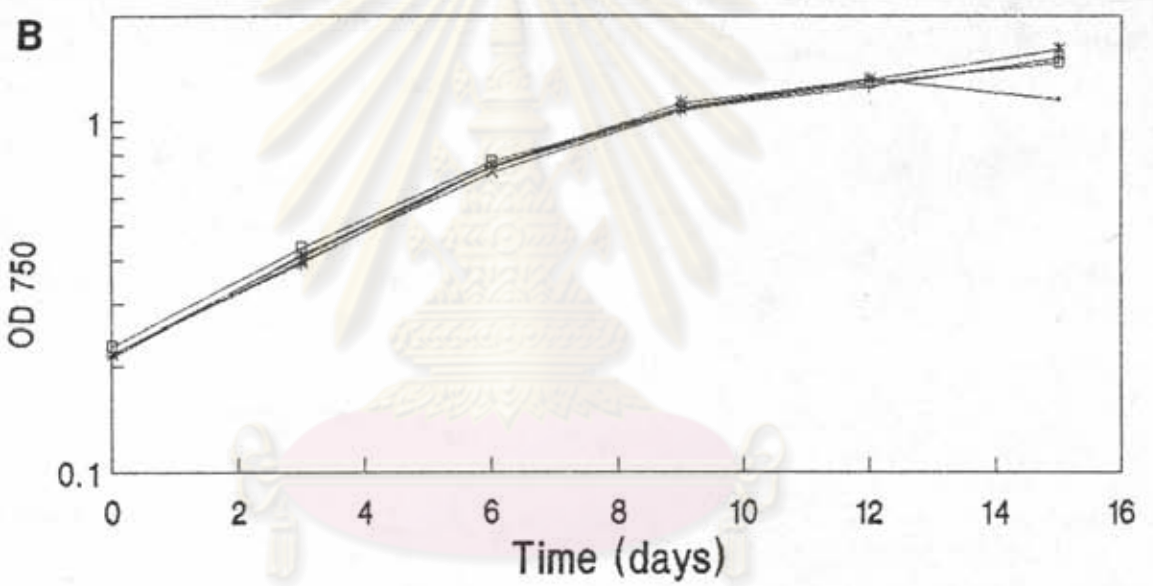
(—×—) $NaNO_3$ 5.0 g/l

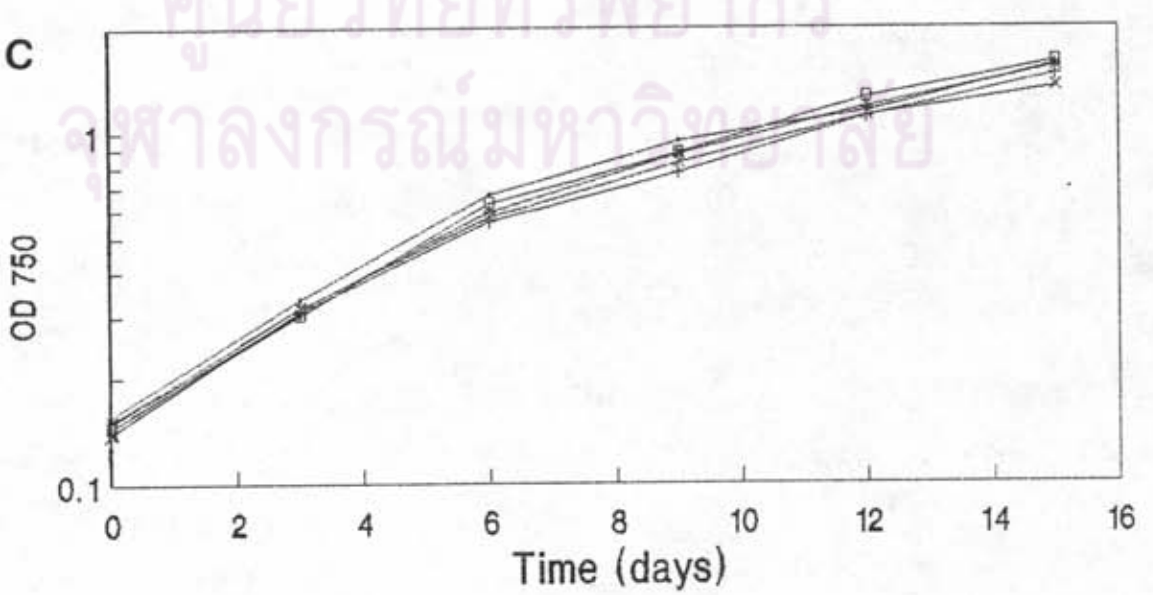
A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)







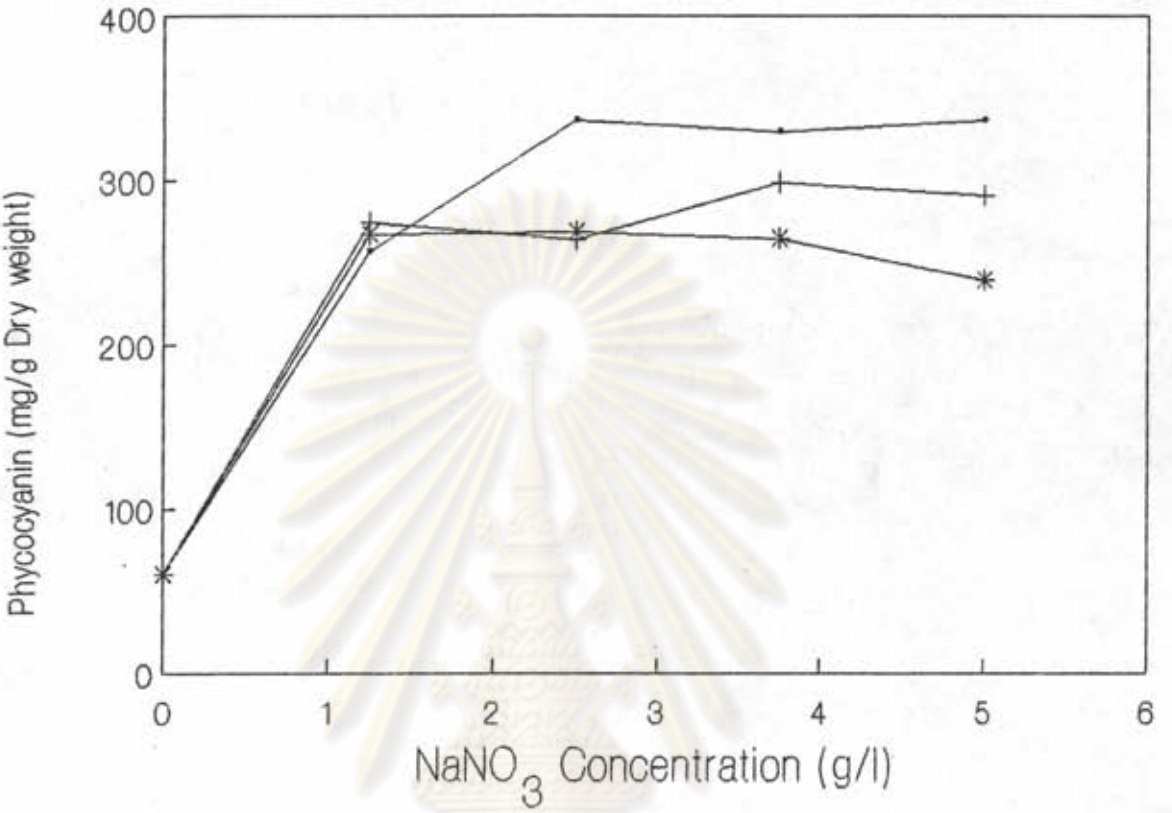


Figure 17 Effect of $NaNO_3$ on phycocyanin at day 12 of three strains of Spirulina.

(—•—) Spirulina (BP)

(—+—) Spirulina (NIFI)

(—*—) Spirulina (MP)

Figure 18 Growth of Spirulina in Zarrouk medium containing various K_2HPO_4 concentrations

(—•—) K_2HPO_4 0.093 g/l

(—+—) K_2HPO_4 0.186 g/l

(—*—) K_2HPO_4 0.370 g/l

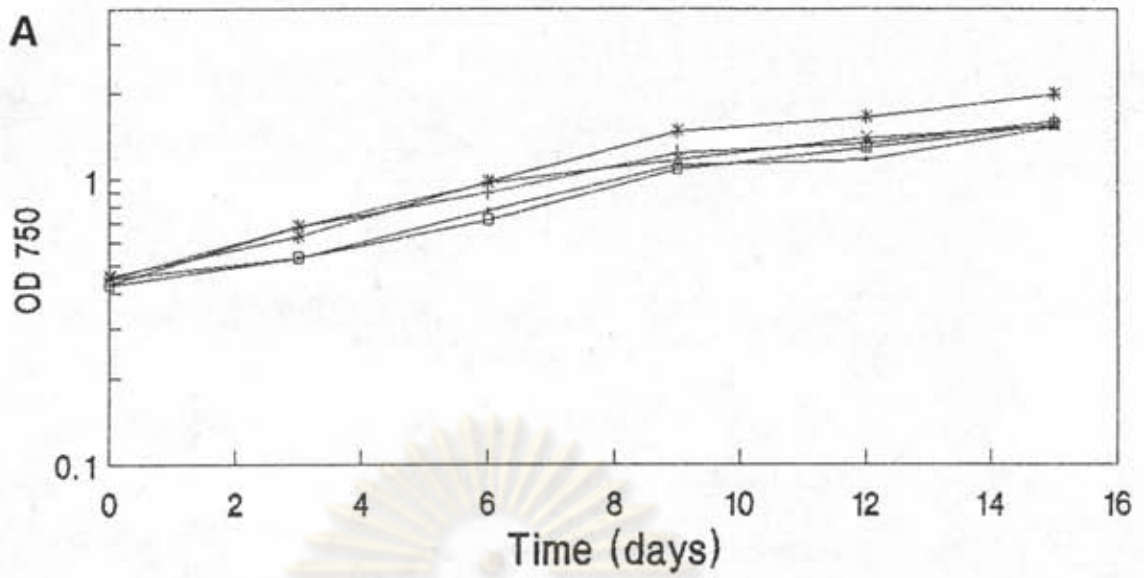
(—□—) K_2HPO_4 0.560 g/l

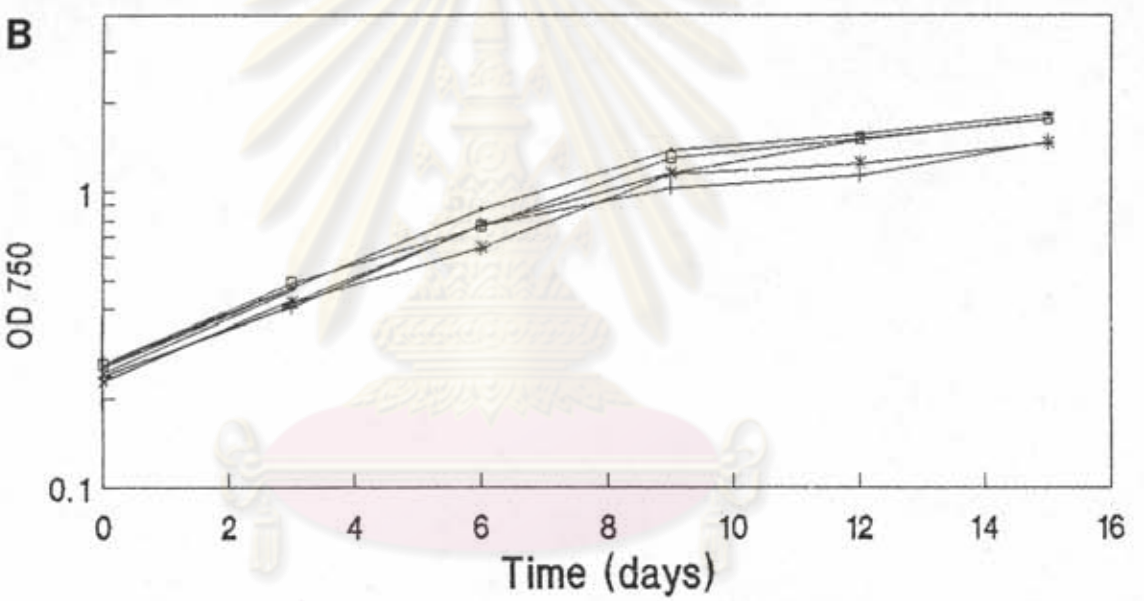
(—×—) K_2HPO_4 0.740 g/l

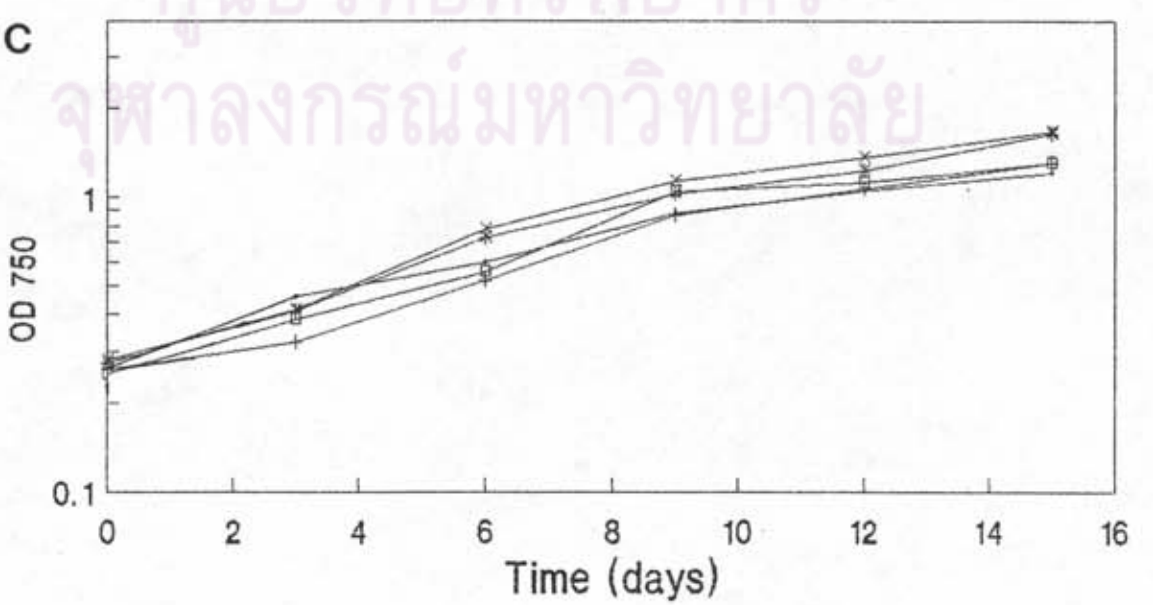
A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)







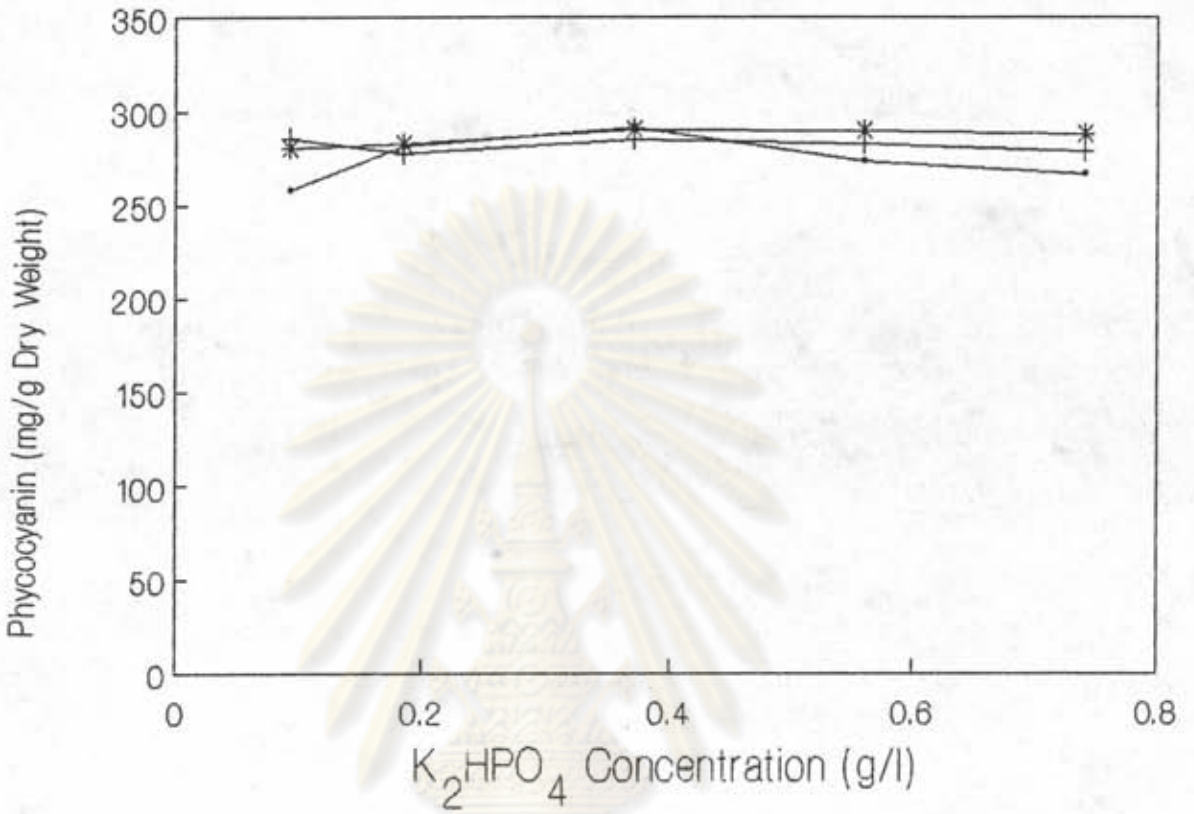


Figure 19 Effect of K_2HPO_4 on phycocyanin at day 12 of three strains of Spirulina

(——) Spirulina (BP)

(—+—) Spirulina (NIFI)

(—*—) Spirulina (MP)

3.4 Effect of NaCl

At various NaCl concentrations, there were no differences in terms of growth in Spirulina (NIFI) whereas Spirulina (BP) and Spirulina (MP) had slight difference of growth (Figure 20). All 3 strains of Spirulina could grow at NaCl as high as 20 g/l. Figure 21 showed that different concentrations of NaCl did not affect phycocyanin content. We chose NaCl concentration at 1 g/l to be the optimum condition for later experiments.

3.5 Effect of light intensity on phycocyanin content

In all 3 strains of Spirulina, the lowest growth occurred at 1500 Lux (Figure 22). Optimum light intensities for growth of all 3 strains of Spirulina were found to be 5000 and 6500 Lux. At 8,000 lux slightly reduced growth occurred. Phycocyanin content was also affected by light intensity. The highest phycocyanin content was found at 5,000 lux for Spirulina (BP) and Spirulina(NIFI) and at 6,500 Lux for Spirulina (MP) (Figure 23)

Figure 20 Growth of Spirulina in Zarrouk medium containing various NaCl concentrations

(——) NaCl 1 g/l

(—+—) NaCl 5 g/l

(—*—) NaCl 10 g/l

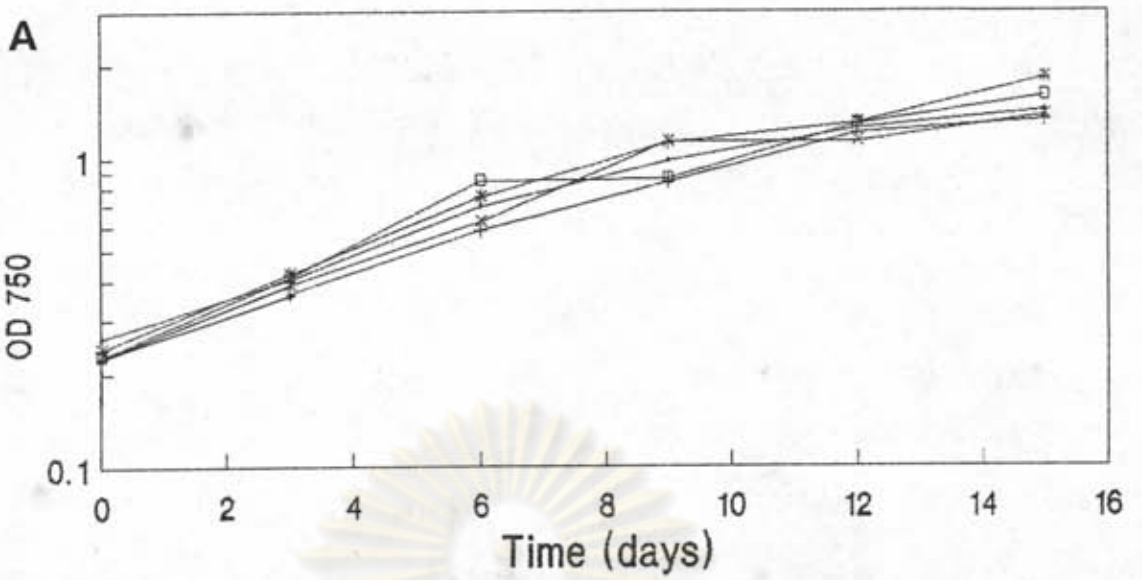
(—□—) NaCl 15 g/l

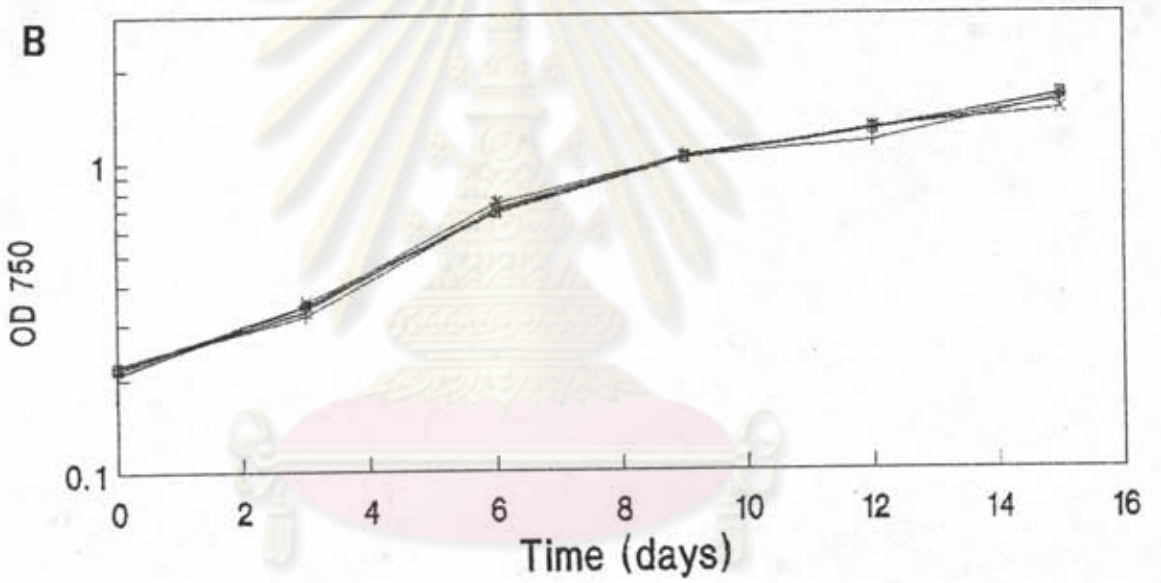
(—×—) NaCl 20 g/l

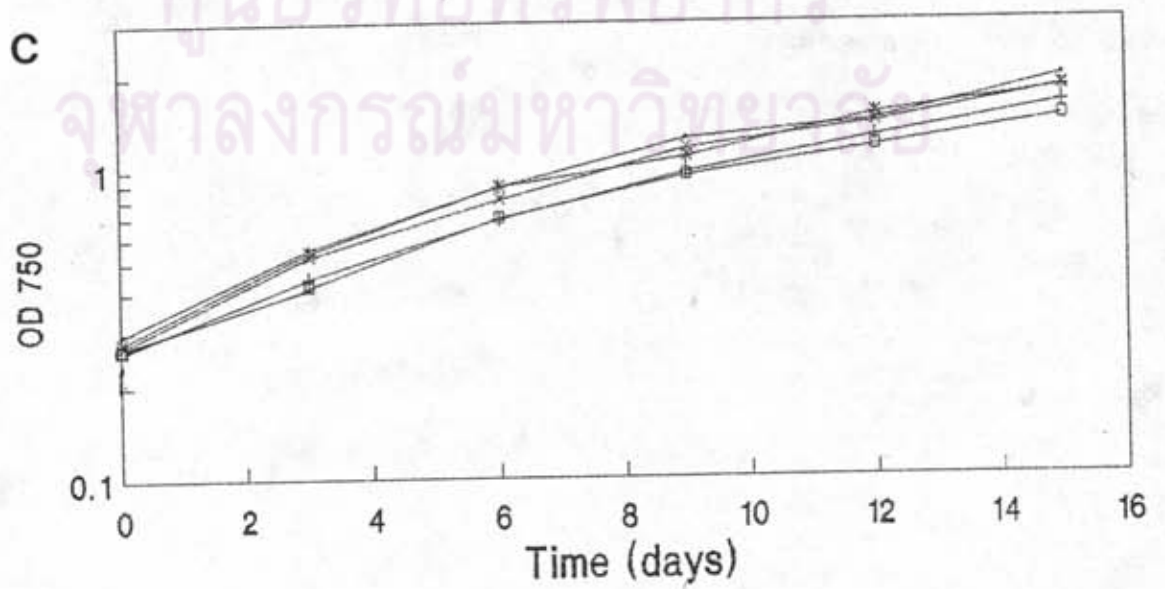
A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)







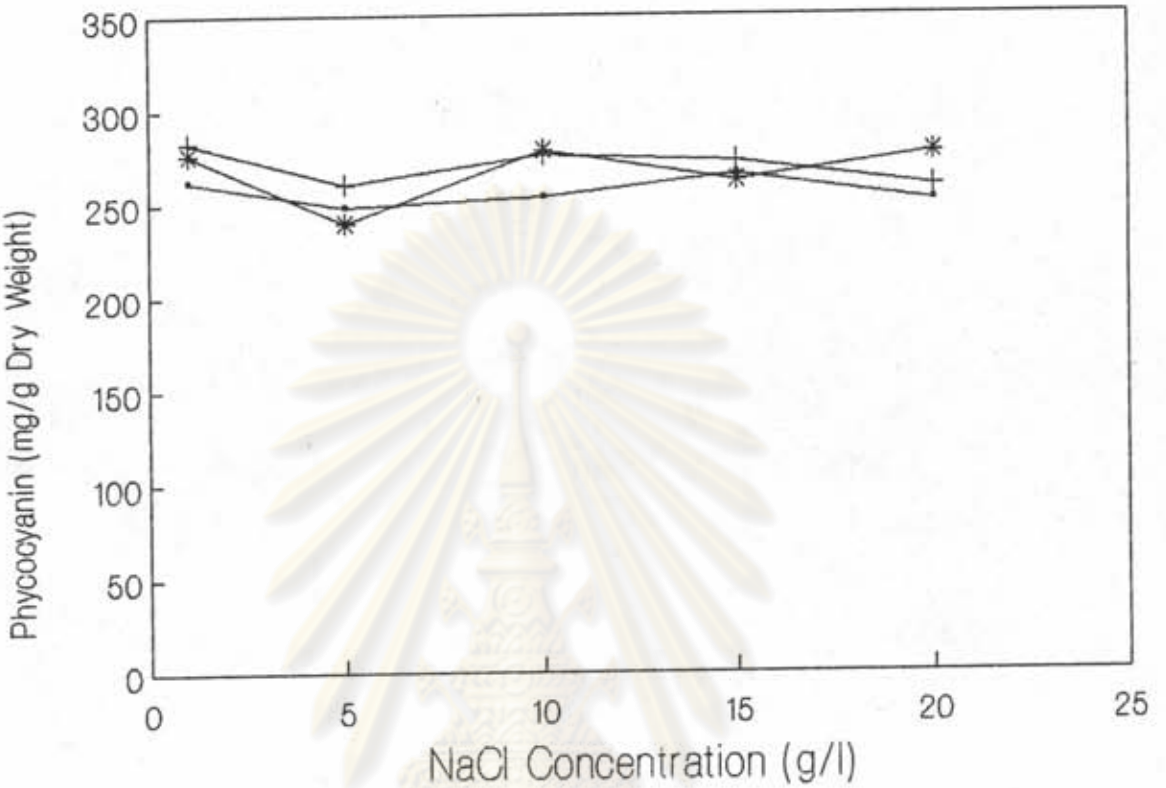


Figure 21 Effect of NaCl on phycocyanin at day 12 of three strains of Spirulina

(—•—) Spirulina (BP)

(—+—) Spirulina (NIFI)

(—*—) Spirulina (MP)

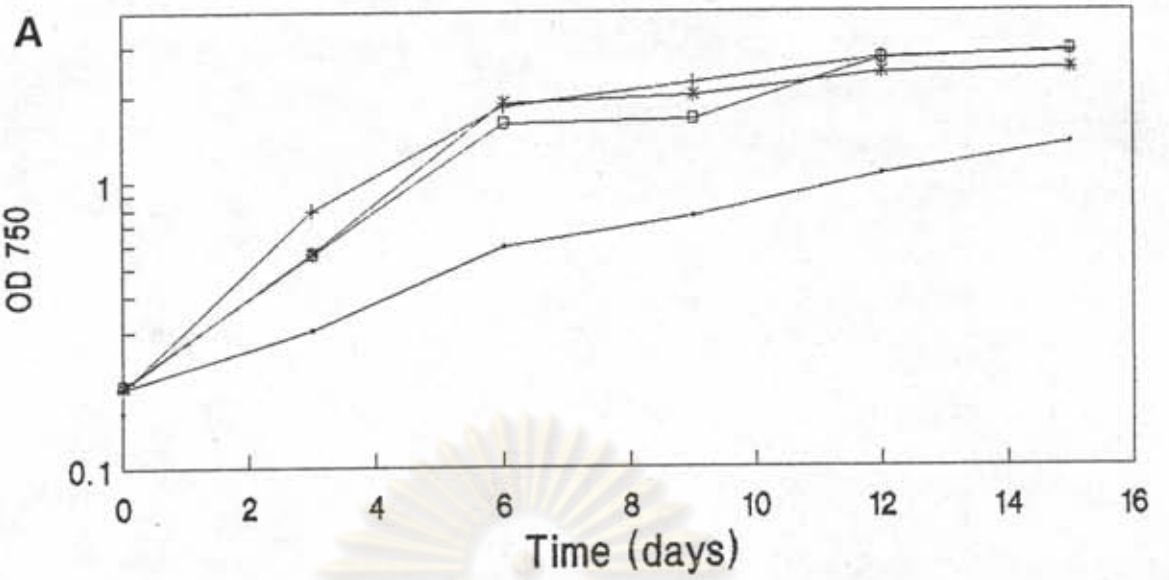
Figure 22 Growth of Spirulina in Zarrouk medium under various light intensities

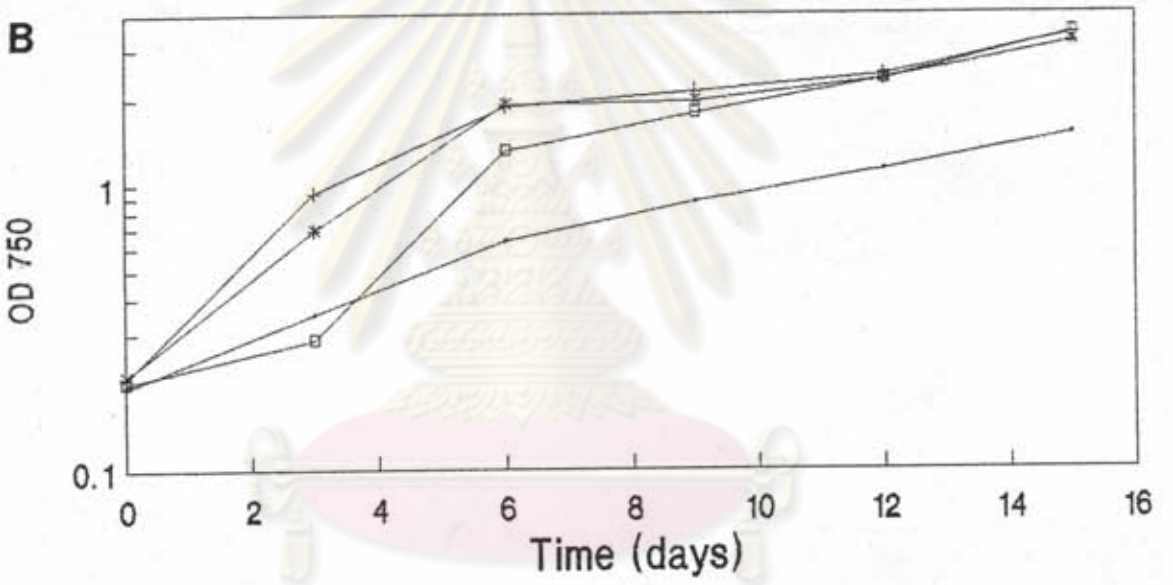
(—•—) 1500 lux
(—+—) 5000 lux
(—*—) 6500 lux
(—o—) 8000 lux

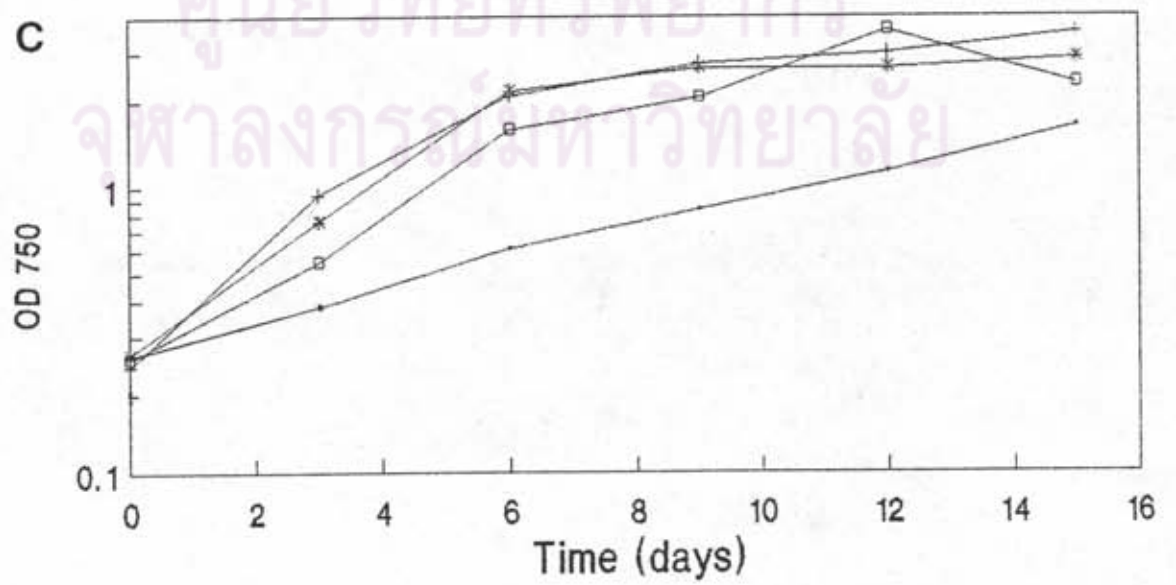
A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)







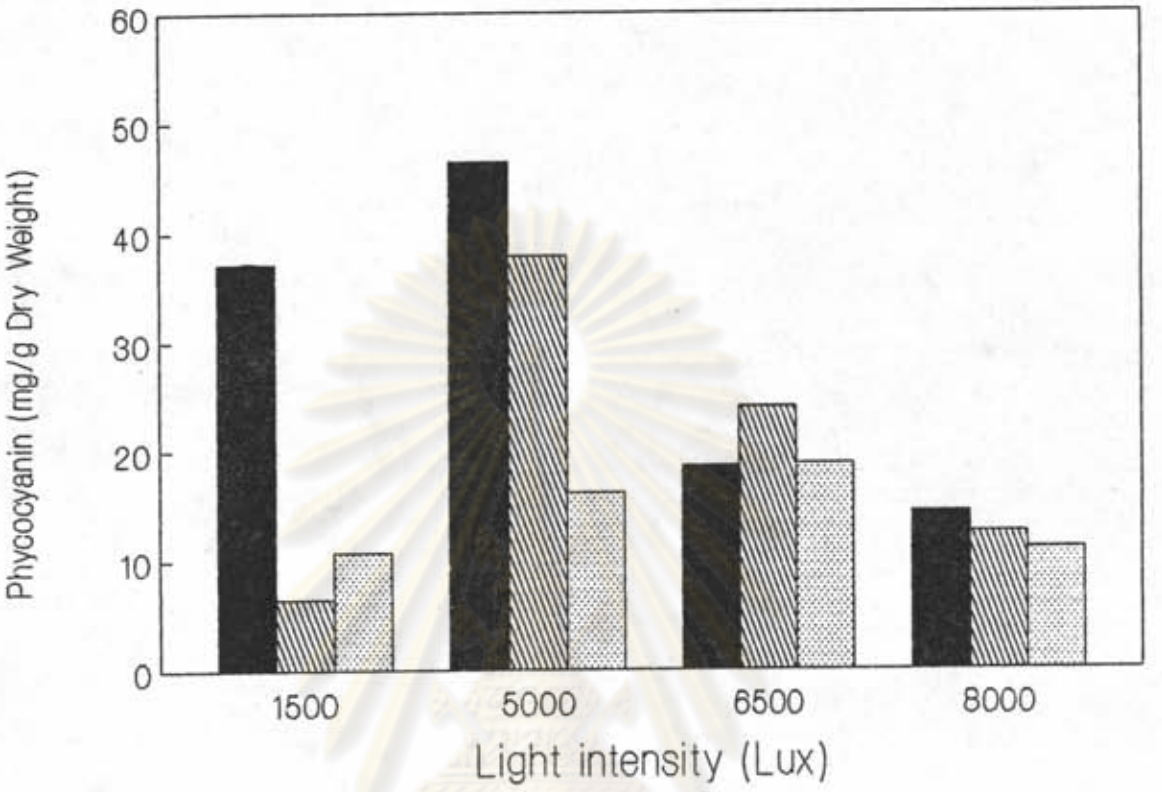


Figure 23 Effect of light intensity on phycocyanin at day 12 of three strains of Spirulina.

3.6 Results of Spirulina grown under optimized condition versus Zarrouk condition

In all 3 strains of Spirulina optimized condition of Zarrouk medium gave both higher growth and higher phycocyanin than in normal Zarrouk (Figures 24 and 25). In Spirulina (BP) the highest yield of phycocyanin occurred at day 12. However, in Spirulina (NIFI) and Spirulina (MP) the highest yield of phycocyanin was detected after 6 days cultivation (Figure 25). Furthermore, the highest yield of phycocyanin under optimized Zarrouk condition for all 3 strains of Spirulina accounted for approximately 290 mg/g dry weight.

4 Comparative study of the effect of light quality on phycocyanin content

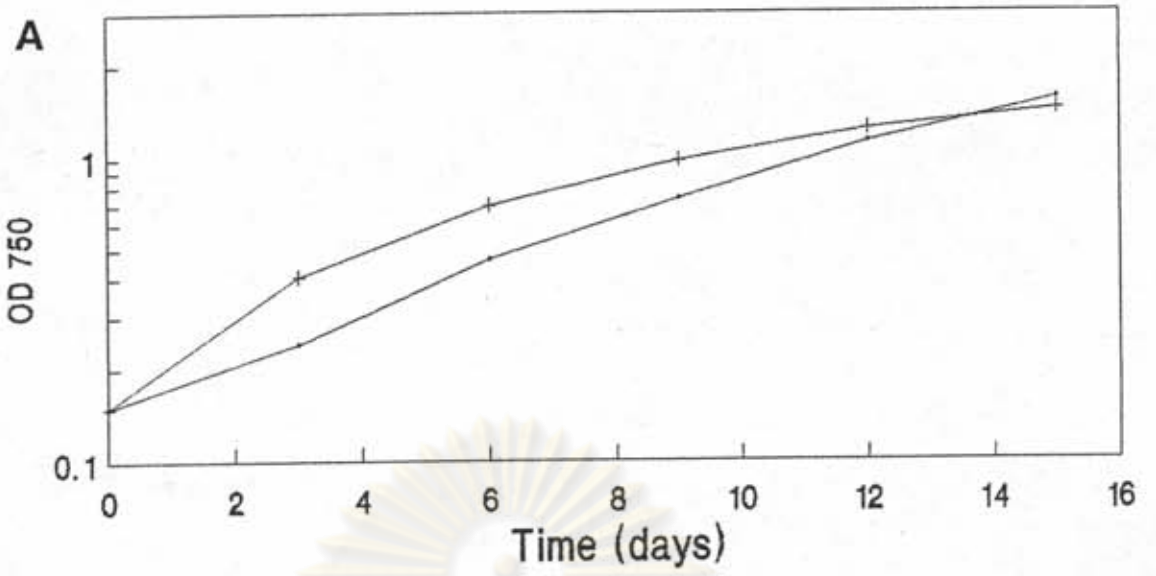
Three strains of Spirulina were grown in green light (35 $\mu Es^{-1}m^{-2}$) ,red light (35 $\mu Es^{-1}m^{-2}$) and white light (270 $\mu Es^{-1}m^{-2}$). Measurement of the photon flux was performed with a Li-cor quantum meter Li-189. The light source is a fluorescent lamp, specific chromatic illumination being provided by the interposition of plastic filters (cellulose acetate colored filters available in arts supply stores). Typical transmission spectra of green and red filters routinely used are shown in Figure 26 (Tandeau De Marsac,1977).

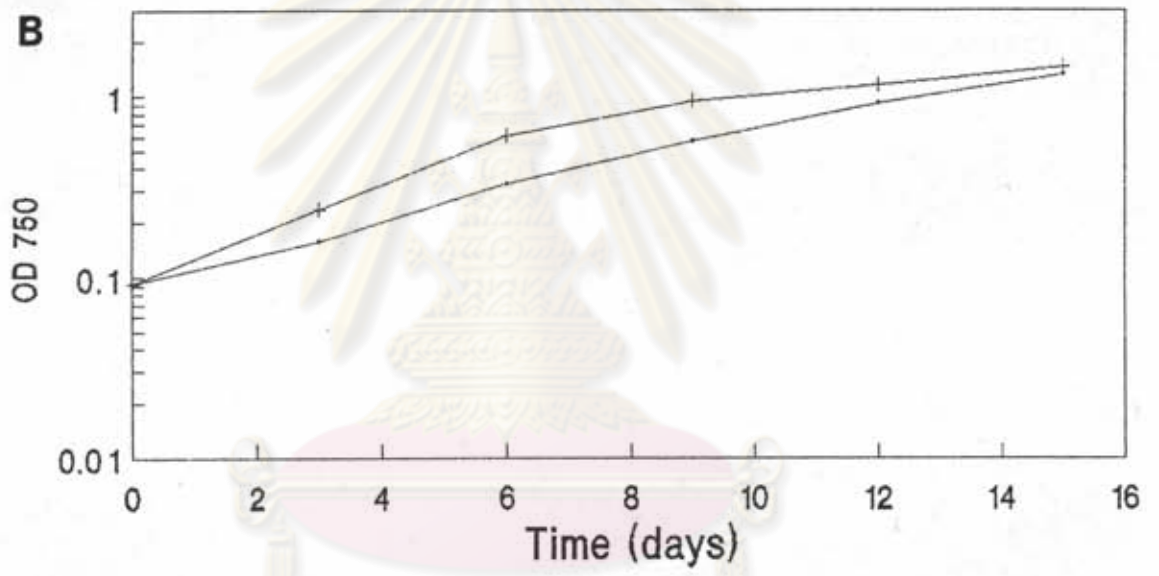
Figure 24 Growth of Spirulina in Zarrouk medium (—•—)and in optimized Zarrouk medium (—+—)

A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)





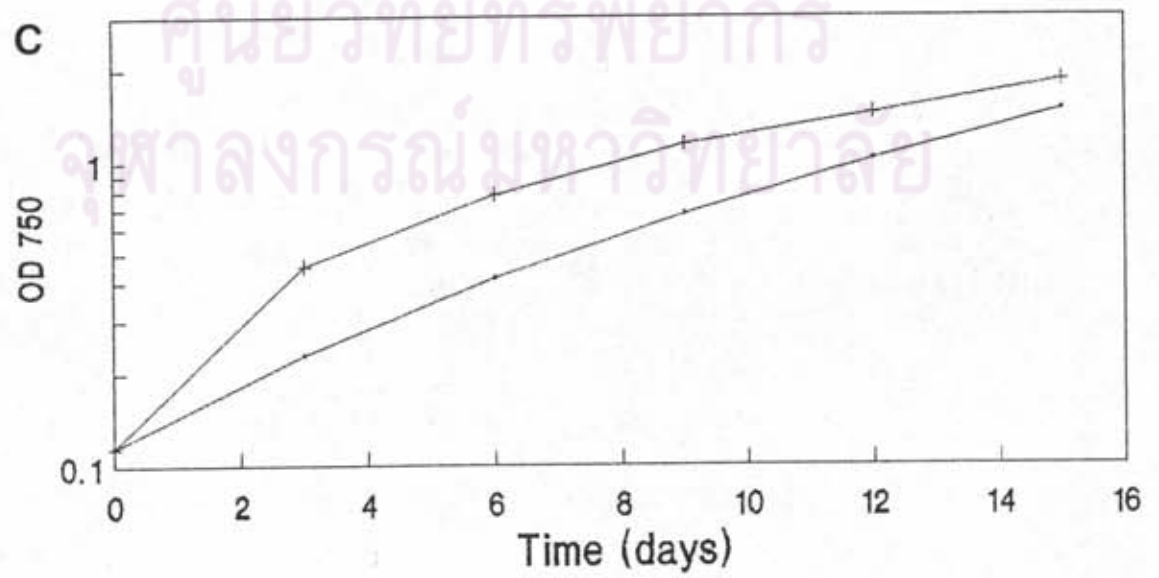
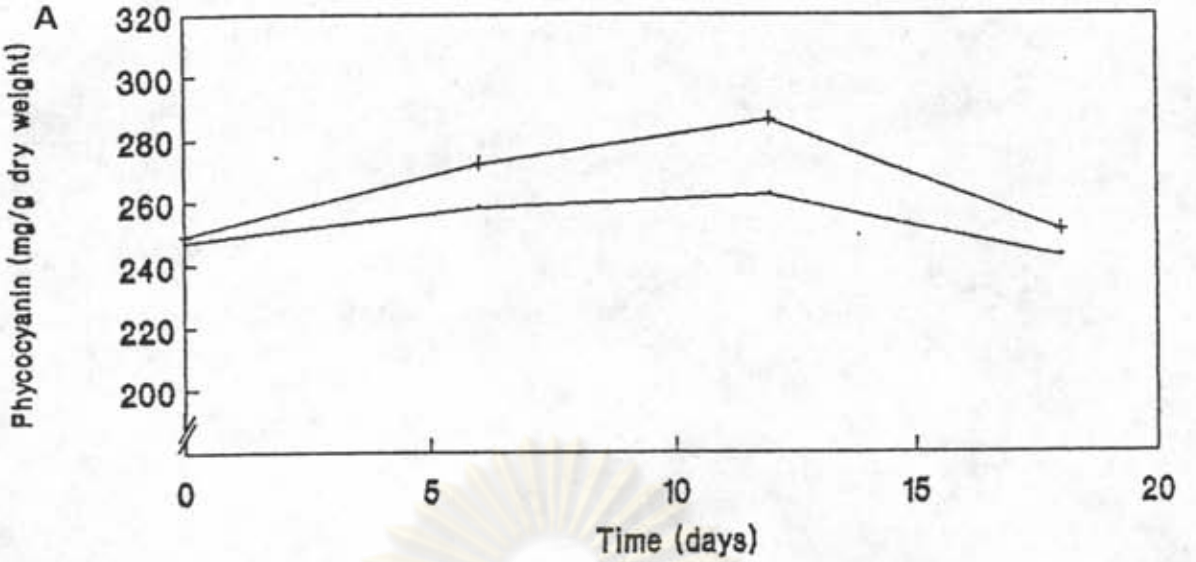


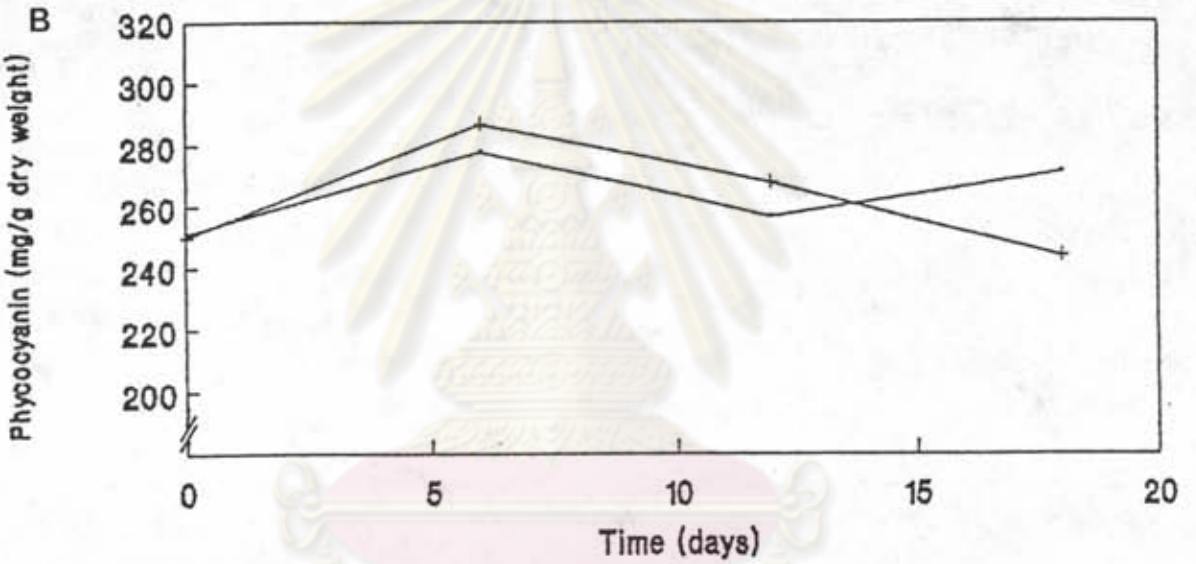
Figure 25 Phycocyanin content of Spirulina grown in Zarrouk medium (—·—) and optimized Zarrouk medium (—+—)

A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)





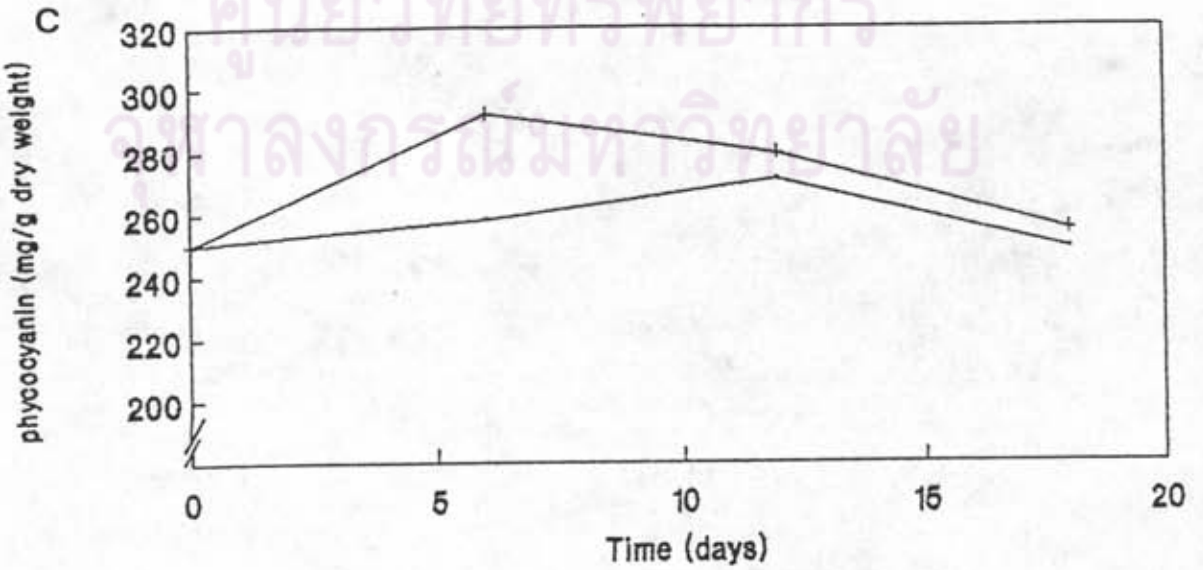
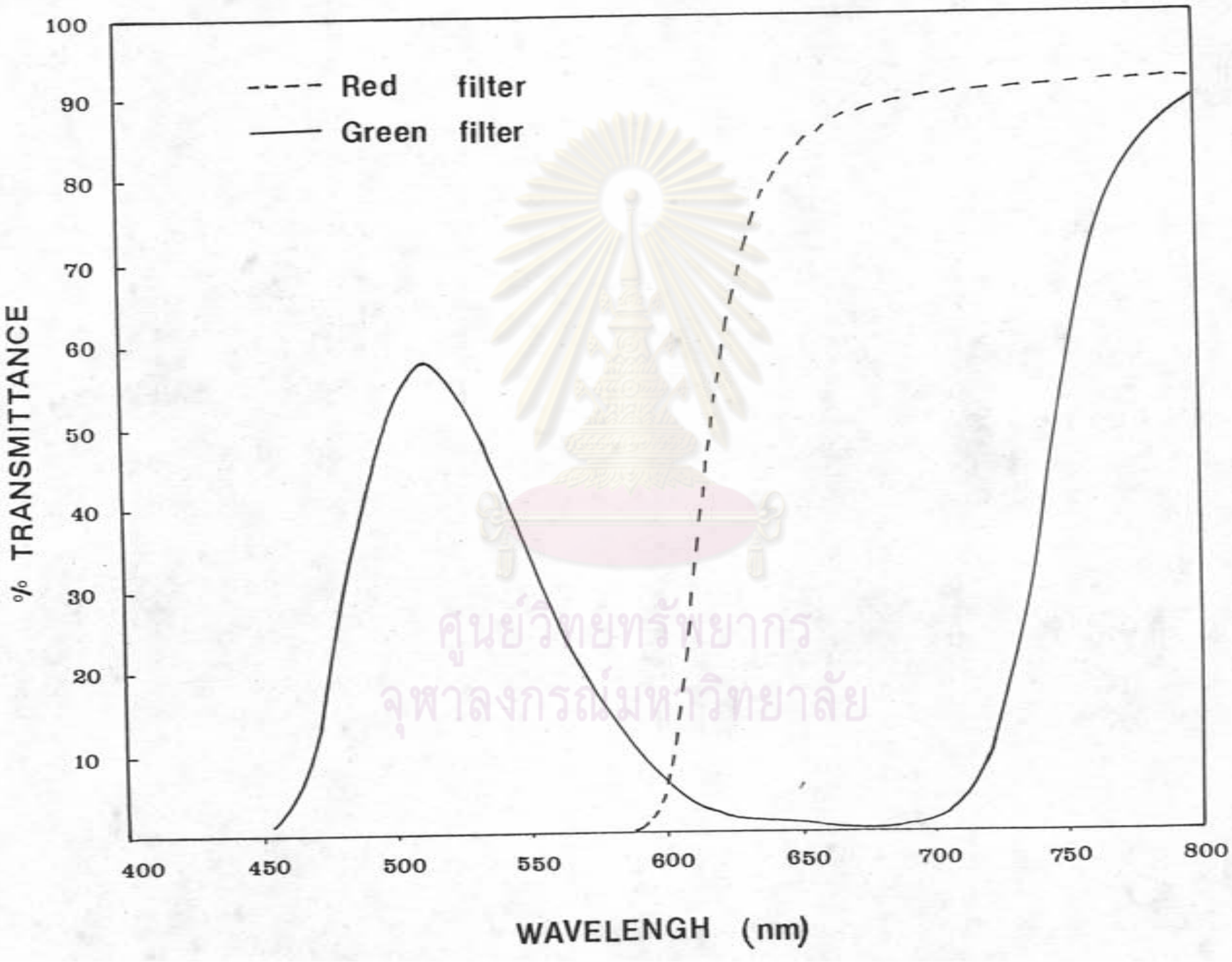


Figure 26 Optical characteristics of the red and green filters



4.1 Effect of red light compared with white light

Growth of all 3 strains of Spirulina in white light was higher than in red light (Figure 27). Figure 29 showed that in red light phycocyanin content was higher than in white light.

4.2 Effect of green light compared with white light

White light also stimulated better growth than green light for all 3 strains of Spirulina (Figure 28). Figure 29 showed that in green light phycocyanin content was higher than in white light.

5. Effect of changing level of $NaNO_3$ concentration

5.1 Changing level of $NaNO_3$ from low to that of Zarrouk medium

Growth of Spirulina (BP) and (NIFI) were not different from the control but in Spirulina (MP) growth was lower than that of the control (Figures 30,31 and 32). In Figures 33, 34 and 35 phycocyanin contents were slightly changed.

5.2 Changing level of $NaNO_3$ from that of Zarrouk medium to high content

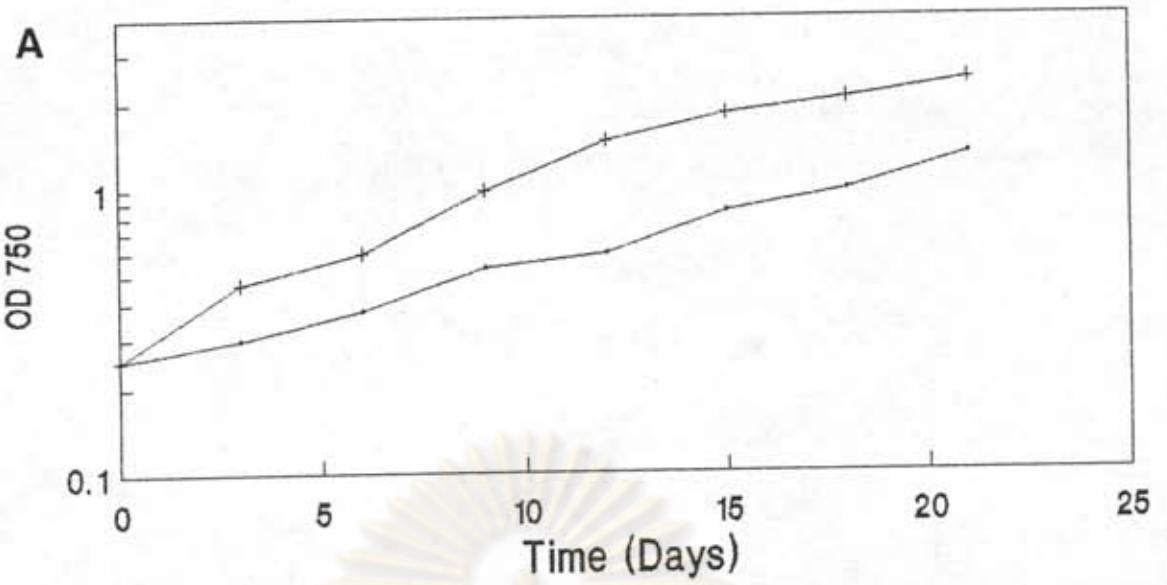
Growth of Spirulina (BP) and (NIFI) were

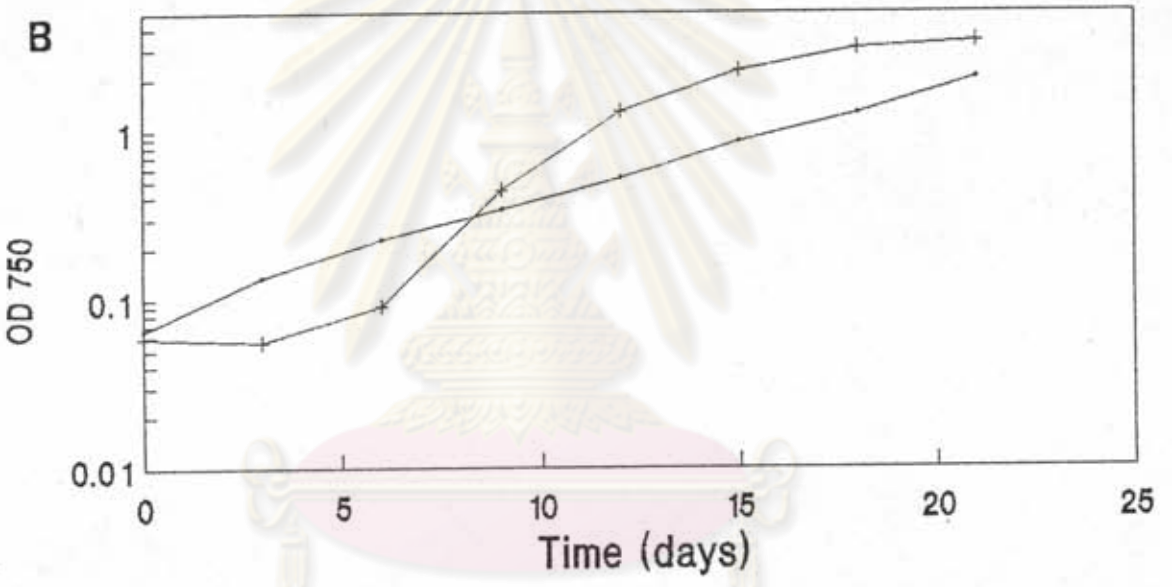
Figure 27 Growth of Spirulina in Zarrouk medium under red light (—·—) and white light (—+—)

A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)





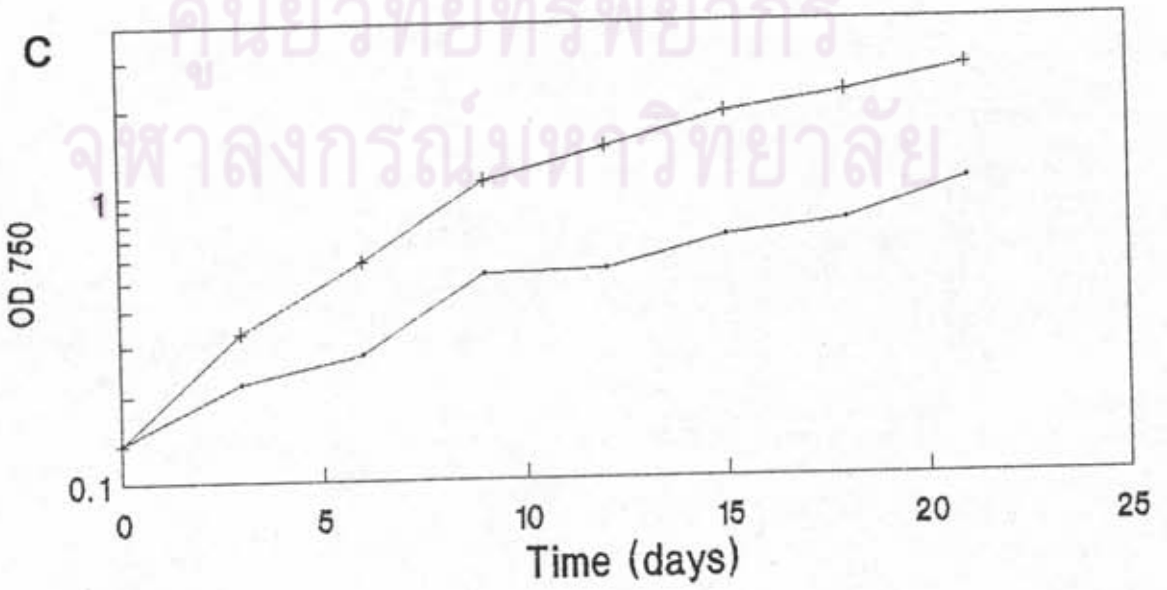
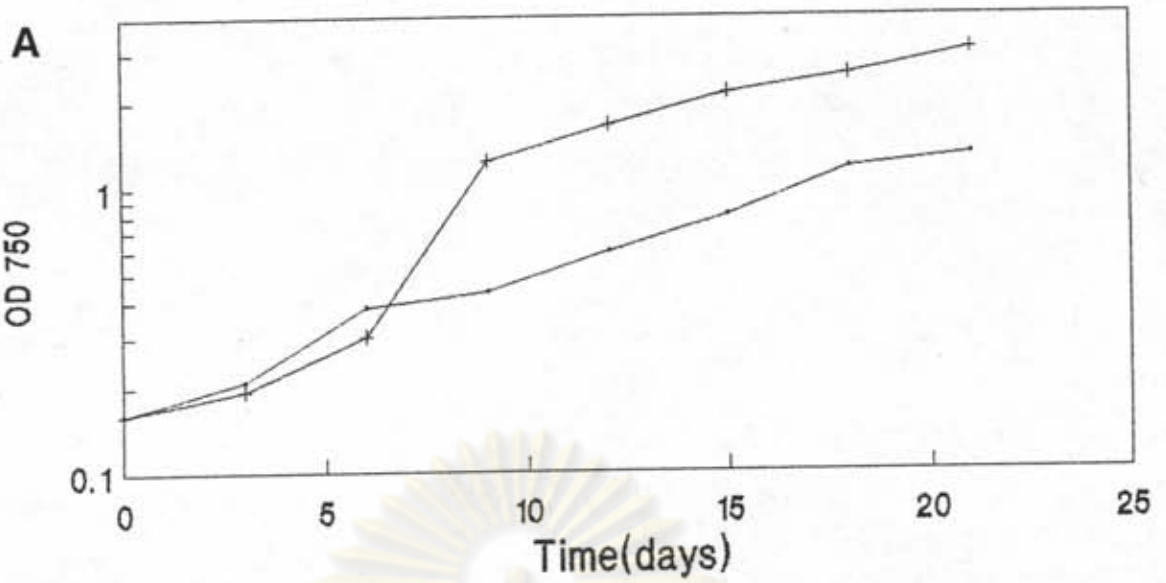


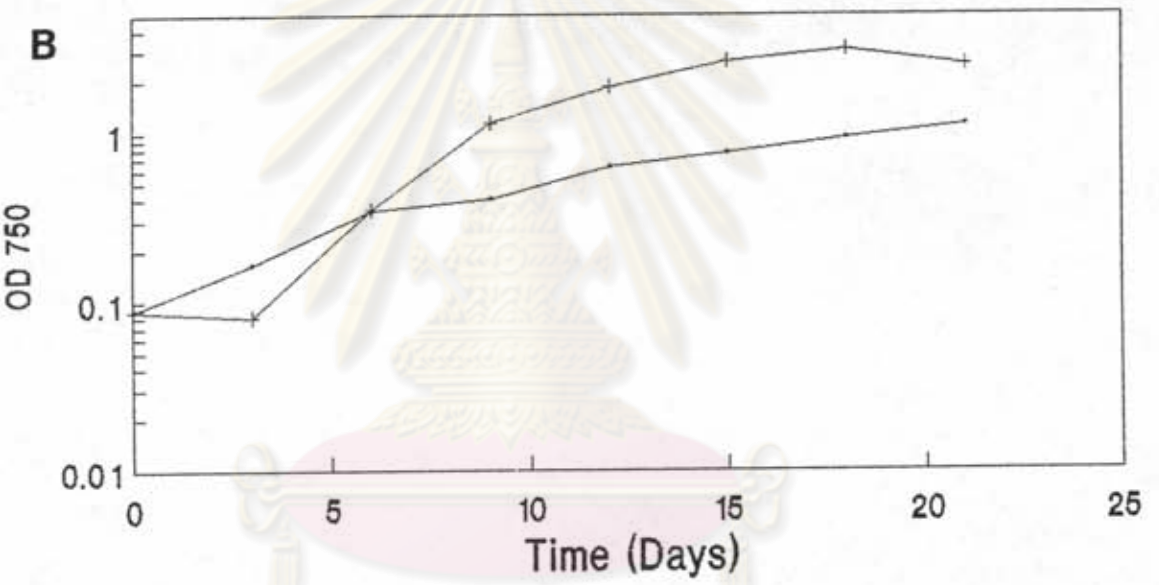
Figure 28 Growth of Spirulina in Zarrouk medium under green light (—•—) and white light (—+—)

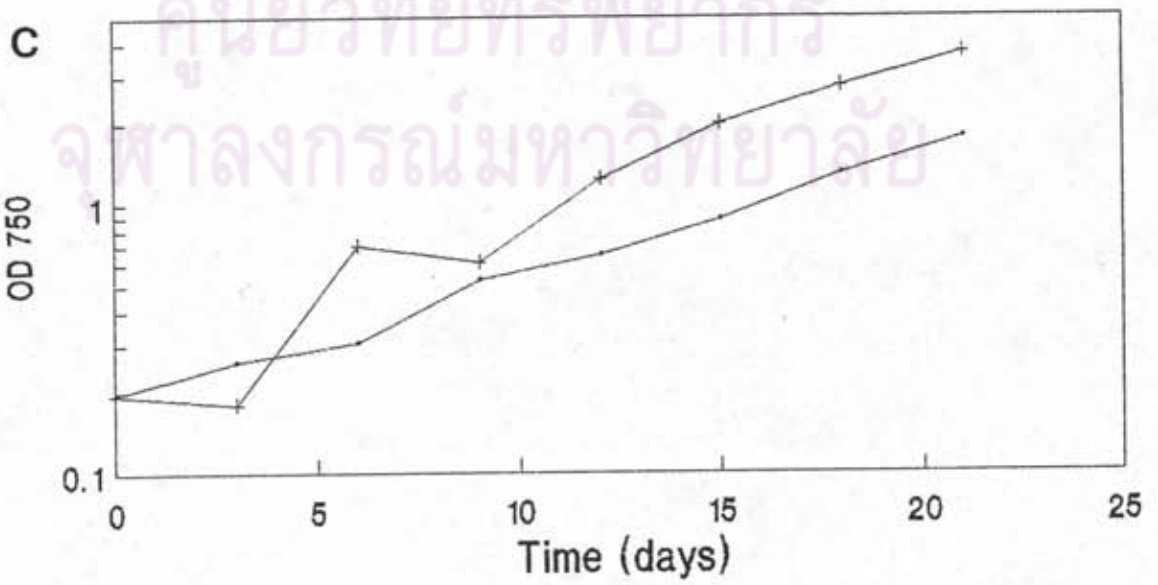
A. Spirulina (BP)

B. Spirulina (NIFI)

C. Spirulina (MP)







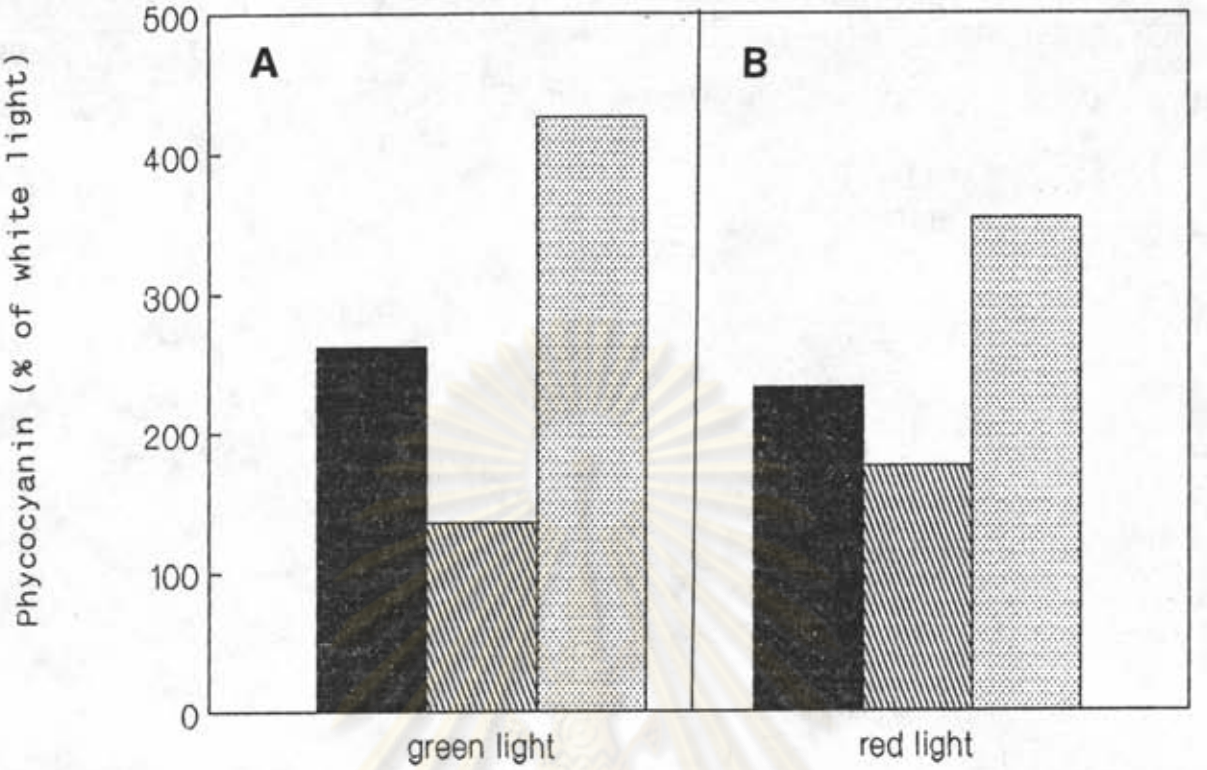


Figure 29 Effect of light quality on phycocyanin at day 12 of three strains of Spirulina

A. Green light

100 % of white light for Spirulina (BP), Spirulina (NIFI) and Spirulina (MP) were 26.1 , 22.2 and 21.9 mg per g dry weight respectively.

B. Red light

100 % of white light for Spirulina (BP), Spirulina (NIFI) and Spirulina (MP) were 29.3 ,22.7 and 15.5 mg per g dry weight respectively.

not different from the control but in Spirulina (MP) growth was lower than the control (Figures 30,31 and 32). In Spirulina (MP) and (NIFI) phycocyanin contents were higher than the control but in Spirulina (BP) the higher content was observed only at 12 and 15 hours culture (Figures 33,34 and 35).

6. Partial purification of phycocyanin

6.1 Methods employed for the extraction of phycocyanin from Spirulina

Many preliminary experiments were done to obtain the best method of extracting phycocyanin from Spirulina . The methods used were 1) lysozyme digestion (Boussiba and Richmond,1979), 2) sonication ,3) homogenization (Kao,Edwards and Berns,1979) , 4) French pressure and 5) freeze thaw (Boussiba and Richmond,1979) . The results indicated that when cells were frozen at -70° C and thawed in a water bath at 37°C for 3 cycles , nearly all of phycocyanin could be extracted .This method was relatively easy and convenient and thus chosen for the extraction of phycocyanin in later experiments.

6.2 Ammonium sulfate precipitation

5 mg wet weight of Spirulina were suspended in 500 ml of 0.1 M sodium phosphate buffer pH 7.0. Five cycles of freeze and thaw were done and finally

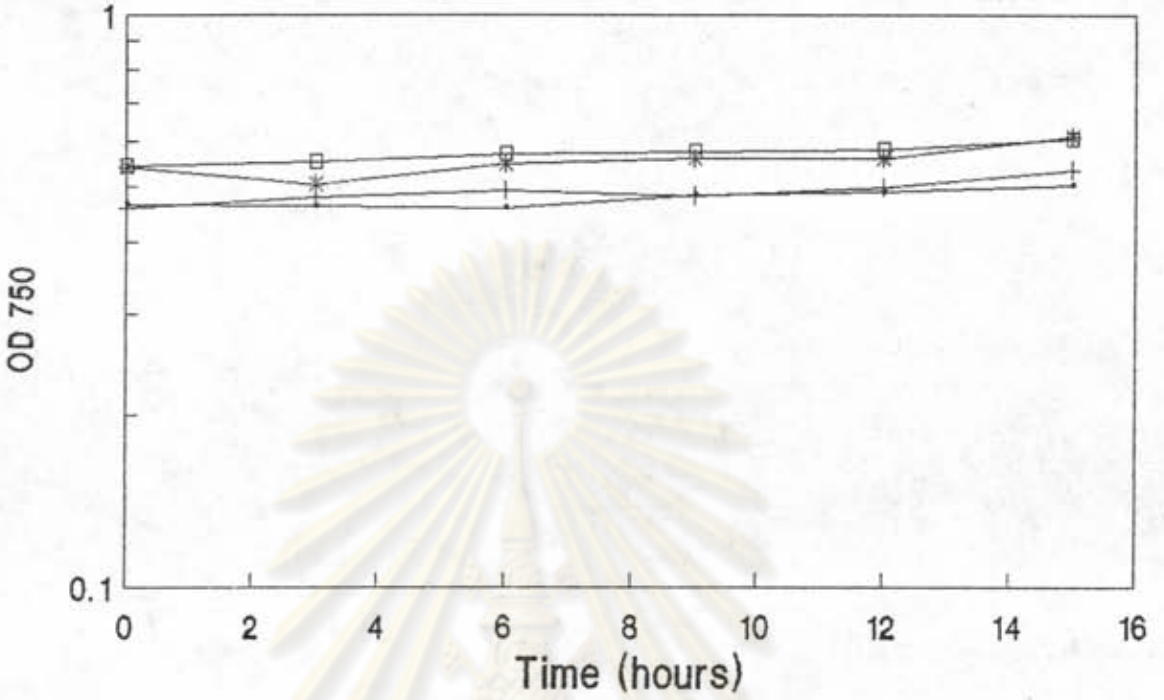


Figure 30 Growth of Spirulina (BP) in changing level of $NaNO_3$ concentrations

(—•—) changing from low $NaNO_3$ (0.33 g/l) to Zarrouk $NaNO_3$ (2.5 g/l)

(—+—) no change (control)

(—*—) changing from Zarrouk $NaNO_3$ (2.5 g/l) to high $NaNO_3$ (10 g/l)

(—□—) no change (control)

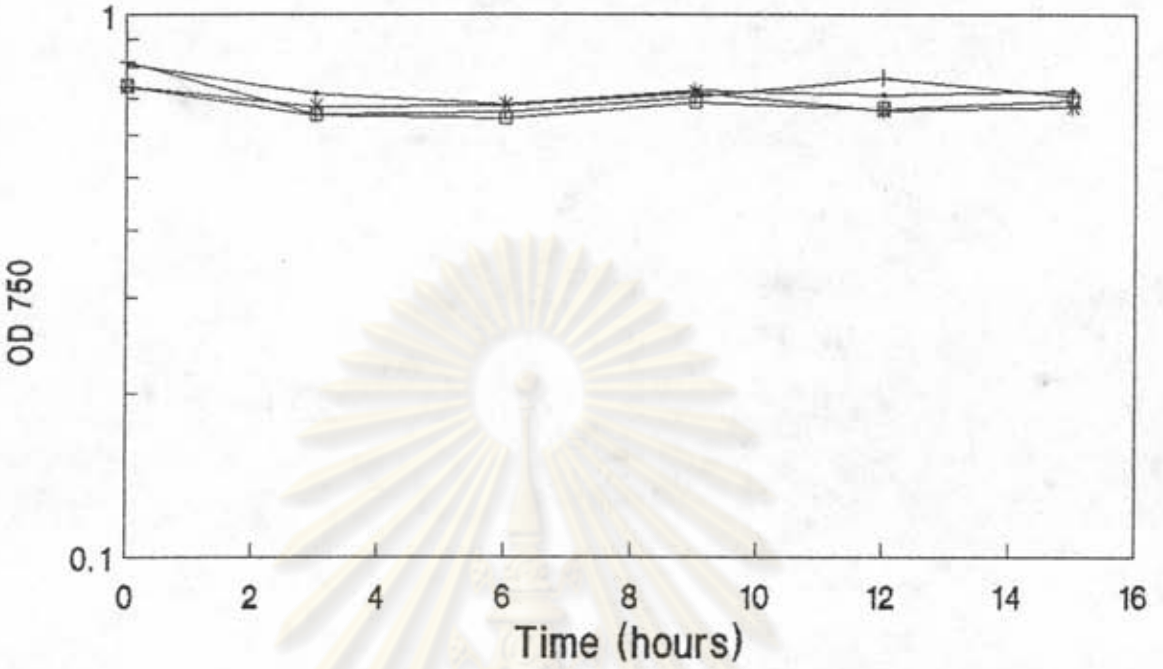


Figure 31 Growth of Spirulina (NIFI) in changing level of $NaNO_3$ concentrations

(—•—) changing from low $NaNO_3$ (0.33 g/l) to Zarrouk $NaNO_3$ (2.5 g/l)

(—+—) no change (control)

(—*—) changing from Zarrouk $NaNO_3$ (2.5 g/l) to high $NaNO_3$ (10 g/l)

(—□—) no change (control)

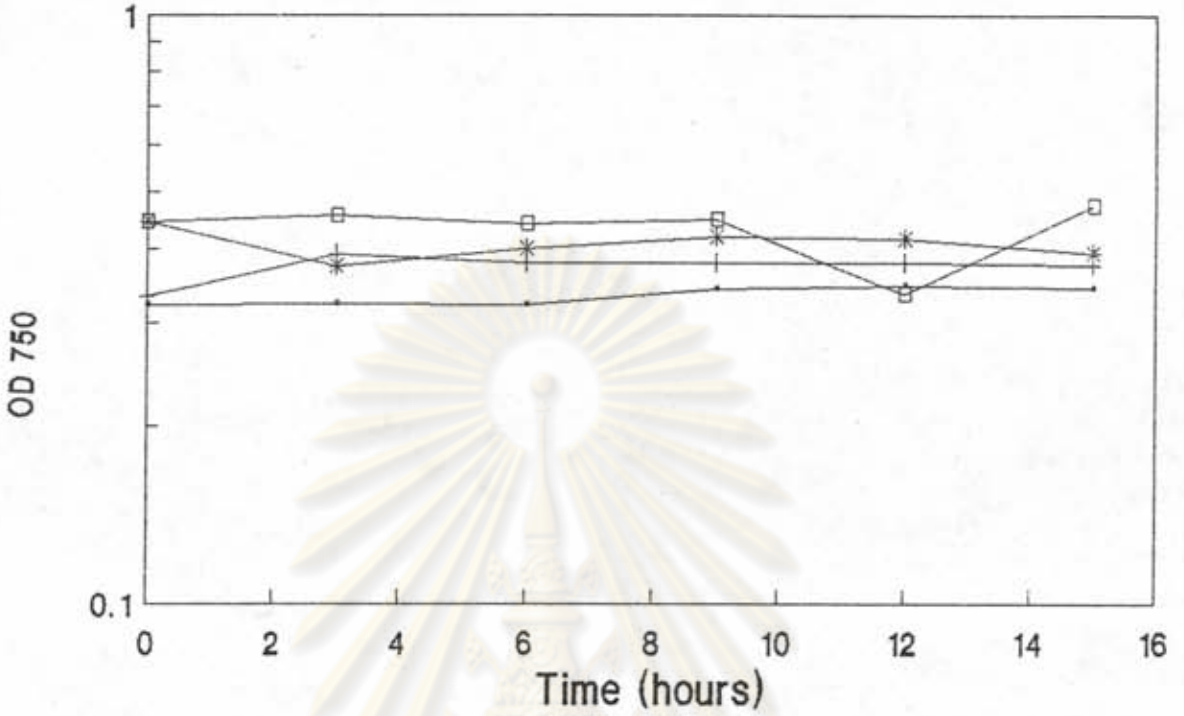


Figure 32 Growth of Spirulina (MP) in changing level of $NaNO_3$ concentrations

(—•—) changing from low $NaNO_3$ (0.33 g/l) to Zarrouk $NaNO_3$ (2.5 g/l)

(—+—) no change (control)

(—*—) changing from Zarrouk $NaNO_3$ (2.5 g/l) to high $NaNO_3$ (10 g/l)

(—□—) no change (control)

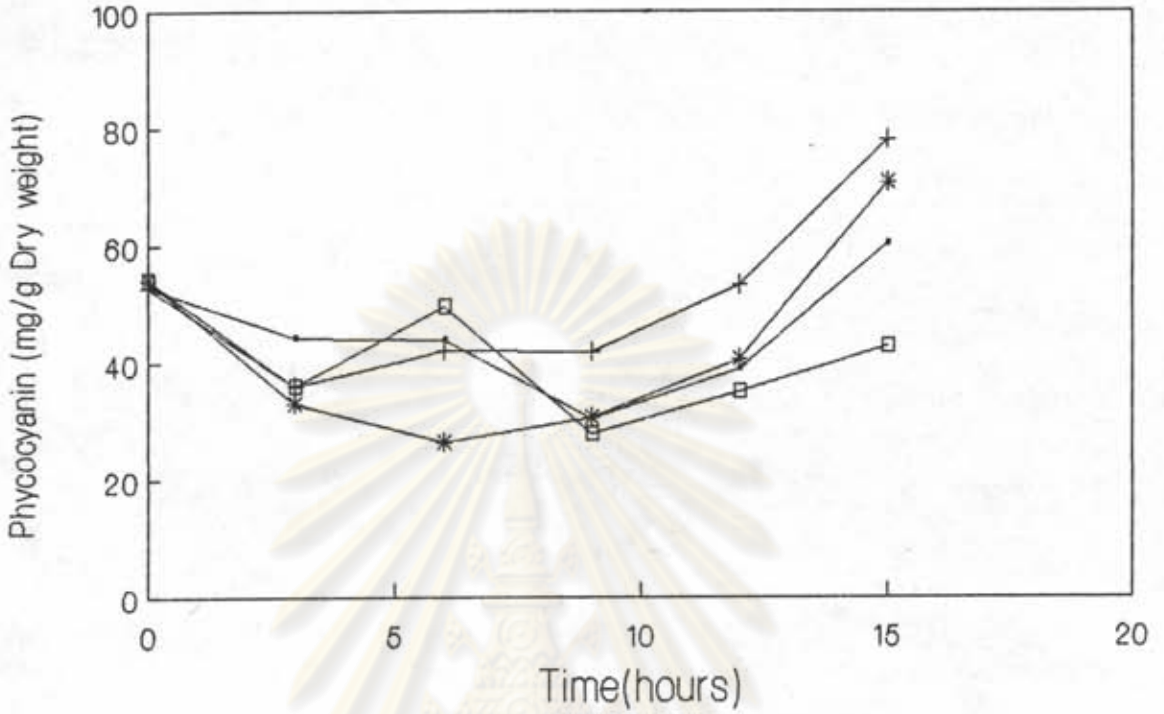


Figure 33 Effect of changing level of $NaNO_3$ on phycocyanin in Spirulina (BP)

(—•—) changing from low $NaNO_3$ (0.33 g/l) to Zarrouk $NaNO_3$ (2.5 g/l)

(—+—) no change (control)

(—*—) changing from Zarrouk $NaNO_3$ (2.5 g/l) to high $NaNO_3$ (10 g/l)

(—□—) no change (control)

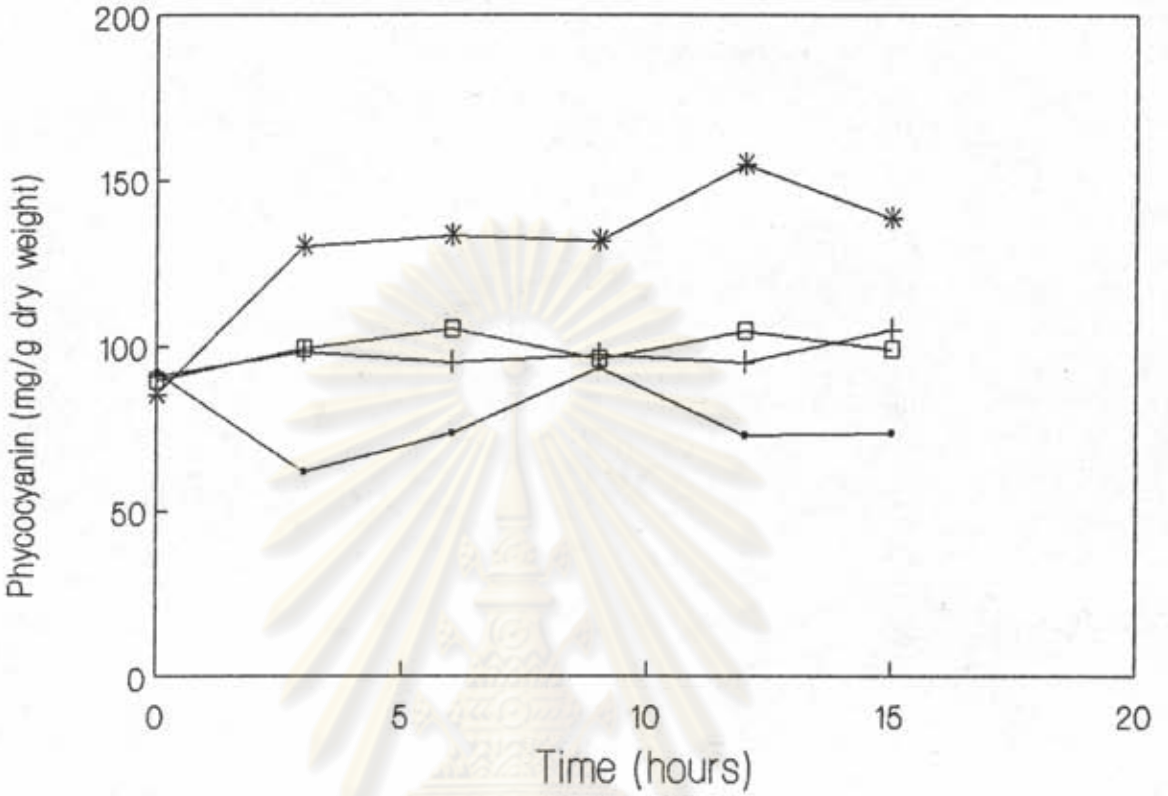


Figure 34 Effect of changing level of $NaNO_3$ on phycocyanin in Spirulina (NIFI)

(—) changing from low $NaNO_3$ (0.33 g/l) to Zarrouk $NaNO_3$ (2.5 g/l)

(—+—) no change (control)

(—*—) changing from Zarrouk $NaNO_3$ (2.5 g/l) to high $NaNO_3$ (10 g/l)

(—□—) no change (control)

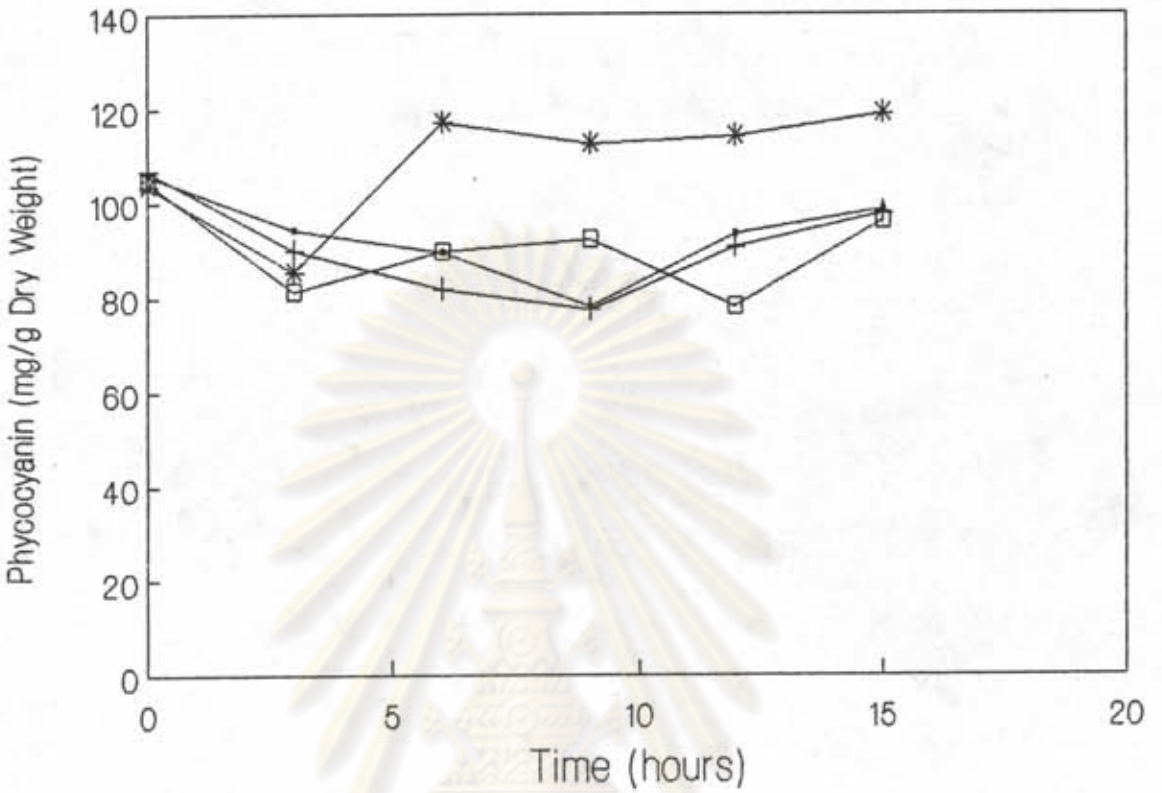


Figure 35 Effect of changing level of $NaNO_3$ on phycocyanin in Spirulina (MP)

(—•—) changing from low $NaNO_3$ (0.33 g/l) to Zarrouk $NaNO_3$ (2.5 g/l)

(—+—) no change (control)

(—*—) changing from Zarrouk $NaNO_3$ (2.5 g/l) to high $NaNO_3$ (10 g/l)

(—□—) no change (control)

the suspension was centrifuged at 2,000 x g 20 min to remove cell debris. The supernatant was precipitated with 0-50 % , 0-75 %, 20-45 %, 20-65 % and 20-75 % ammonium sulfate.The suspension was centrifuged at 2,000 x g, 20 min. The pellet was suspended in 0.1 M sodium phosphate buffer pH 7.0 , the absorbance was measured at 620 nm and phycocyanin content was calculated by using $E^{1\%}_{1\,cm} = 73$ (Boussiba and Richmond,1979)

The results in Table 2 showed that 20-65 % ammonium sulfate was the optimum condition for phycocyanin precipitation because of the highest yield of phycocyanin. The discarded supernatant was clear (no blue color of non precipitated phycocyanin) indicating that phycocyanin was completely precipitated.

6.3 Partial purification of phycocyanin from Spirulina

10 g wet weight of Spirulina was suspended in 100 ml of 0.02 M sodium phosphate buffer pH 7.5 and freeze thawed 5 cycles followed by centrifugation at 2,000 x g, 20 min. The supernatant was precipitated with 20-65 % ammonium sulfate and dialyzed against the same buffer overnight.

Procedure of phycocyanin purification for three strains of Spirulina was as described by Boussiba and Richmond (1979). Phycocyanin from each strain was

Table 2 Result of ammonium sulfate precipitation

% Ammonium sulfate	Phycocyanin (mg/100 ml extract)
0–50	1.34
0–75	1.76
20–45	0.19
20–65	2.31

purified by Ion exchange chromatography on DEAE-cellulose column and gel filtration on Sephadex G-150 column. Results of purification were summarized in Table 3 . Details of purification step for each strain are described below.

Spirulina (BP)

1. DEAE-cellulose chromatography

240 mg protein fraction from 20-65 % ammonium sulfate suspended in 25 ml 0.02 M sodium phosphate buffer pH 7.5 was loaded on DEAE-cellulose column (I) (2.5 x 30 cm).The unbound proteins were washed with the same buffer. Phycocyanin was eluted by 300 ml of linear gradient of 0 - 0.5 M NaCl prepared in 0.02 M sodium phosphate buffer pH 7.5. Four ml fractions were collected and the absorbances were measured at 280 and 620 nm. The elution profile after DEAE cellulose column (I) (Figure 36) showed a single peak with a small shoulder. Phycocyanin (OD 620) was eluted between NaCl concentration of 0 - 0.3 M. Absorption in the ultraviolet region (OD 280) coincided with the elution profile of the coloured fraction (OD 620) .The protein peak fractions (79-107) were pooled and precipitated by 20-65 % ammonium sulfate before rechromatography on DEAE-cellulose column .The elution profile in Figure 37 showed a single peak of protein coincident with phycocyanin peak which was eluted at 0 - 0.3 M NaCl. The phycocyanin peak

Table 3 Partial purification of phycocyanin from 3 strains of Spirulina. All purification steps (except centrifugation and dialysis) were performed at 25°C. Phycocyanin was calculated by using $E^{1\%}_{1cm} = 73$

A. Spirulina (BP)

Fraction	Spirulina (BP)		
	Total Phycocyanin (mg)	% Yield of Phycocyanin	Absorbance Ratio (nm) A620/A280
Crude Phycocyanin	84.0	100	0.84
DEAE Cellulose (I)	63.1	75	2.81
DEAE Cellulose (II)	37.3	44	4.44
Sephadex G-150	21.8	26	4.04

B. Spirulina (NIFI)

Fraction	Spirulina (NIFI)		
	Total Phycocyanin (mg)	% Yield of Phycocyanin	Absorbance Ratio (nm) A620/A280
Crude Phycocyanin	107.1	100	1.20
DEAE Cellulose (I)	67.5	63	1.30
DEAE Cellulose (II)	42.1	39	1.41
Sephadex G-150	23.9	22	3.49

C. Spirulina (MP)

Fraction	Spirulina (MP)		
	Total Phycocyanin (mg)	% Yield of Phycocyanin	Absorbance Ratio (nm) A620/A280
Crude Phycocyanin	117.7	100	0.96
DEAE Cellulose (I)	65.5	56	1.36
DEAE Cellulose (II)	56.6	48	4.73
Sephadex G-150	28.5	24	3.14

fractions (81-96) were pooled and precipitated by 20-65 % ammonium sulfate before loading on Sephadex G-150 column.

2 Sephadex G-150

8.5 mg of protein (3 ml) was loaded on Sephadex G-150 column and eluted with 0.02 M sodium phosphate buffer pH 7.5. Two ml fractions were collected and the recovery of phycocyanin was 26 % (Table 3 A). The elution profile was shown in figure 38.

Spirulina (NIFI)

1.DEAE-cellulose chromatography

140 mg protein (20 ml) was loaded on DEAE cellulose column (I) and the linear gradient was performed. The elution profile after DEAE-cellulose column was shown in Figure 39 and the % recovery was 63 %. Rechromatography was also done on DEAE-cellulose column (II). Forty-nine mg protein (9 ml) was loaded on DEAE-cellulose column (II). The elution profile was shown in Figure 40 and the % recovery was 39 %.

2.Sephadex G-150

66 mg protein (4 ml) was loaded on Sephadex G-150 column. The elution profile after Sephadex G-150 column was shown in Figure 41 and the % recovery was 22 % (Table 3 B).

Figure 36 Chromatographic profile of 65 % ammonium sulfate fraction from Spirulina (BP) on DEAE-cellulose column.A 2.5 x 30 cm column and a linear gradient of 0 - 0.5 M NaCl in 0.02 M sodium phosphate buffer pH 7.5 was used. The flow rate was maintained at 30 ml/hr.The 4 ml fractions were collected and the absorbances were measured at 620 and 280 nm.

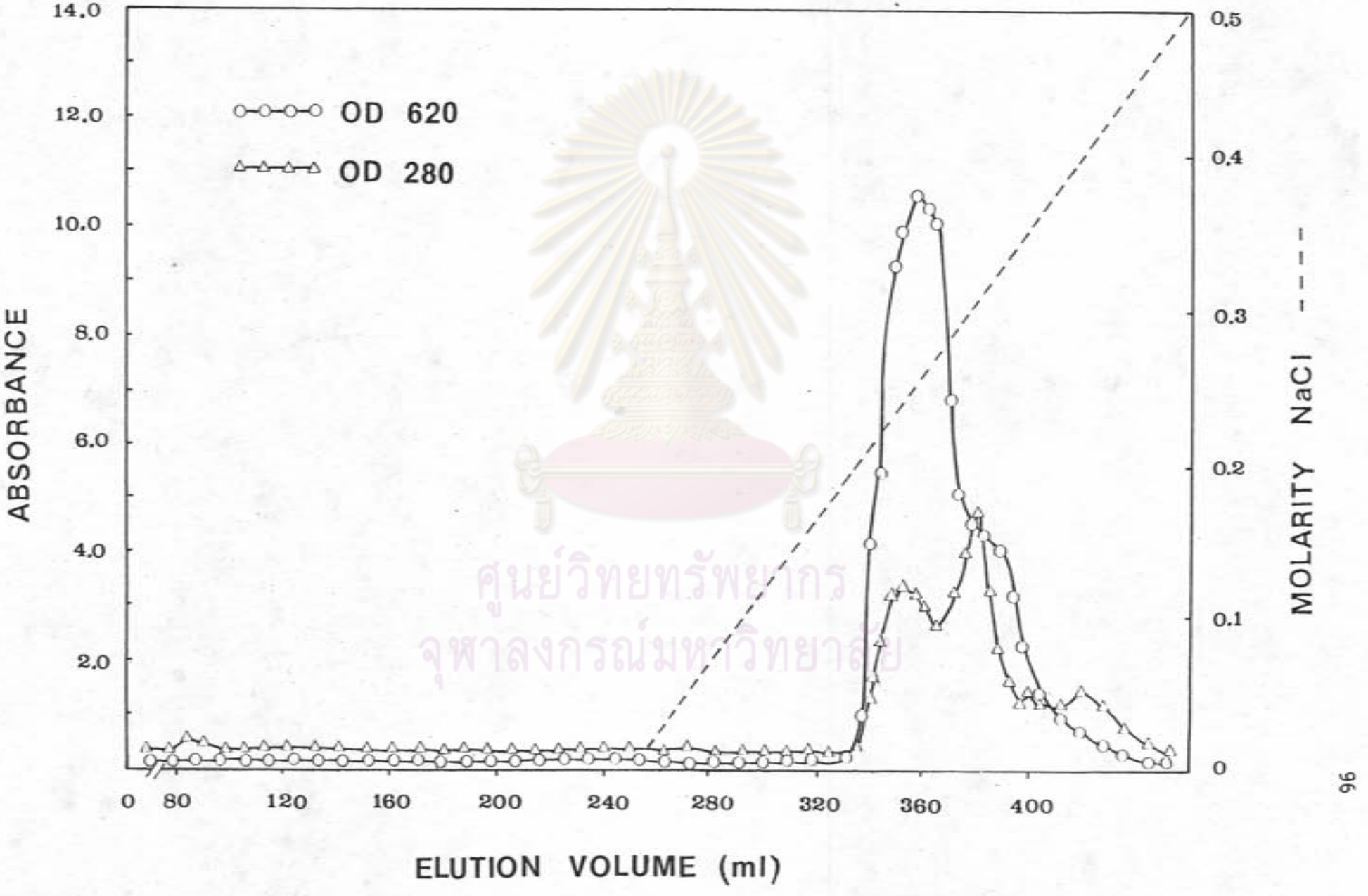


Figure 37 Rechromatography of the first DEAE-cellulose fraction from Spirulina (BP) on another DEAE-cellulose column.The protocol was the same as that in Figure 36.

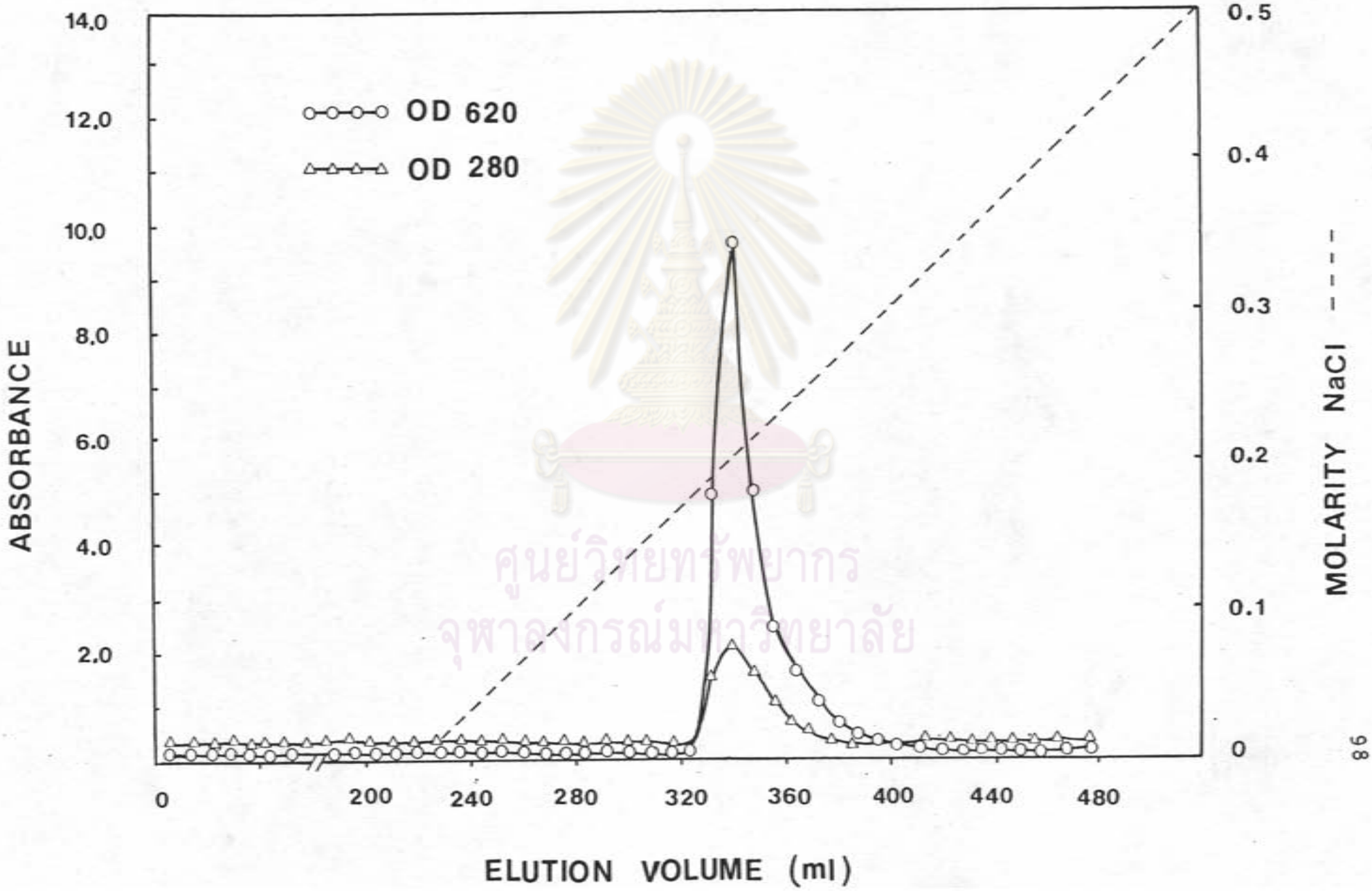


Figure 38 Chromatographic profile of pooled fractions after the second DEAE-cellulose column from Spirulina (BP) on Sephadex G-150 column (1.5x60 cm).The flow rate was maintained at 15 ml/hr. The 2 ml fractions were collected and the absorbances were measured at 620 and 280 nm.

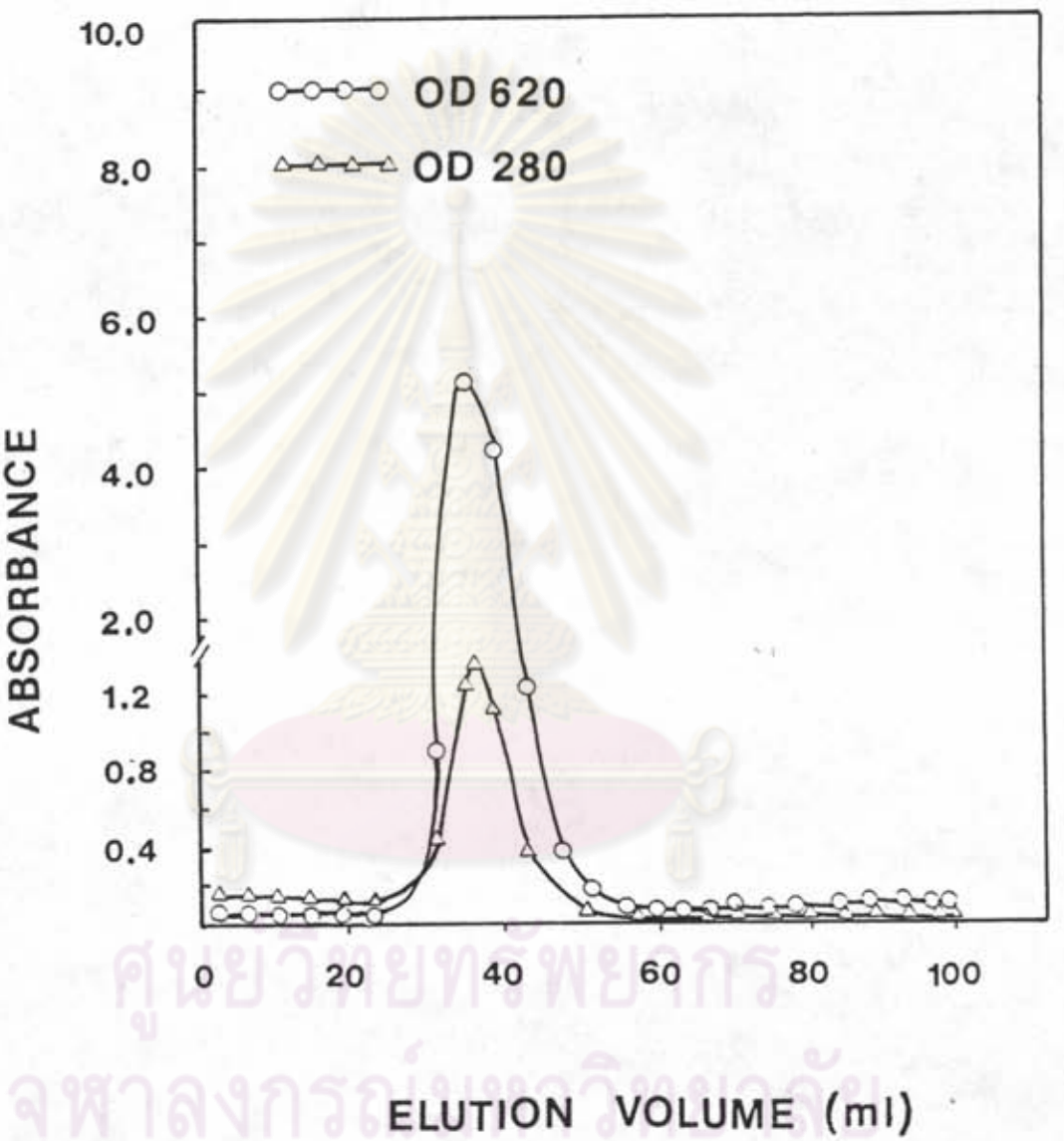


Figure 39 Chromatographic profile of 65 % ammonium sulfate fraction from Spirulina (NIFI) on DEAE-cellulose column.A 2.5 x 30 cm column and a linear gradient of 0 - 0.5 M NaCl in 0.02 M sodium phosphate buffer pH 7.5 was used. The flow rate was maintained at 30 ml/hr.The 4 ml fractions were collected and the absorbances were measured at 620 and 280 nm.

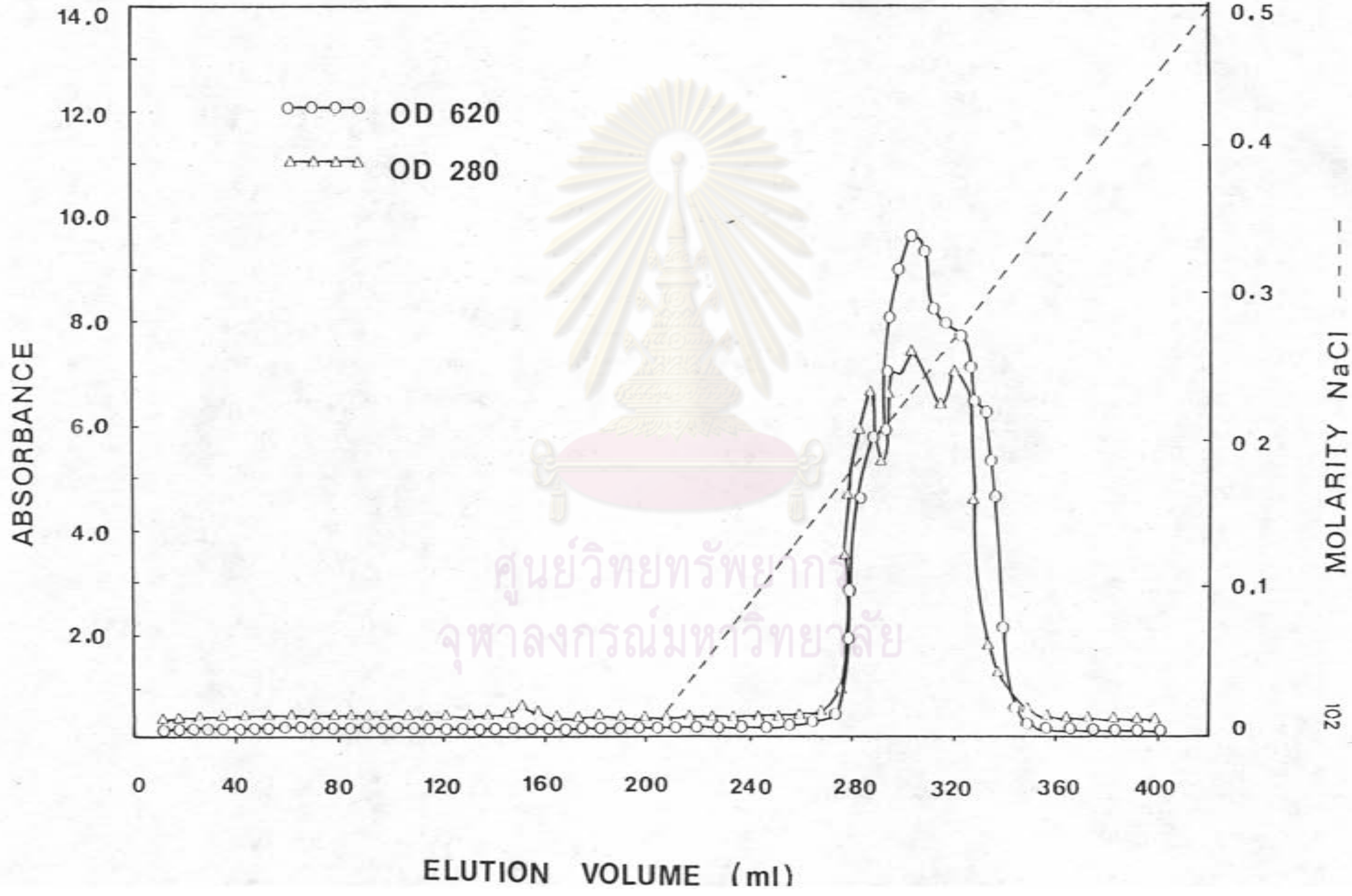


Figure 40 Rechromatography of the first DEAE - cellulose fraction from Spirulina (NIFI) on another DEAE-cellulose column. The protocol was the same as that in Figure 36.

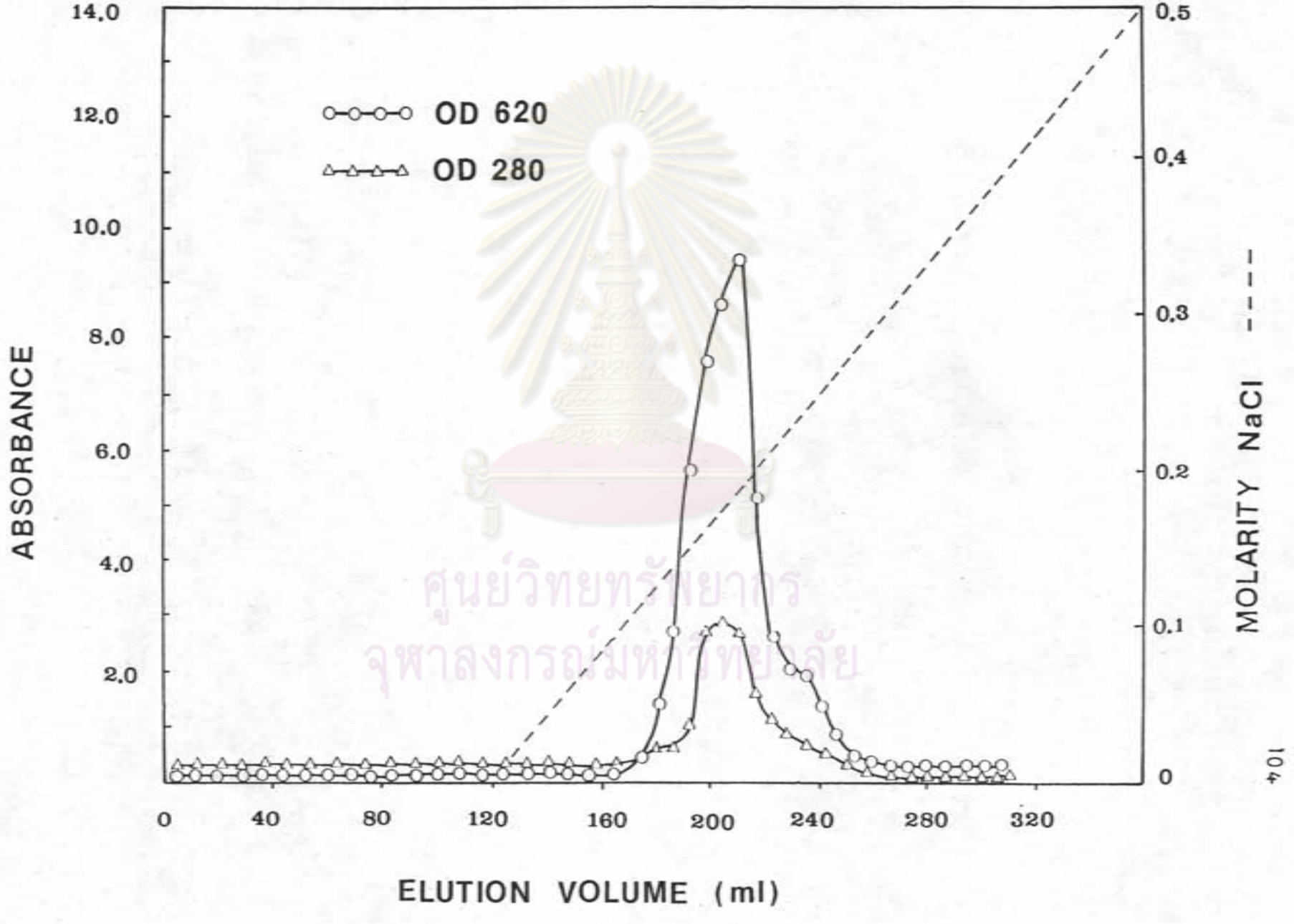
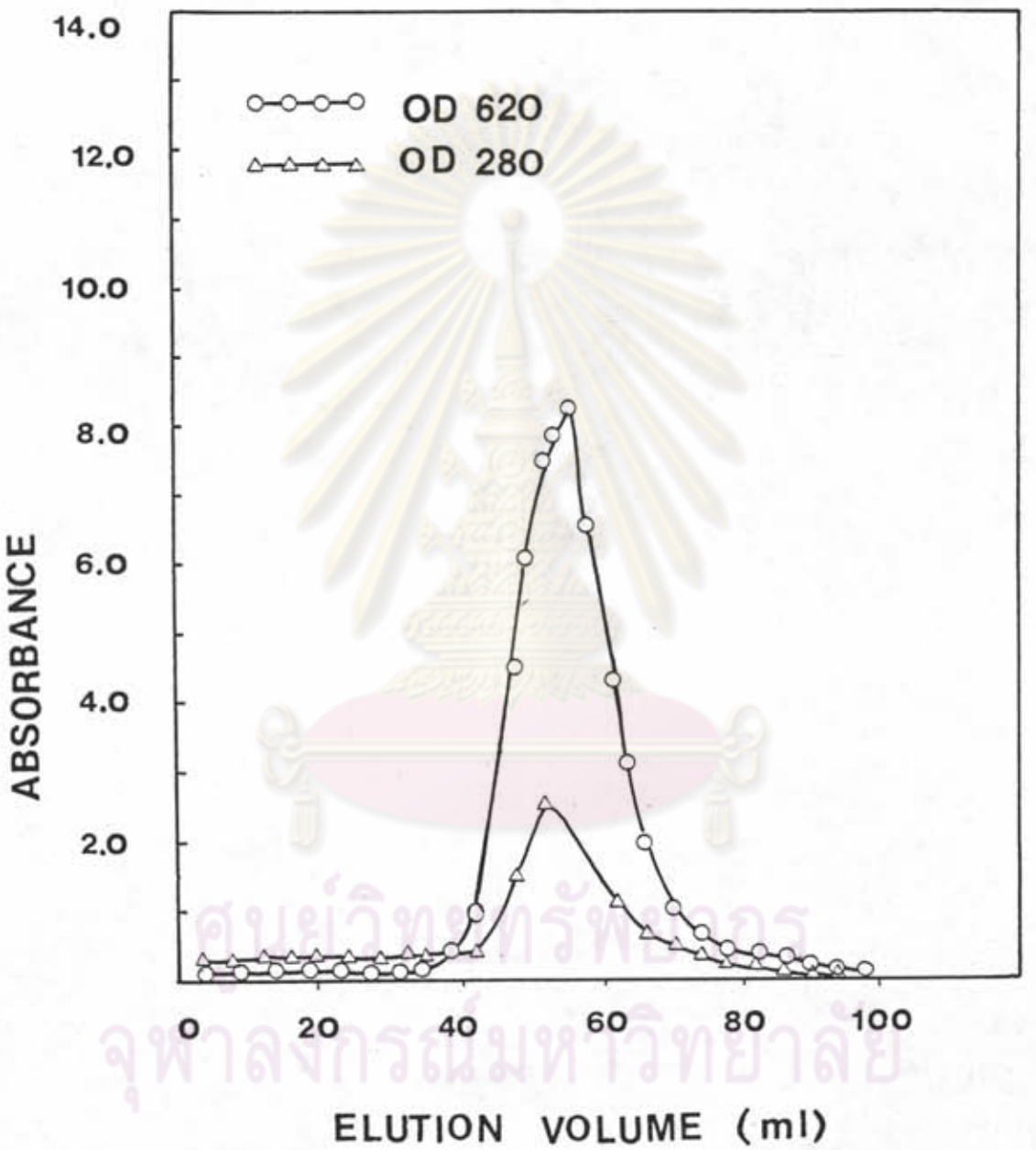


Figure 41 Chromatographic profile of pooled fractions after the second DEAE-cellulose column from Spirulina (NIFI) on Sephadex G-150 column (1.5x60 cm).The flow rate was maintained at 15 ml/hr. The 2 ml fractions were collected and the absorbances were measured at 620 and 280 nm.



Spirulina (MP)

1. DEAE-cellulose chromatography

162 mg protein (20 ml) was loaded on DEAE-cellulose column (I) and the linear gradient was performed. The elution profile after DEAE-cellulose column was shown in Figure 42 and the % recovery was 56 %. Rechromatography was also done on DEAE-cellulose column (II). Ninety-eight mg protein (7 ml) was loaded on DEAE-cellulose column (II). The elution profile was shown in Figure 43 and the % recovery was 48 %.

2. Sephadex G-150

25 mg protein (3.5 ml) was loaded on Sephadex G-150 column. The elution profile after Sephadex G-150 column was shown in Figure 44 and the % recovery was 24 % (Table 3 C).

6.4 Polyacrylamide gel electrophoresis

Fractions from Sephadex G-150 column were dialyzed over night and the purities of phycocyanin from three strains of Spirulina were checked by polyacrylamide gel electrophoresis. The results showed that only one single band was present (Figures 45,46 and 47) when the amount of the sample loaded was low but when increasing the amount of the sample another faint band was also observed.

Figure 42 Chromatographic profile of 65 % ammonium sulfate fraction from Spirulina (MP) on DEAE-cellulose column.A 2.5 x 30 cm column and a linear gradient of 0 - 0.5 M NaCl in 0.02 M sodium phosphate buffer pH 7.5 was used. The flow rate was maintained at 30 ml/hr.The 4 ml fractions were collected and the absorbances were measured at 620 and 280 nm.

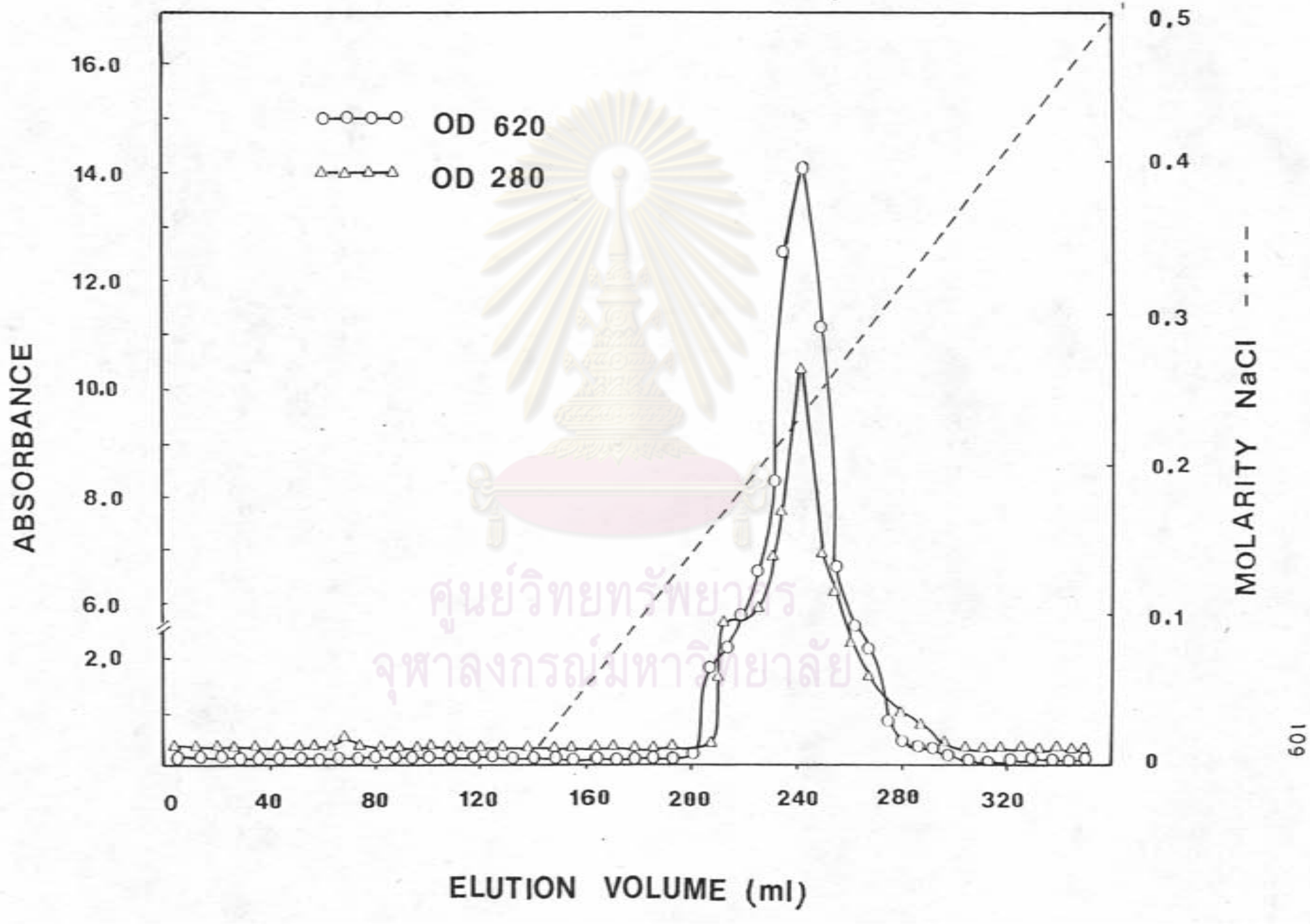


Figure 43 Rechromatography of the first DEAE - cellulose fraction from Spirulina (MP) on another DEAE-cellulose column.The protocol was the same as that in Figure 36.

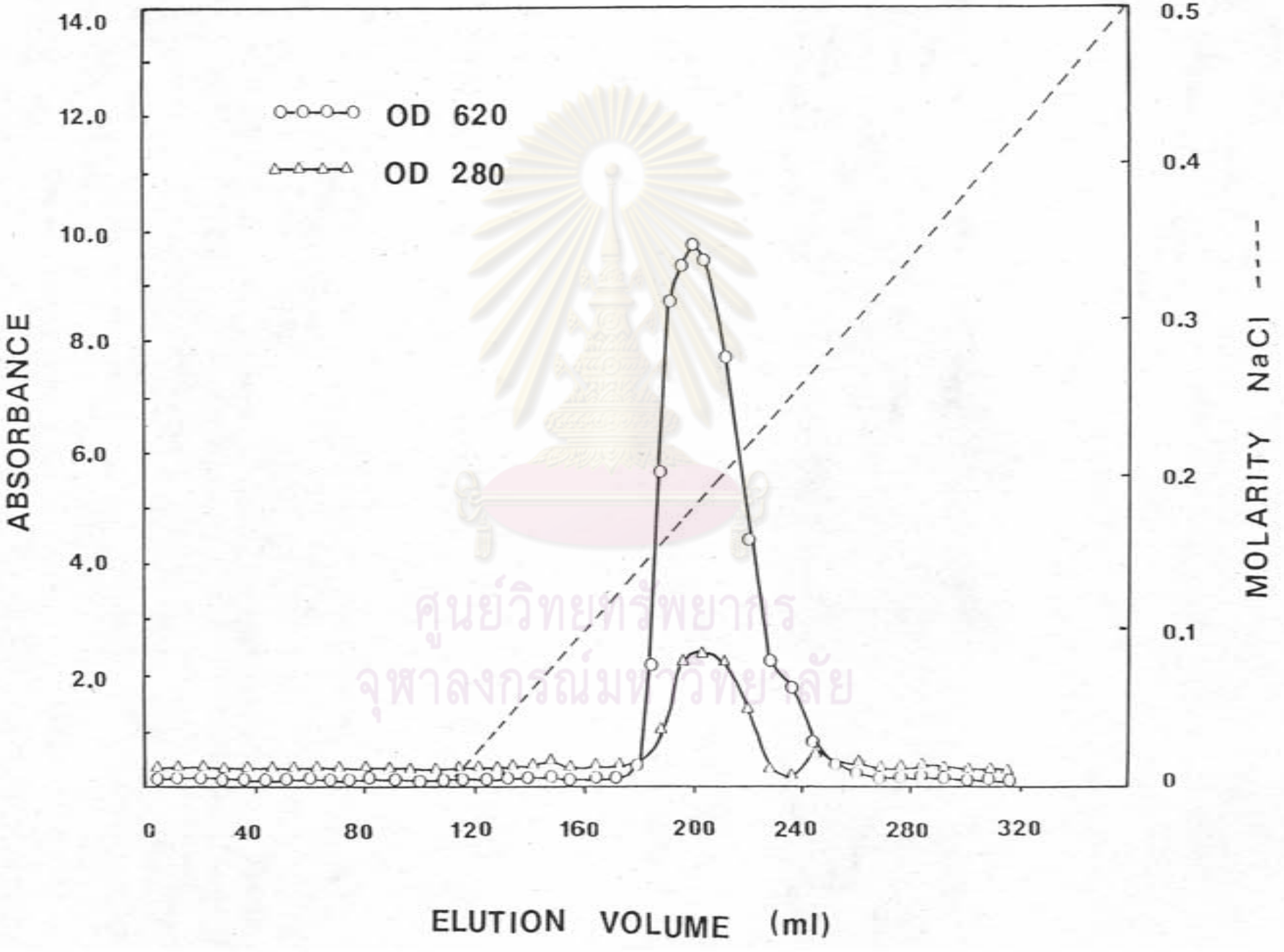


Figure 44 Chromatographic profile of pooled fractions after the second DEAE-cellulose column from Spirulina (MP) on Sephadex G-150 column (1.5x60 cm).The flow rate was maintained at 15 ml/hr. The 2 ml fractions were collected and the absorbances were measured at 620 and 280 nm.

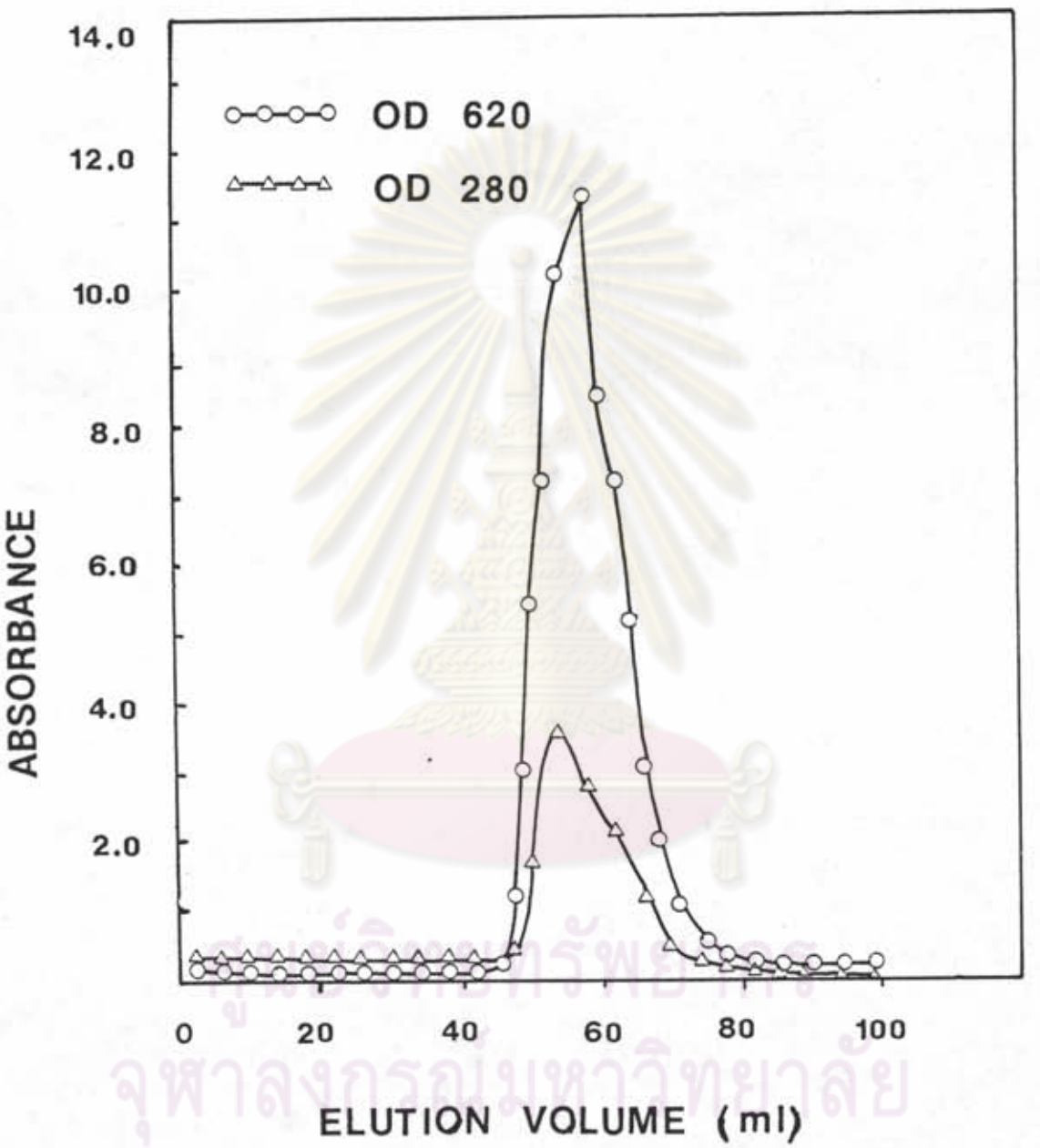


Figure 45 Polyacrylamide gel electrophoresis of phycocyanin from Spirulina (BP)

A. 5 µg protein
B. 10 µg protein
C. 15 µg protein
D. 20 µg protein
E. 25 µg protein

Figure 46 Polyacrylamide gel electrophoresis of phycocyanin from Spirulina (NIFI)

A. 5 µg protein
B. 10 µg protein
C. 15 µg protein
D. 20 µg protein
E. 25 µg protein

Figure 47 Polyacrylamide gel electrophoresis of phycocyanin from Spirulina (MP)

A. 5 µg protein
B. 10 µg protein
C. 15 µg protein
D. 20 µg protein
E. 25 µg protein

Determination of molecular weight of phycocyanin

6.5 SDS-polyacrylamide gel electrophoresis

The fractions having the highest phycocyanin content from DEAE-cellulose (I),DEAE-cellulose (II) and Sephadex G-150 column were analyzed by SDS-PAGE. The separation patterns were shown in Figures 48,50 and 52. Two bands were detected and the upper one was more intense than the lower one suggesting that these two bands represented two different proteins of differing quantities. The band pattern also indicated that repeated DEAE-cellulose column followed by Sephadex G-150 column effectively removed other contaminating proteins. By means of standard curves (Figures 49,51 and 53), the 2 proteins of Spirulina (BP) were determined to have molecular weights of 14,000 and 13,000 daltons whereas for Spirulina (NIFI) and Spirulina (MP) these values were 13,000 daltons and 12,000 daltons. The higher molecular weight protein was likely to be phycocyanin as it corresponded to the intense band of standard phycocyanin (Linablue).

To further check and identify the lower molecular weight protein, the peak fractions eluted from 3 chromatographic columns were scanned for the spectrum from 400 to 700 nm. As shown in Figures 54,55 and 56 for all 3 strains of Spirulina ,apart from one main peak at

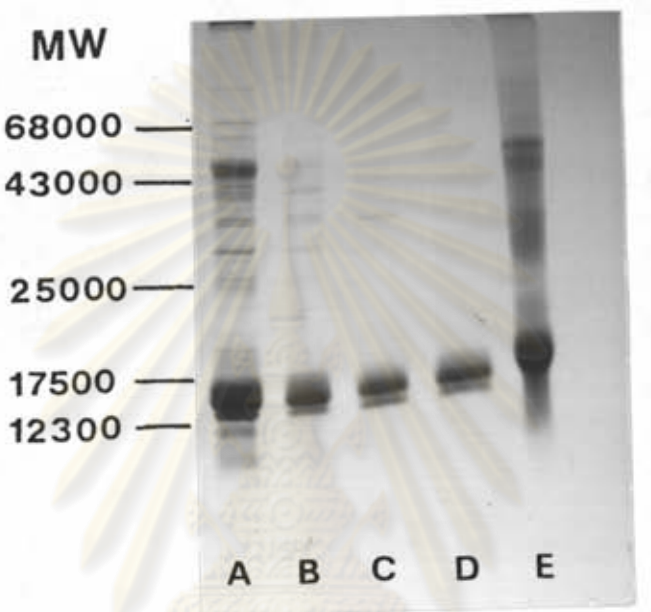


Figure 48 SDS-Polyacrylamide gel electrophoresis of phycocyanin from Spirulina (BP). Standard molecular weights are shown on the left.

A. 50 µg crude phycocyanin

B. 25 µg DEAE-cellulose (I) fraction

C. 25 µg DEAE-cellulose (II) fraction

D. 25 µg Sephadex G-150 fraction

E. 50 µg of standard phycocyanin (Linablue)

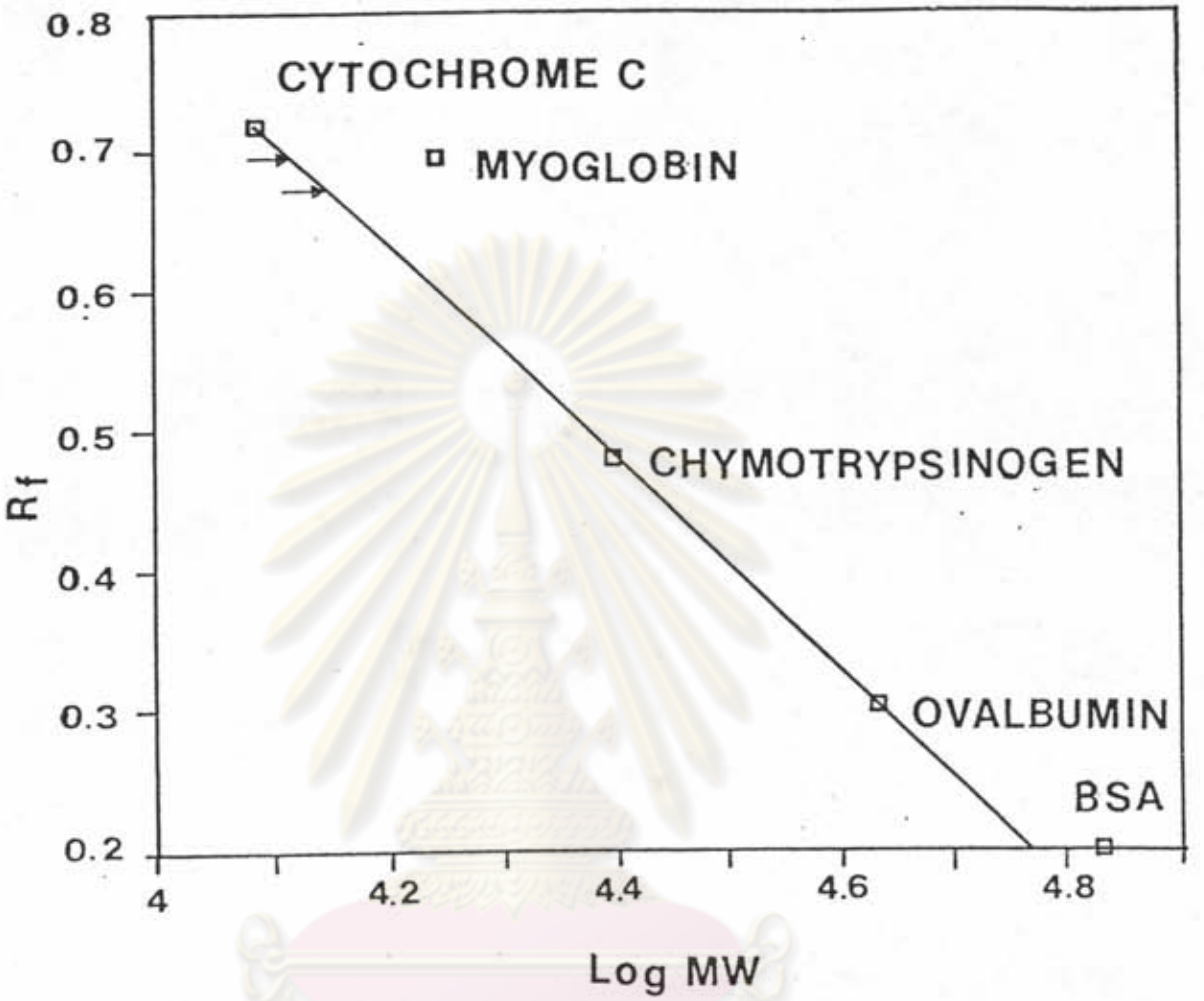


Figure 49 Determination of the molecular weight of phycocyanin from Spirulina (BP).Standard proteins were bovine serum albumin (68000),ovalbumin (43000), chymotrypsinogen (25000), myoglobin (17500) and cytochrome c (12300)

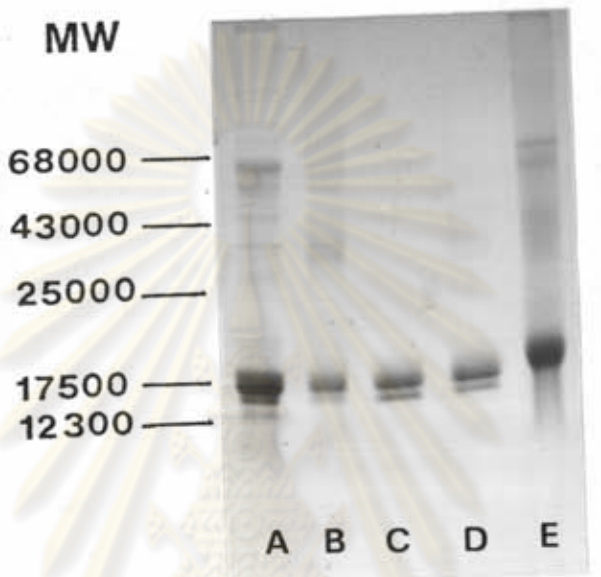


Figure 50 SDS-Polyacrylamide gel electrophoresis of phycocyanin from Spirulina (NIFI). Standard molecular weightS are shown on the left .

A. 50 µg crude phycocyanin

B. 25 µg DEAE-cellulose (I) fraction

C. 25 µg DEAE-cellulose (II) fraction

D. 25 µg Sephadex G-150 fraction

E. 50 µg of standard phycocyanin (Linablue)

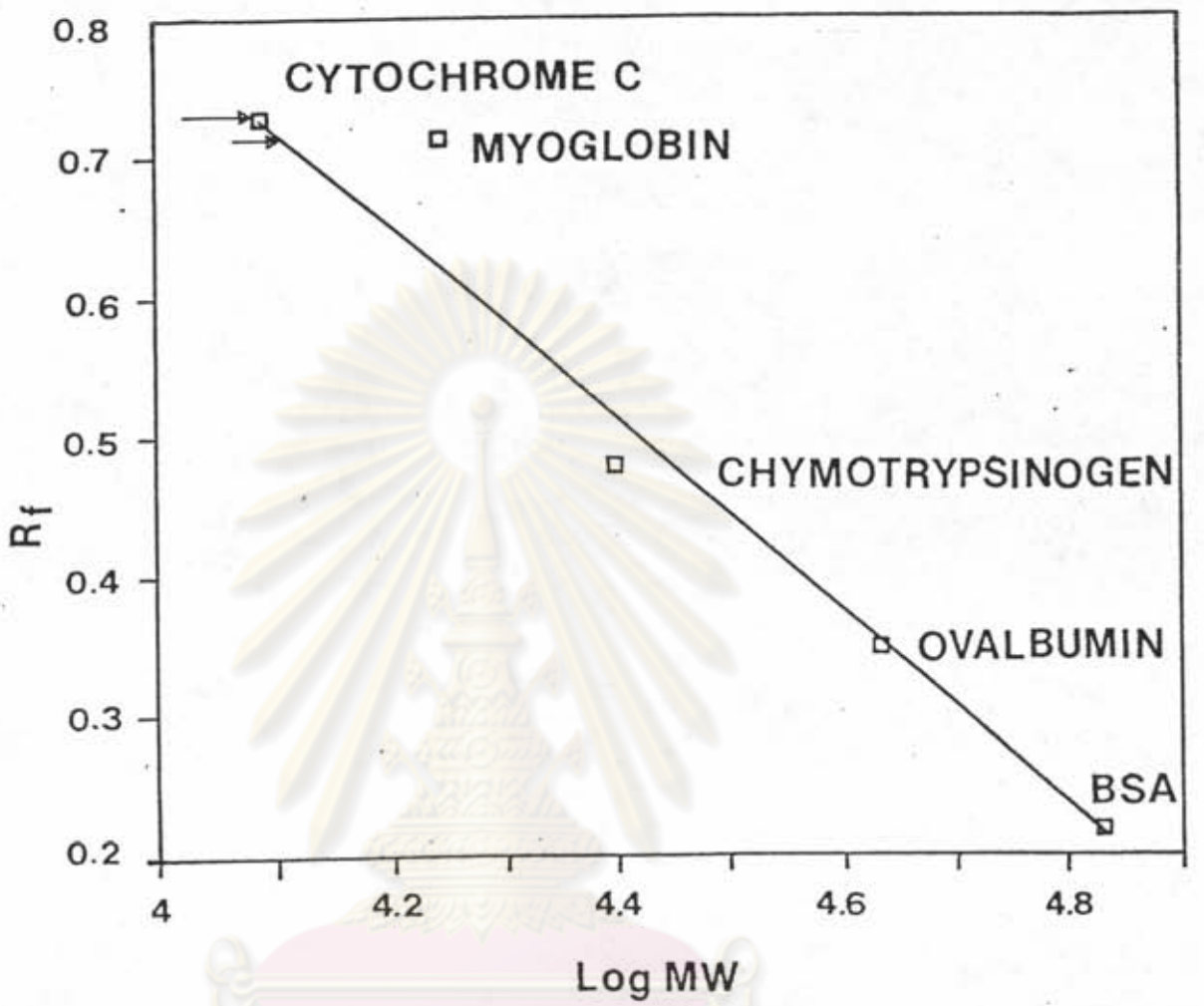


Figure 51 Determination of the molecular weight of phycocyanin from Spirulina (NIFI).Standard proteins were bovine serum albumin (68000),ovalbumin (43000), chymotrypsinogen (25000), myoglobin (17500) and cytochrome c (12300)

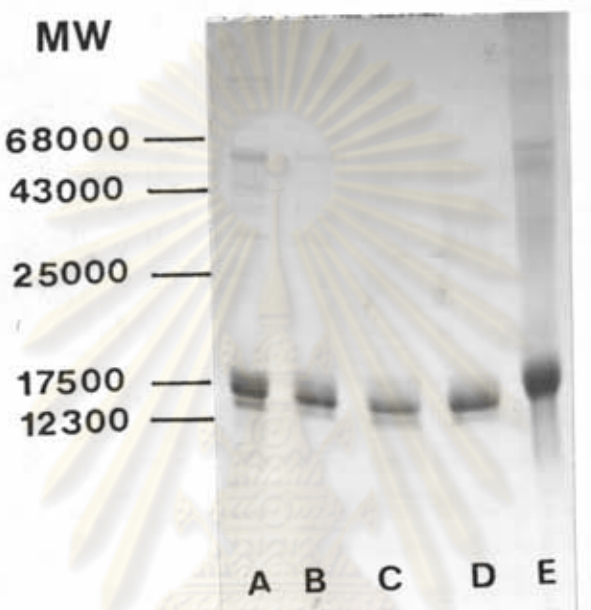


Figure 52 SDS-Polyacrylamide gel electrophoresis of phycocyanin from Spirulina (MP). Standard molecular weightS are shown on the left .

A. 50 µg crude phycocyanin

B. 25 µg DEAE-cellulose (I) fraction

C. 25 µg DEAE-cellulose (II) fraction

D. 25 µg Sephadex G-150 fraction

E. 50 µg of standard phycocyanin (Linablue)

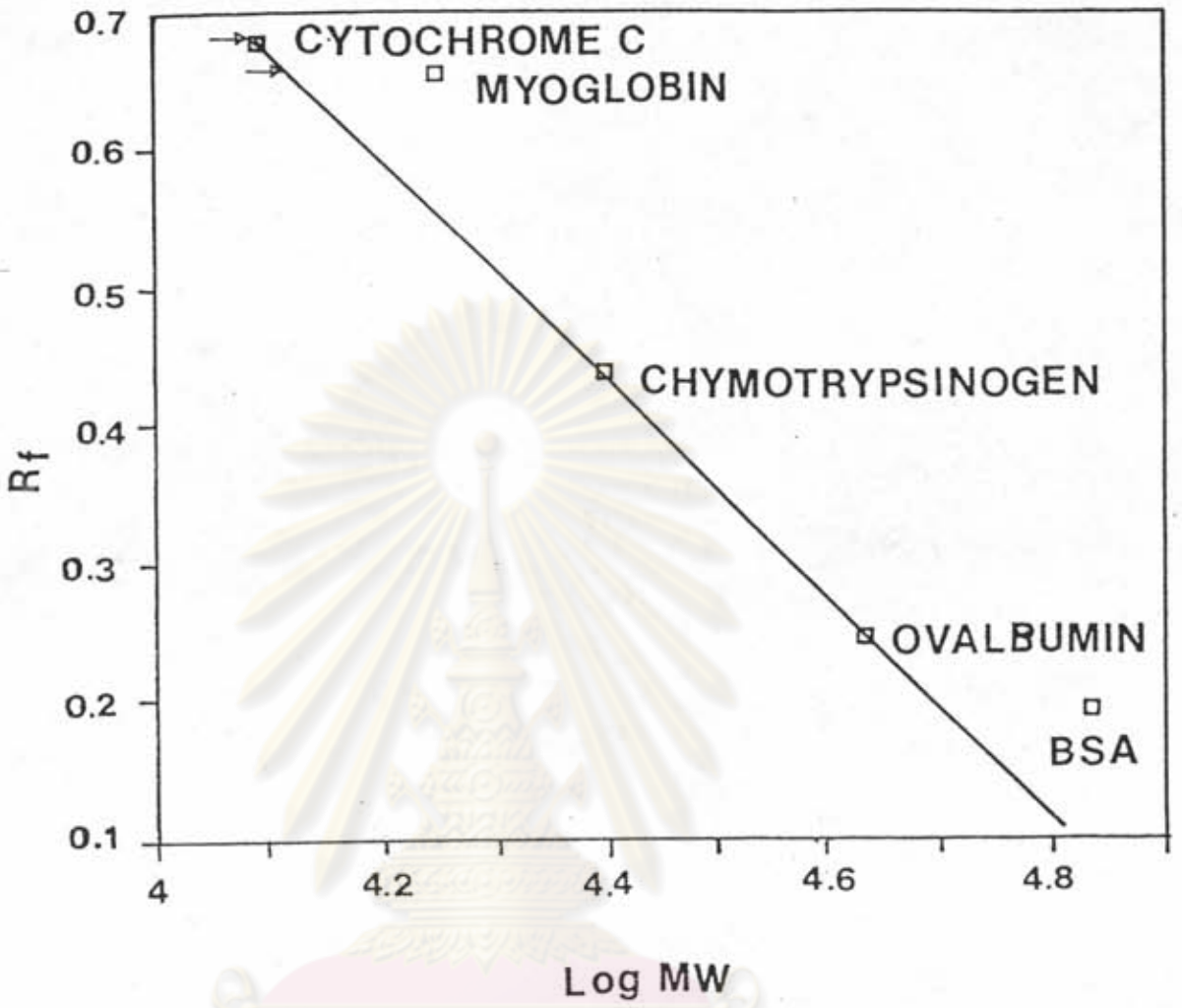


Figure 53 Determination of the molecular weight of phycocyanin from Spirulina (MP).Standard proteins were bovine serum albumin (68000),ovalbumin (43000),chymotrypsinogen (25000), myoglobin (17500) and cytochrome c (12300)

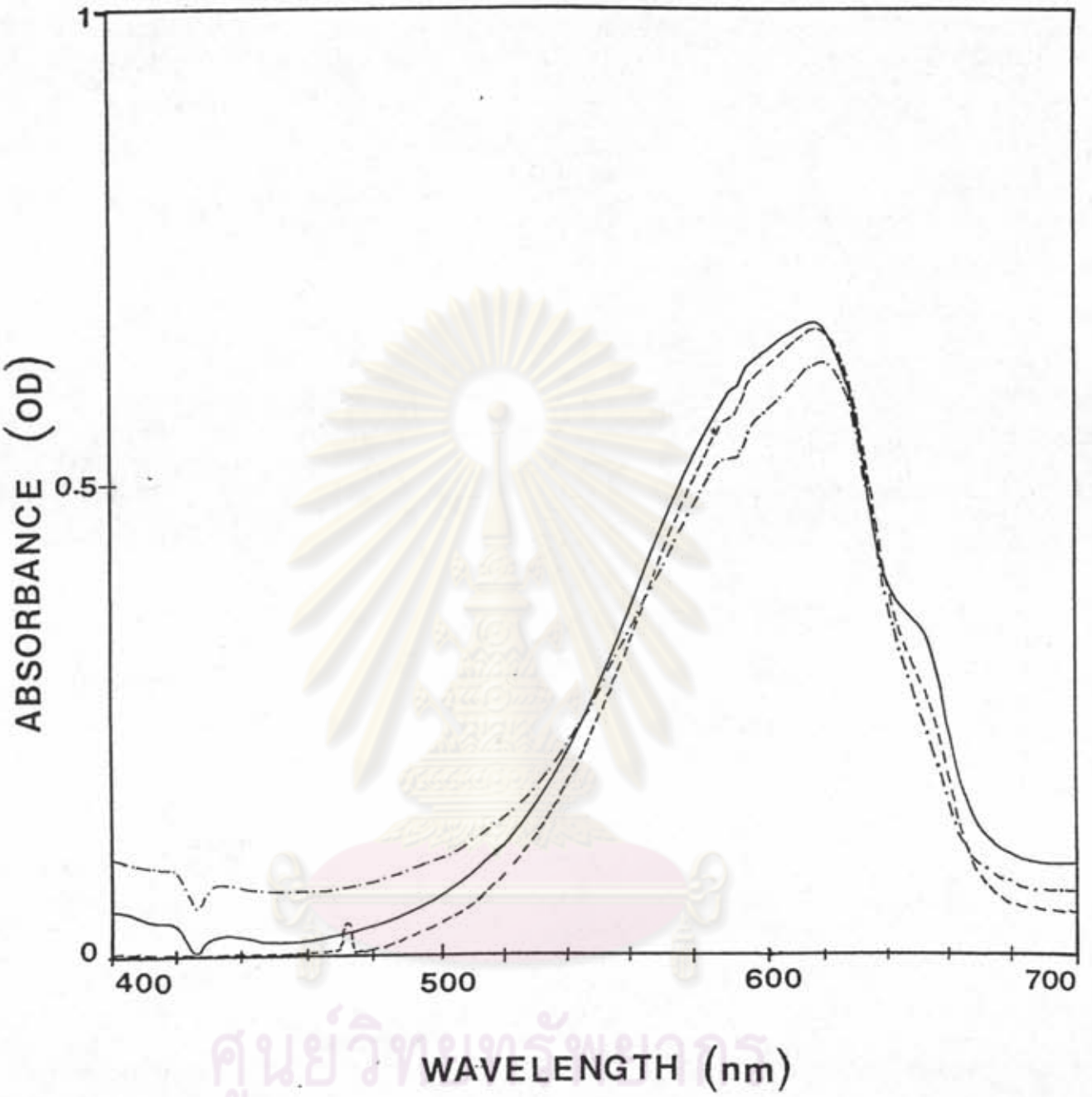


Figure 54 Absorption spectra of fraction from DEAE-cellulose column I (——), DEAE-cellulose column II (-----) and Sephadex G-150 column (-·-·-·) of Spirulina (BP)

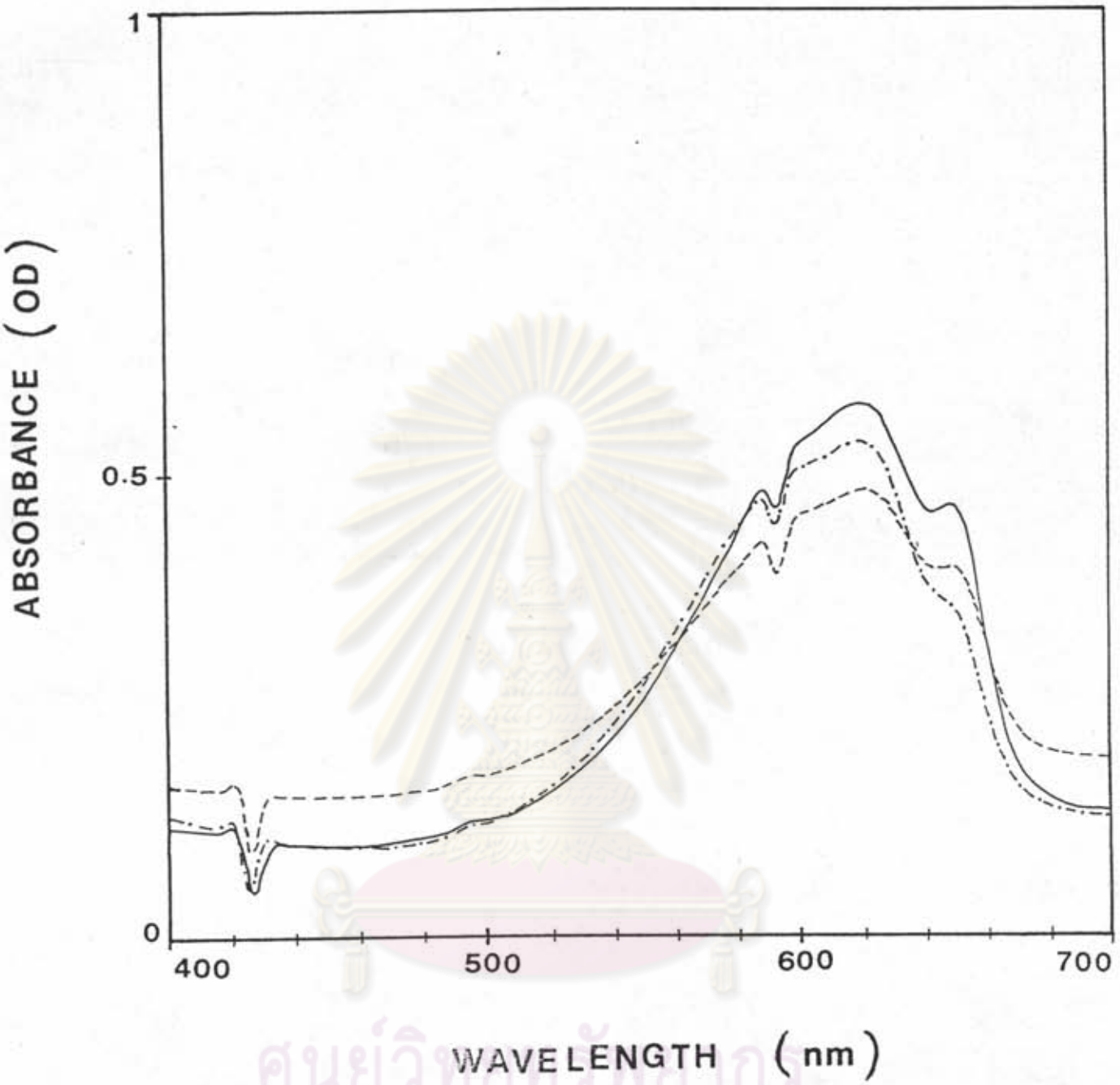


Figure 55 Absorption spectra of fraction from DEAE-cellulose column I (———), DEAE-cellulose column II (-----) and Sephadex G-150 column (—·—·—) of Spirulina (N1FI)

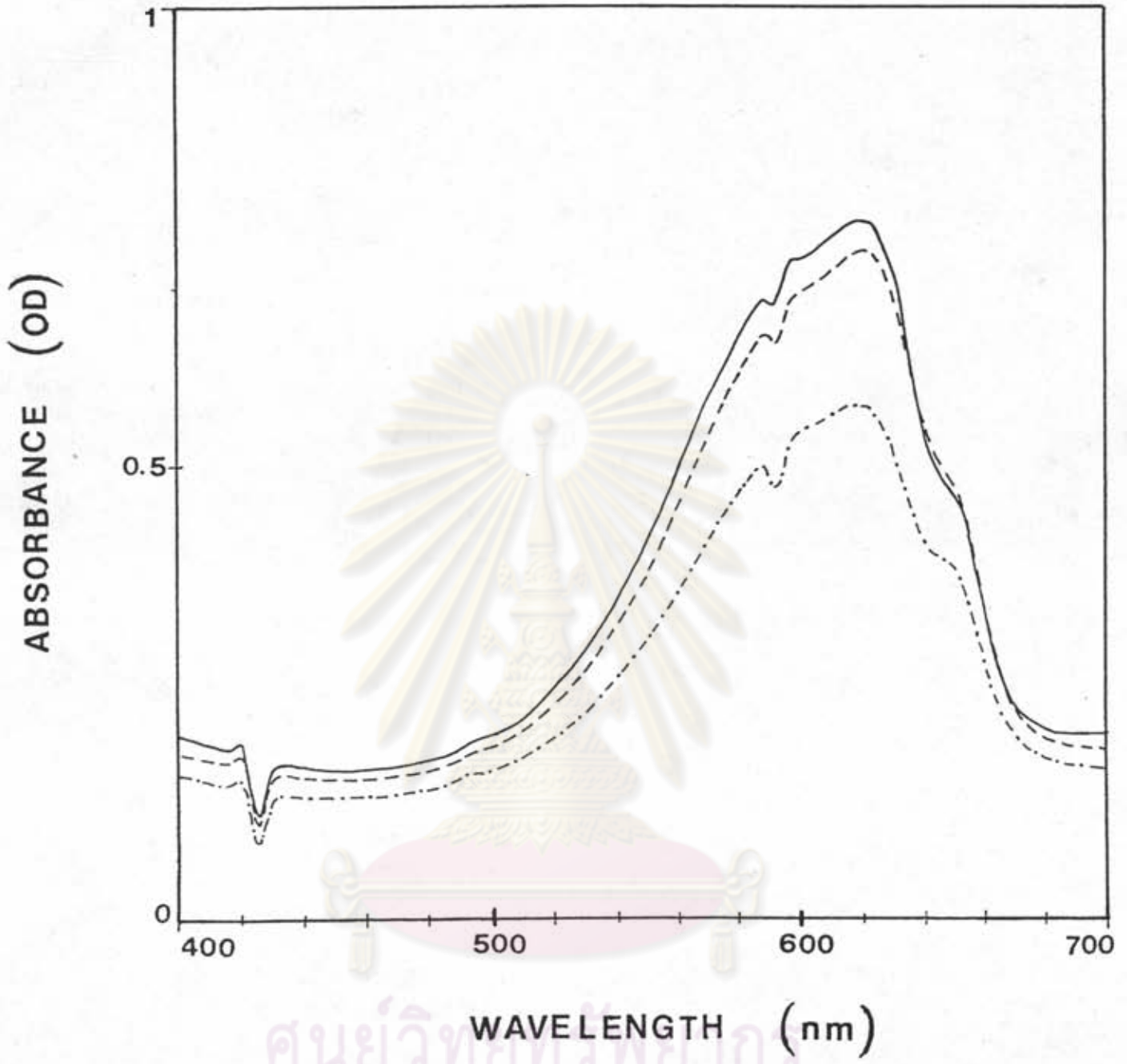


Figure 56 Absorption spectra of fraction from DEAE-cellulose column I (———), DEAE-cellulose column II (-----) and Sephadex G-150 column (—·—·—·) of Spirulina (MP)

620 nm there existed a shoulder at 650 nm. The shoulder peak at 650 nm was attributed to the presence in small amount of another class of pigment called allophycocyanin. This allophycocyanin was absent in the standard phycocyanin (Linablue) as shown in Figure 57,ie., no absorption peak occurred at 650 nm.

6.6 Hydroxylapatite column chromatography

About 7-18 mg protein was loaded on Hydroxylapatite column and eluted by stepwise gradient of phosphate. The elution profile showed two peaks. The first one, phycocyanin (OD 620), was eluted between phosphate concentration of 30-50 mM and the second one, allophycocyanin (OD 650), was eluted between phosphate concentration of 80 mM (Figures 58,59 and 60). These 2 peaks were further analyzed by SDS-PAGE and the results were shown in Figure 61. The sample from the first peak (phycocyanin) when run on SDS-PAGE gave one band whereas that from the second peak (allophycocyanin) gave 2 bands. It was noted that the extra band from the allophycocyanin sample corresponded to the position of the band from the phycocyanin sample. The spectra of both phycocyanin and allophycocyanin were then run and shown in Figures 62,63 and 64. The allophycocyanin sample contained a shoulder at 620 nm apart from a main peak at 650 nm.

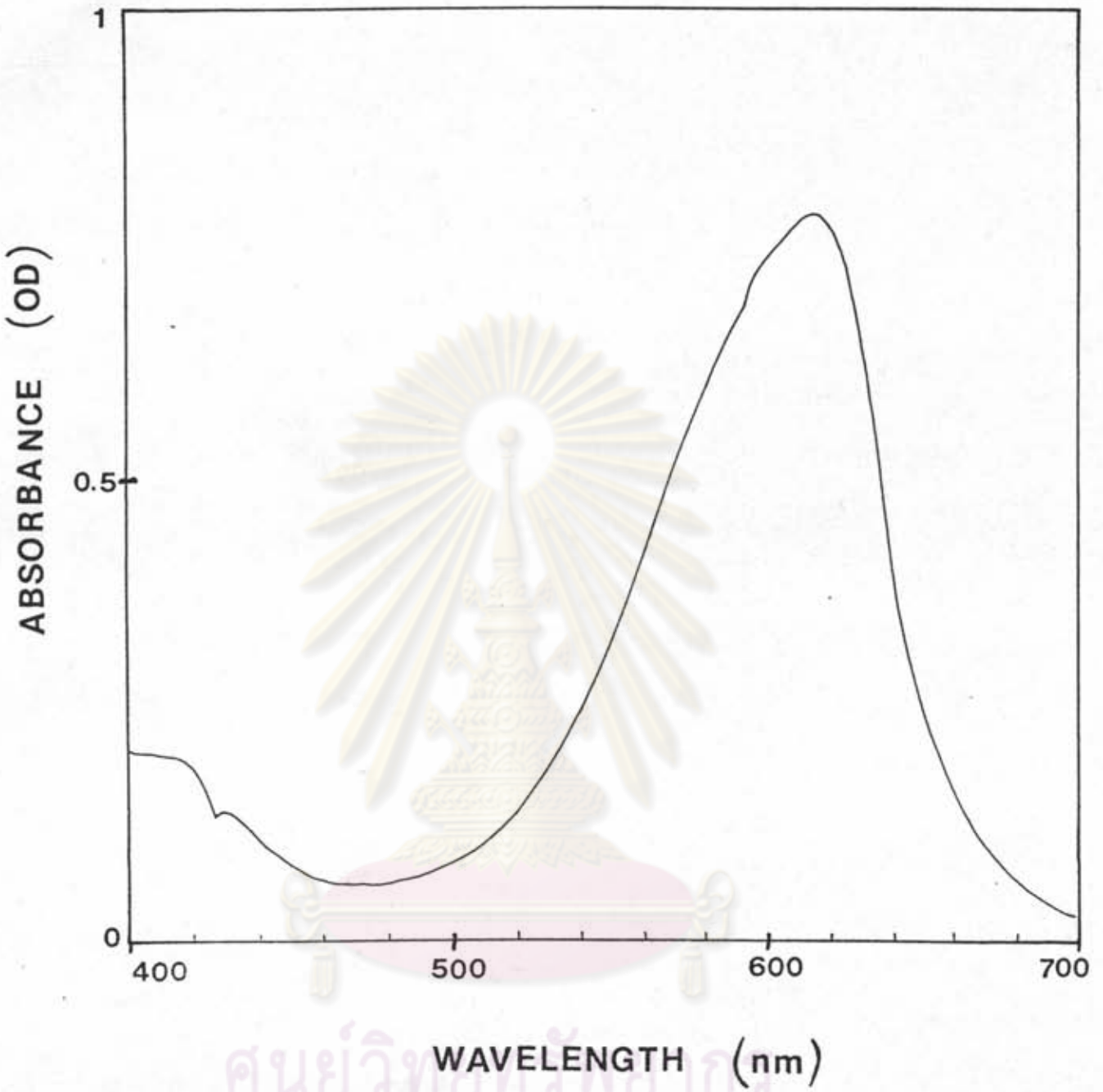


Figure 57 Absorption spectra of " Linablue " in 0.02 M phosphate buffer pH 7.5

Figure 58 Chromatographic profile of peak fraction after Sephadex G-150 column from Spirulina (BP) on Hydroxylapatite column (1.4x9 cm) . The stepwise gradient of 2.5, 10, 30, 50, 80 and 100 mM phosphate buffer at pH 7.0 was used. The flow rate was maintained at 30 ml/hr. The 0.5 ml fractions were collected and the absorbance were measured at 280 , 620 and 650 nm.

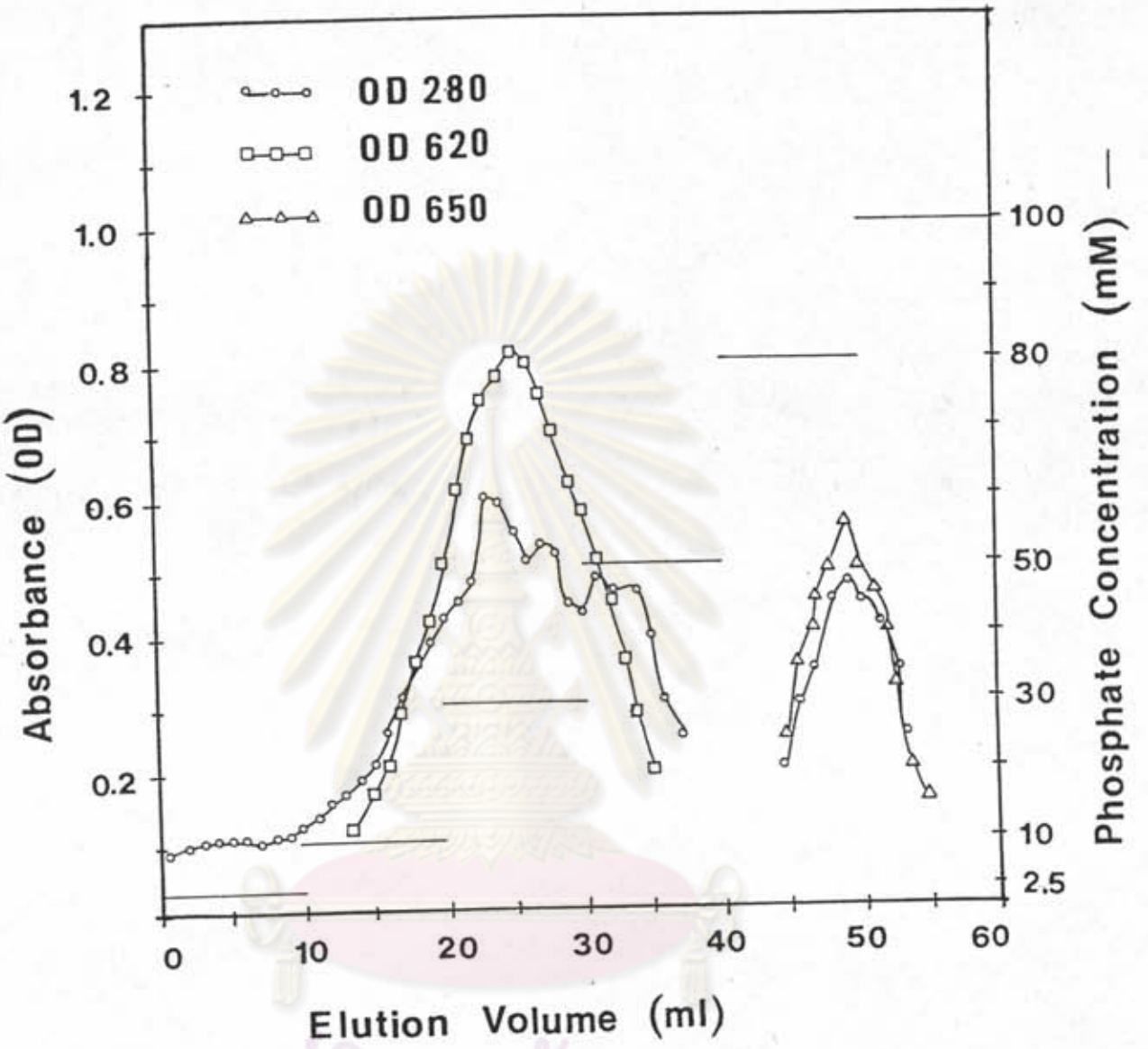


Figure 59 Chromatographic profile of peak fraction after Sephadex G-150 column from Spirulina (NIFI) on Hydroxylapatite column (1.4x9 cm) . The stepwise gradient of 2.5, 10, 30, 50, 80 and 100 mM phosphate buffer at pH 7.0 was used. The flow rate was maintained at 30 ml/hr. The 0.5 ml fractions were collected and the absorbance were measured at 280 , 620 and 650 nm.

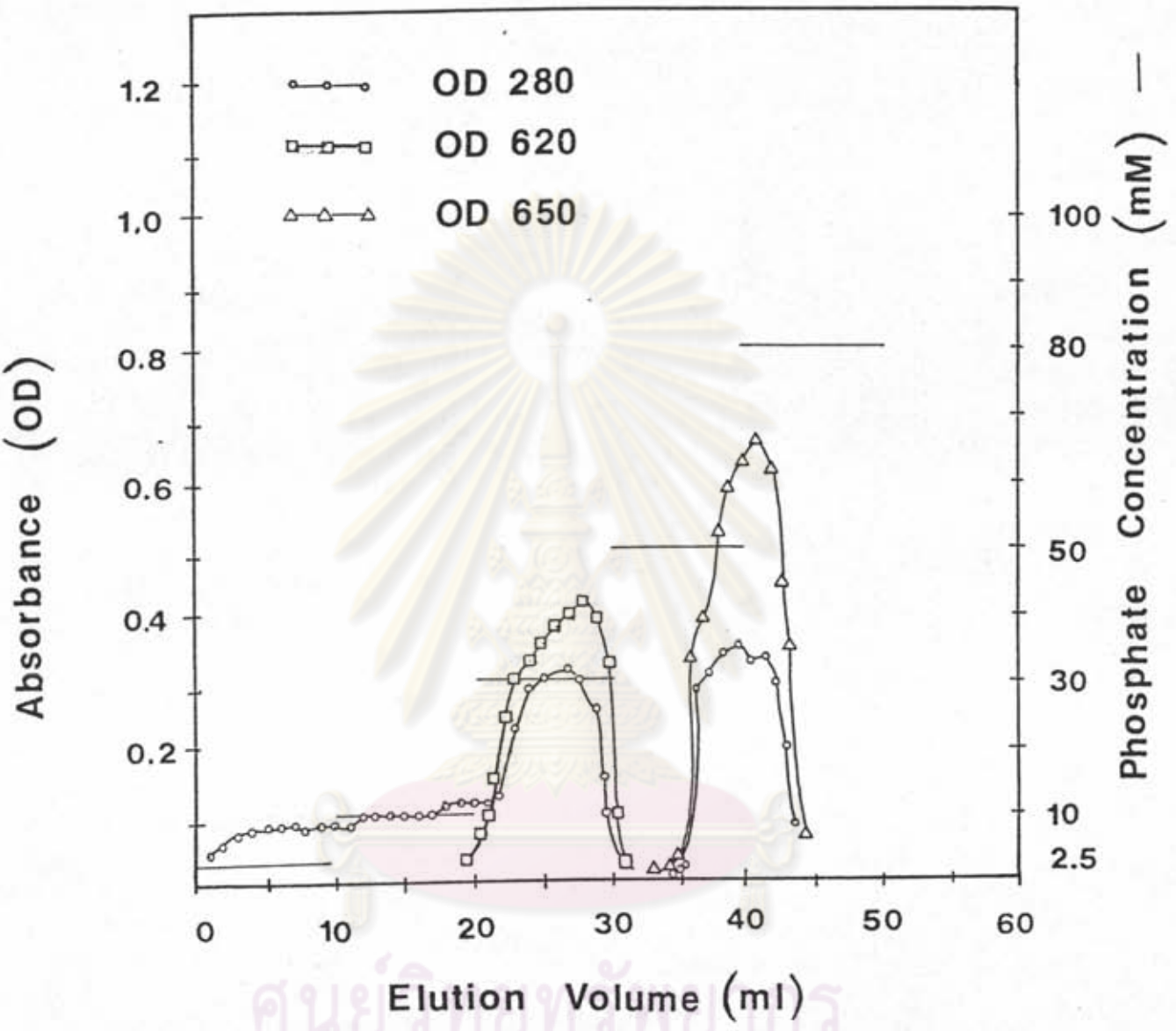
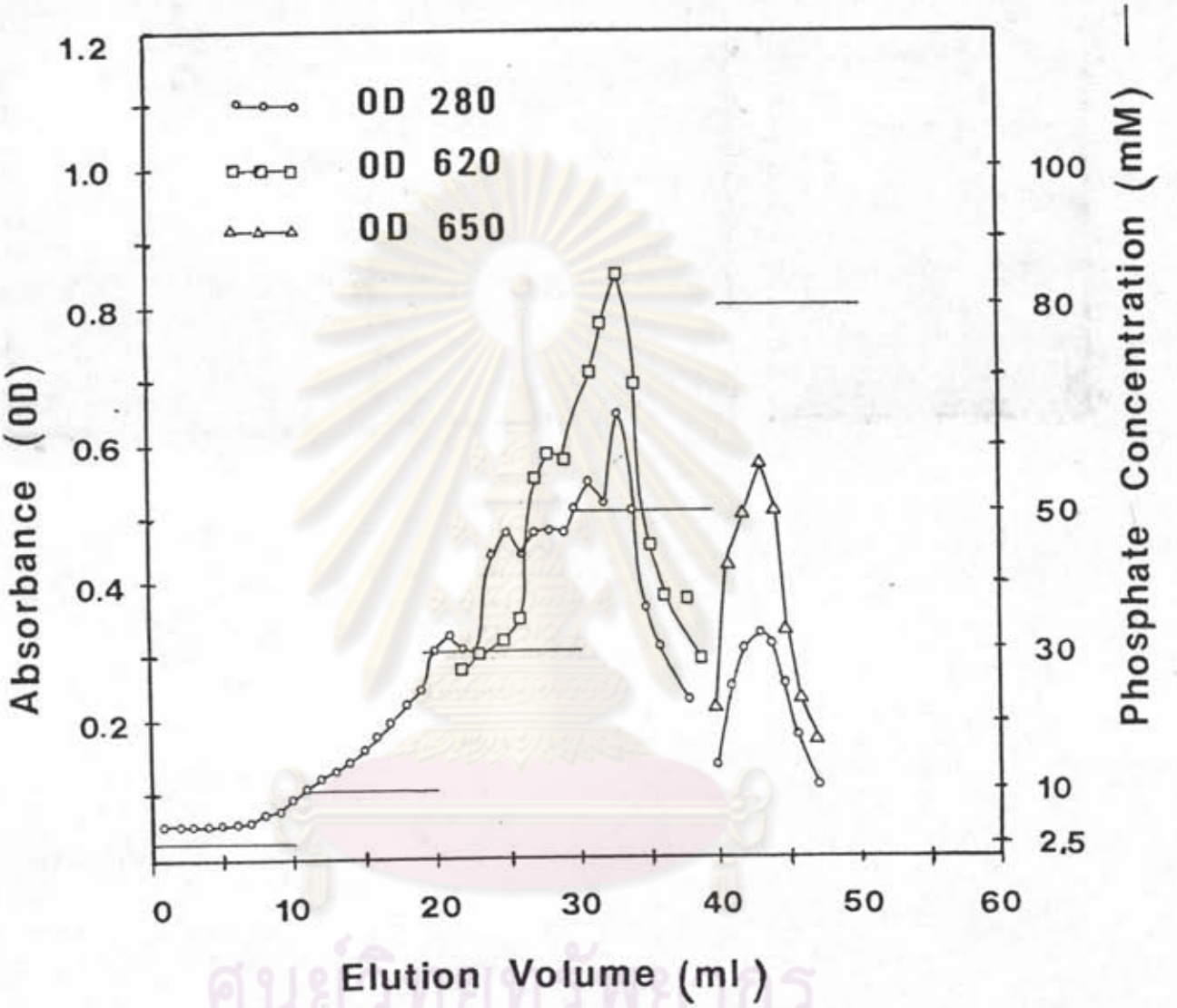


Figure 60 Chromatographic profile of peak fraction after Sephadex G-150 column from Spirulina (MP) on Hydroxylapatite column (1.4x9 cm) . The stepwise gradient of 2.5, 10, 30, 50, 80 and 100 mM phosphate buffer at pH 7.0 was used. The flow rate was maintained at 30 ml/hr. The 0.5 ml fractions were collected and the absorbance were measured at 280 , 620 and 650 nm.



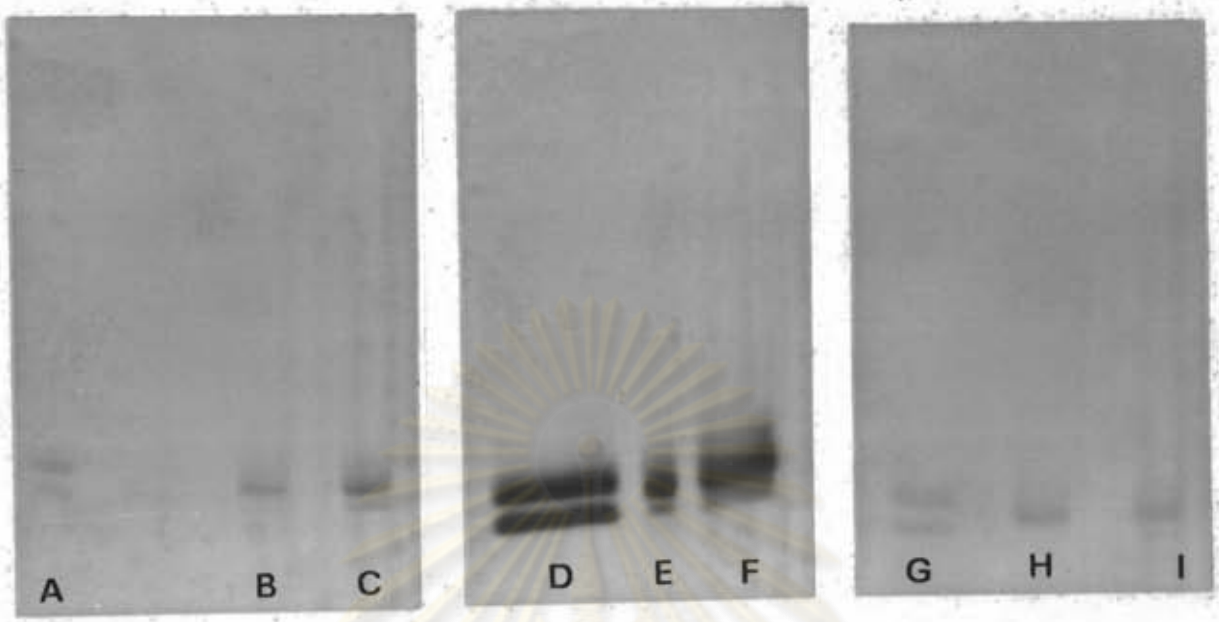


Figure 61 SDS-Polyacrylamide gel electrophoresis of peak fraction after Hydroxylapatite column.

A. 50 µg of fraction absorbing at 650 nm (Spirulina (BP)

B. 50 µg of fraction absorbing at 620 nm (Spirulina (BP)

D. 50 µg of fraction absorbing at 650 nm (Spirulina (NIFI)

F. 50 µg of fraction absorbing at 620 nm (Spirulina (NIFI)

G. 50 µg of fraction absorbing at 650 nm (Spirulina (MP)

H. 50 µg of fraction absorbing at 620 nm (Spirulina (MP)

Lanes C, E and I were 50 µg of samples before loading on Hydroxylapatite from Spirulina (BP), (NIFI) and (MP) respectively.

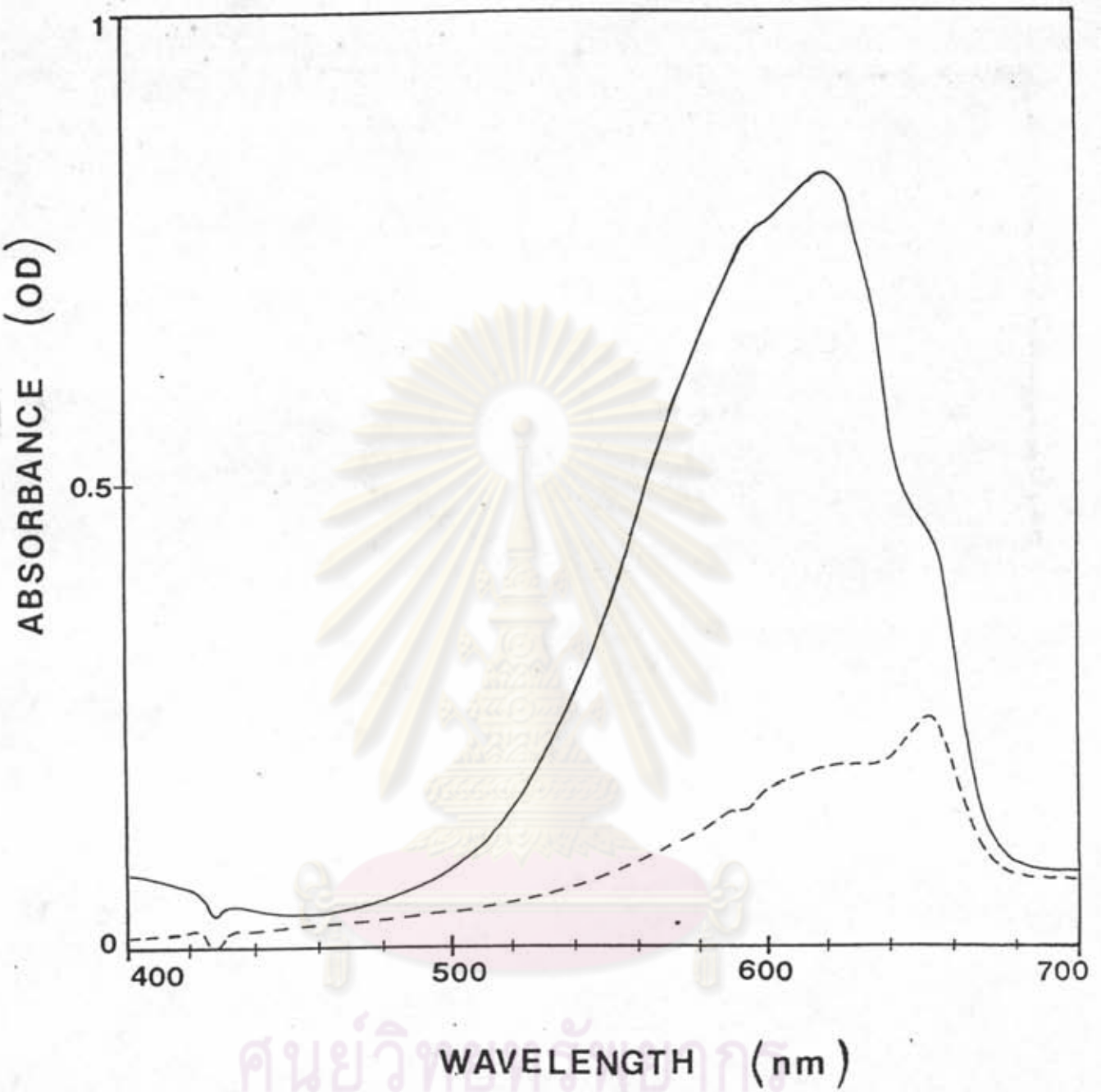


Figure 62 Absorption spectra of the two major phycobiliproteins of Spirulina (BP) after Hydroxylapatite column.

(———) Phycocyanin

(- - - - -) Allophycocyanin

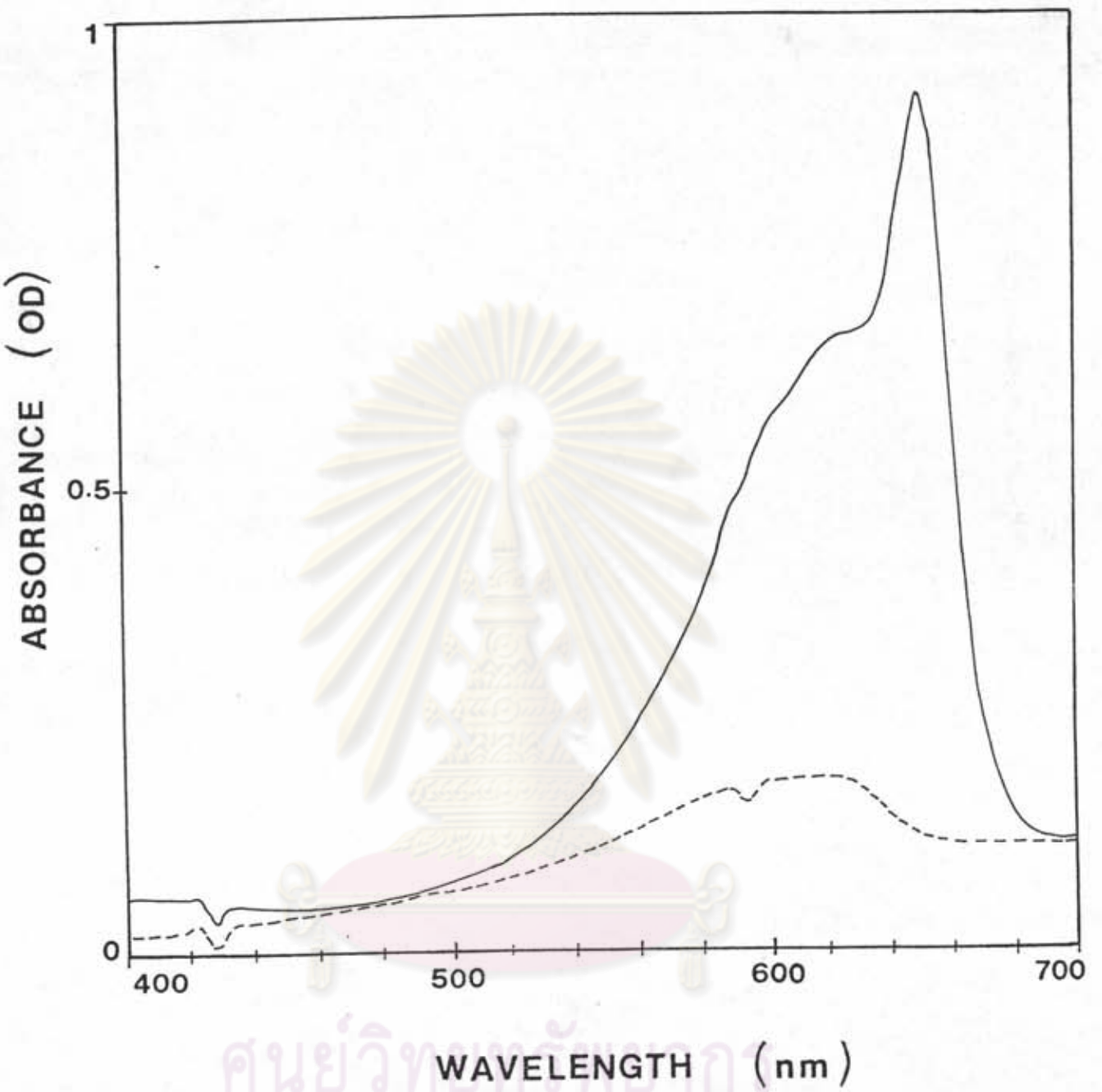


Figure 63 Absorption spectra of the two major phycobiliproteins of Spirulina (NIFI) after Hydroxylapatite column.

(———) Phycocyanin

(- - - -) Allophycocyanin

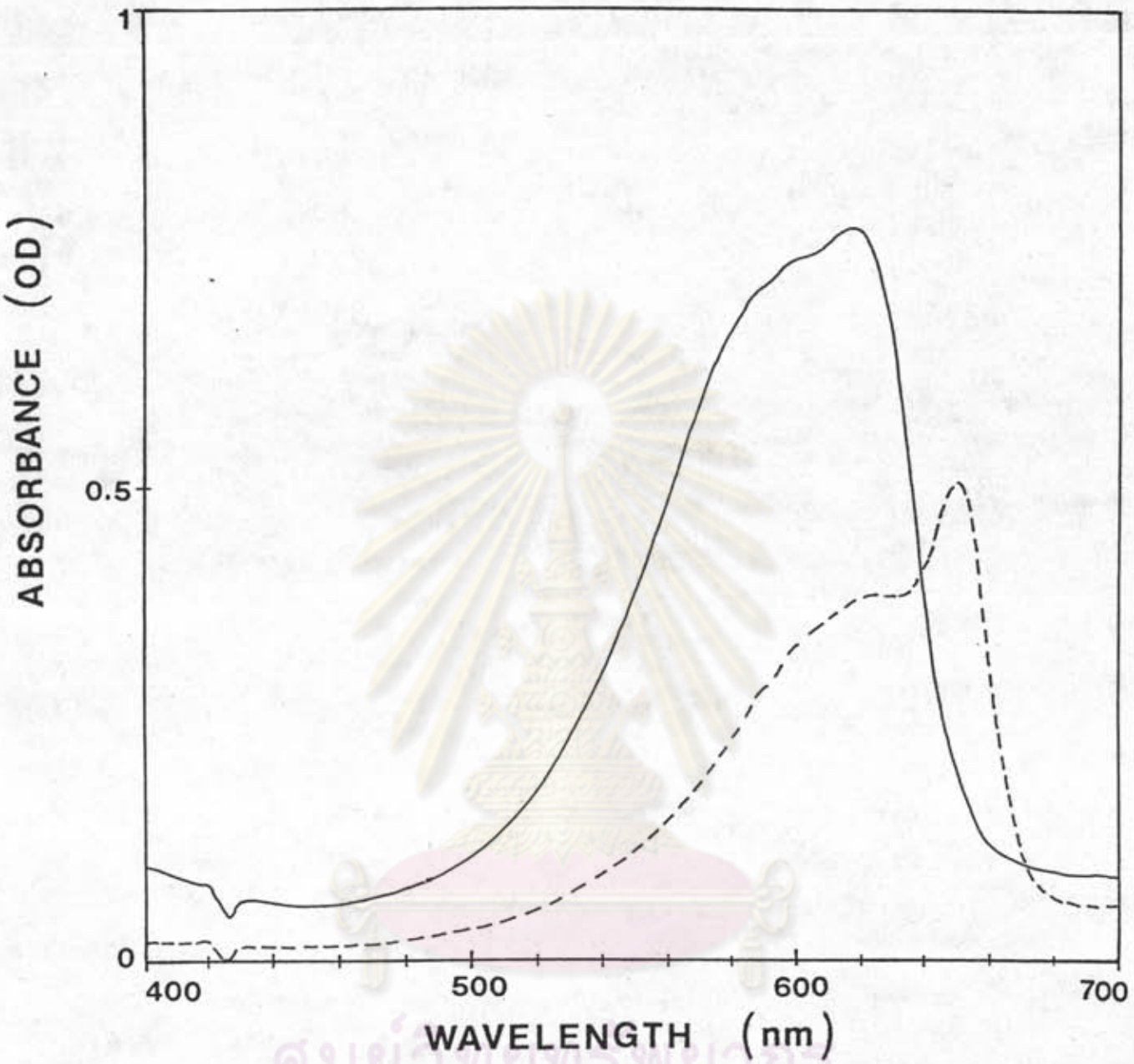


Figure 64 Absorption spectra of the two major phycobiliproteins of Spirulina (MP) after Hydroxylapatite column.

(———) Phycocyanin

(– – – –) Allophycocyanin

From all these results, the upper band and the lower band in Figure 61 were identified as phycocyanin and allophycocyanin respectively and their molecular weights were as described in section 6.5.