CHAPTER III

RESULTS

Growth of *H. pluvialis* NIES 144

H. pluvialis NIES 144 is a unicellular, motile, green alga that accumulated significant amounts of astaxanthin under certain stress conditions as in fig. 5 The growth analysis of *H. pluvialis* NIES 144 was determined by cell number counting with a haemacytometer and the specific growth rates (μ) were determined as the slope on the exponential phase of growth by least squares regression .

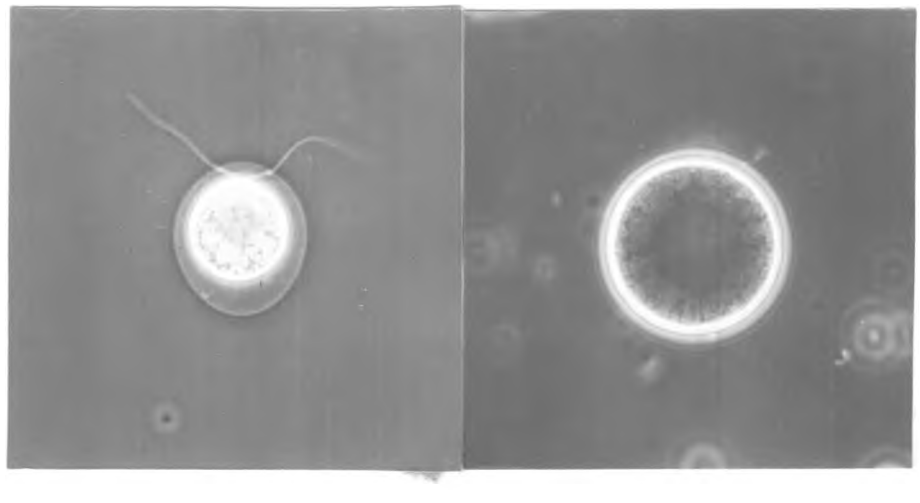


Figure 5 Vegetative green cell and red cyst cell of *H. pluvialis* NIES 144 (X 200)

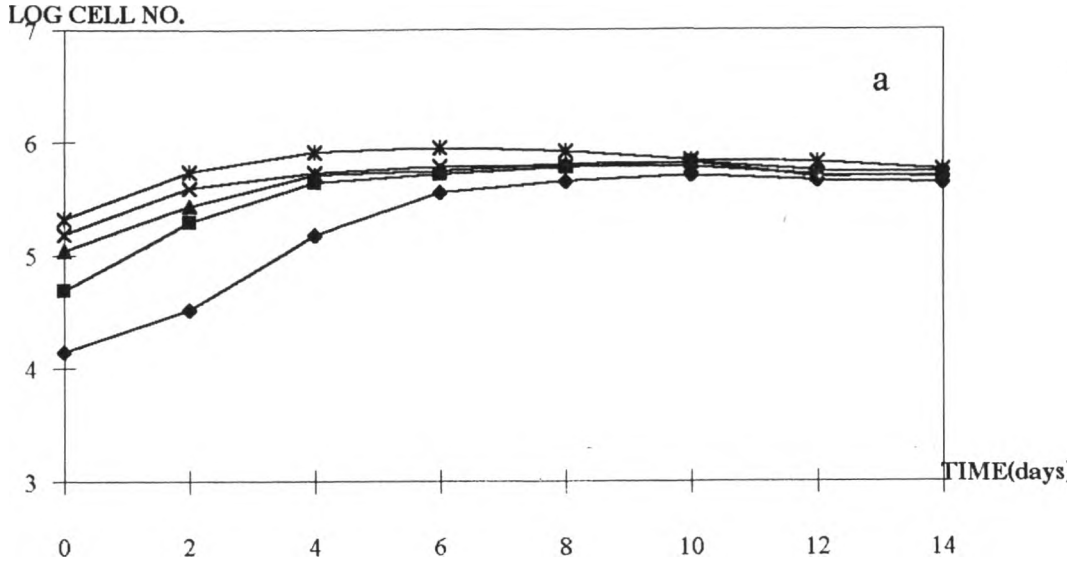
Method Employed for the Extraction of Astaxanthin and Chlorophyll from *H. pluvialis* NIES 144

1 - 3 ml of *H. pluvialis* NIES 144 cultures were extracted by two types of solvent, dimethysulfoxide and 90 % acetone for astaxanthin and chlorophyll determination by spectrophotometry. The result indicated that the astaxanthin content by either dimethysulfoxide or 90 % acetone extraction was not different whereas the chlorophyll content by 90 % acetone extraction was slightly higher than that by dimethylsulfoxide extraction. Therefore, in later experiments dimethyl sulfoxide was employed as the solvent for astaxanthin and chlorophyll extraction.

1. Effect of Environmental Factors on Growth of *H. pluvialis* NIES 144

1.1 Type of medium

Fig. 6 showed the 14 - day growth of *H. pluvialis* NIES 144 in The Basal Medium, Medium for *H. lacustris* ATCC 30453, and Bold Basal Modified Medium with various initial cell numbers of 1, 5, 10, 15, and 20 x 10^4 cells per ml under 20 μ mol $m^{-2}s^{-1}$ (12 h - dark ; 12 h - light) illumination at 21- 23^{O}C. It was found that *H. pluvialis* NIES 144 could grow best in The Basal Medium followed by Medium for *H. lacustris* ATCC 30453 and Bold Basal Modified Medium respectively. Fig. 7 showed growth analysis of *H. pluvialis* NIES 144 in terms of specific growth rate (d^{-1}) at the various initial cell numbers of three types of media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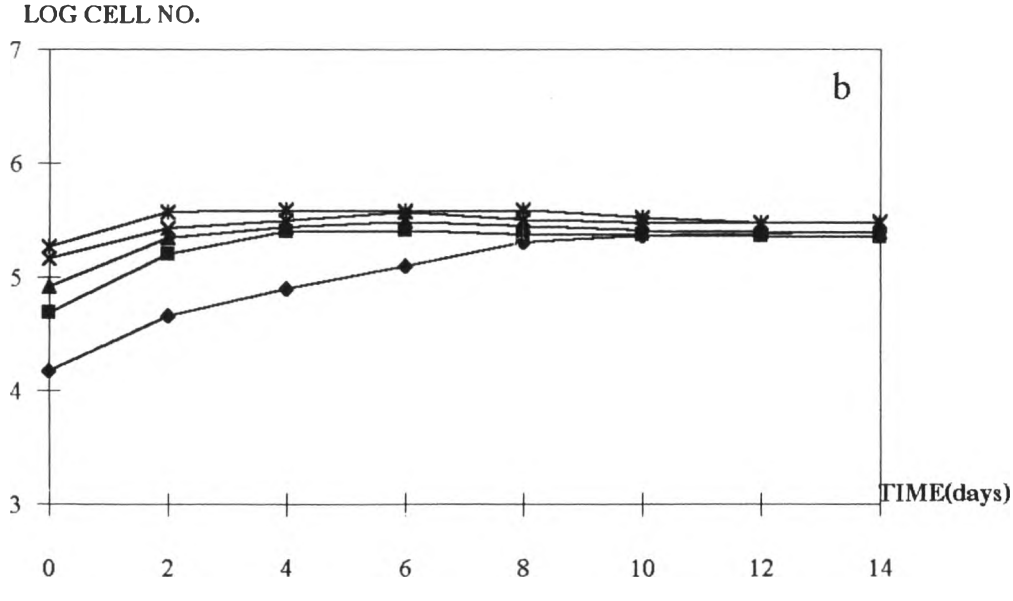
14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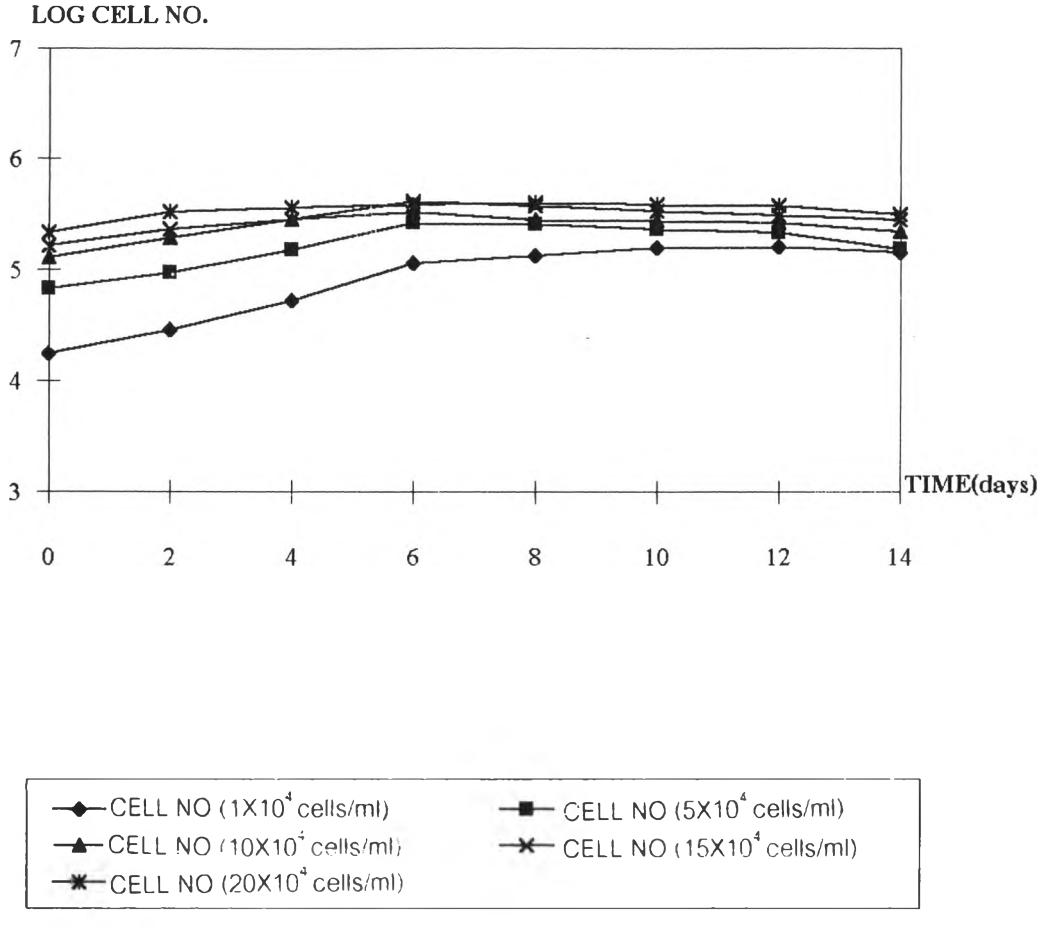


Figure 6 Growth of *H. pluvialis* NIES in three types of media, The Basal medium (a), Medium for *H. lacustris* ATCC 30453 (b) and Modified Basal medium (c) with various initial cell numbers

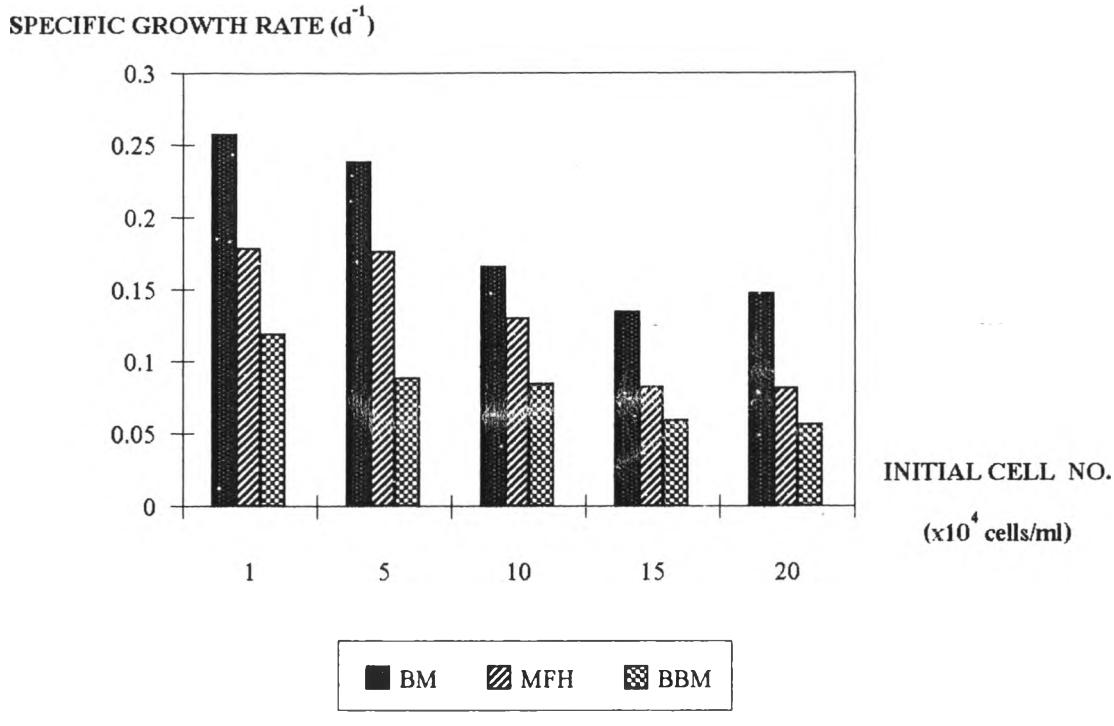


Figure 7 Specific growth rates of *H.pluvialis* NIES 144 with various initial cell numbers in three types of media

1.2 Type and concentration of carbon source

Cells were grown in three media as described in section 1. The modified medium was performed by changing the concentration of carbon and used CO_2 from air (no addition of c-source) and Na_2CO_3 the same concentration of carbon in the Basal medium. (1.2 g / l).

Fig. 8 and 9 showed that the lowest growth occurred in three types of media with Na_2CO_3. The cell growth was gradually decrease when grown in the medium containing CO_2 as carbon source and slightly from that when grown in the range of CH_3COONa concentrations between 1.0 to 1.4 g / l .

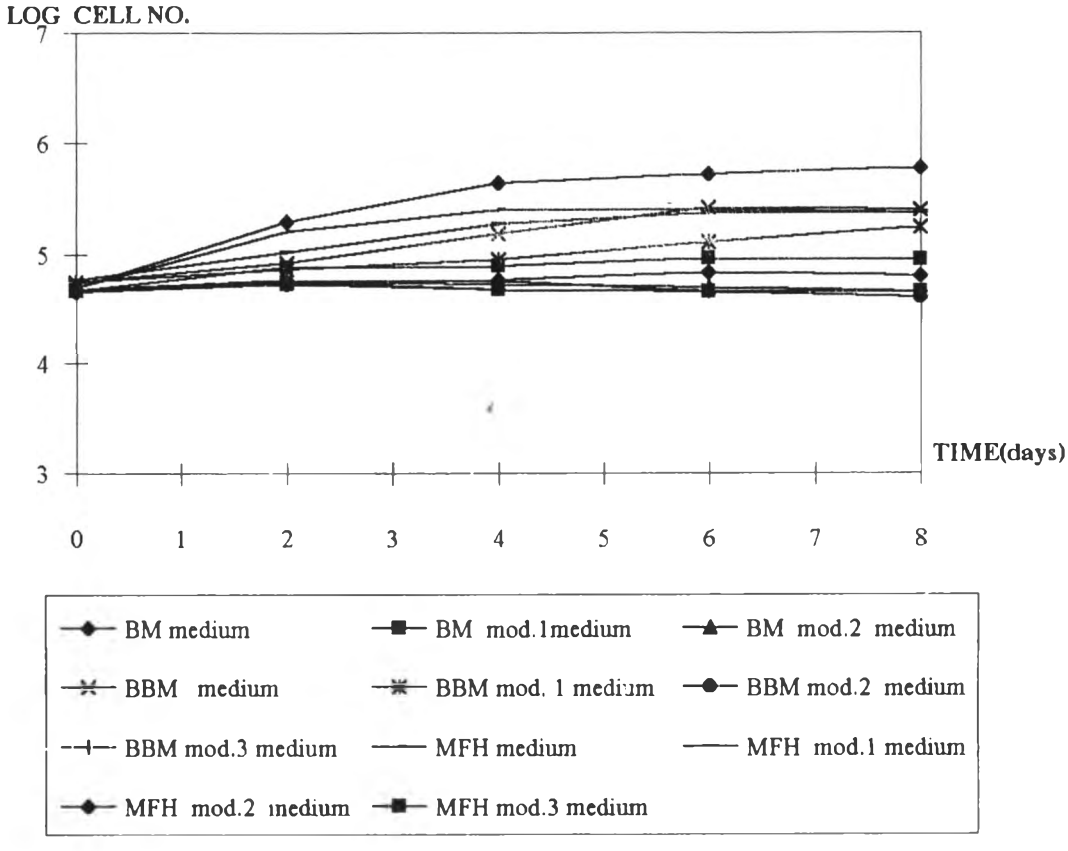


Figure 8 Growth of *H. pluvialis* in three types of media containing various concentrations of carbon source

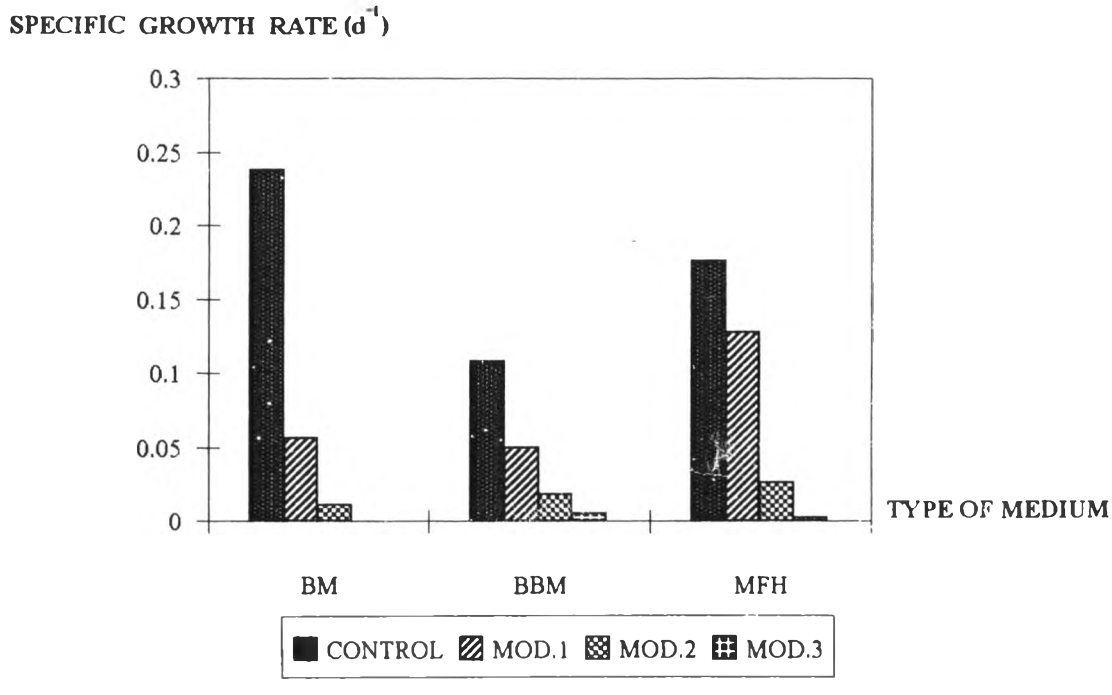


Figure 9 Specific growth rate of *H. pluvialis* in three types of media containing various concentrations of carbon source

1.3 Concentration of nitrogen source

H. pluvialis NIES 144 was grown in The Basal Medium, Medium for *H. lacustris* ATCC 30453 and Basal Modified Medium. The concentrations of nitrogen in the Medium for *H. lacustris* ATCC 30453 and Bold Basal Modified Medium were adjusted to equal to that of the Basal Medium. The cultures were incubated at 20 - 23^{O}C under 20 μ mol $m^{-2}s^{-1}$, 12 h - dark ; 12 h - light illumination for 8 days.

Fig.10 and 11 showed that the specific growth rates of *H. pluvialis* NIES 144 in The Basal Medium were better than both of modified medium for *H. lacustris* ATCC 30453 and modified Bold Basal modified medium. The increase of urea in Bold Basal Modified medium decreased the specific growth rate whereas the increase of $NaNO_3$ in Medium for *H. lacustris* slightly affected the specific growth rates.

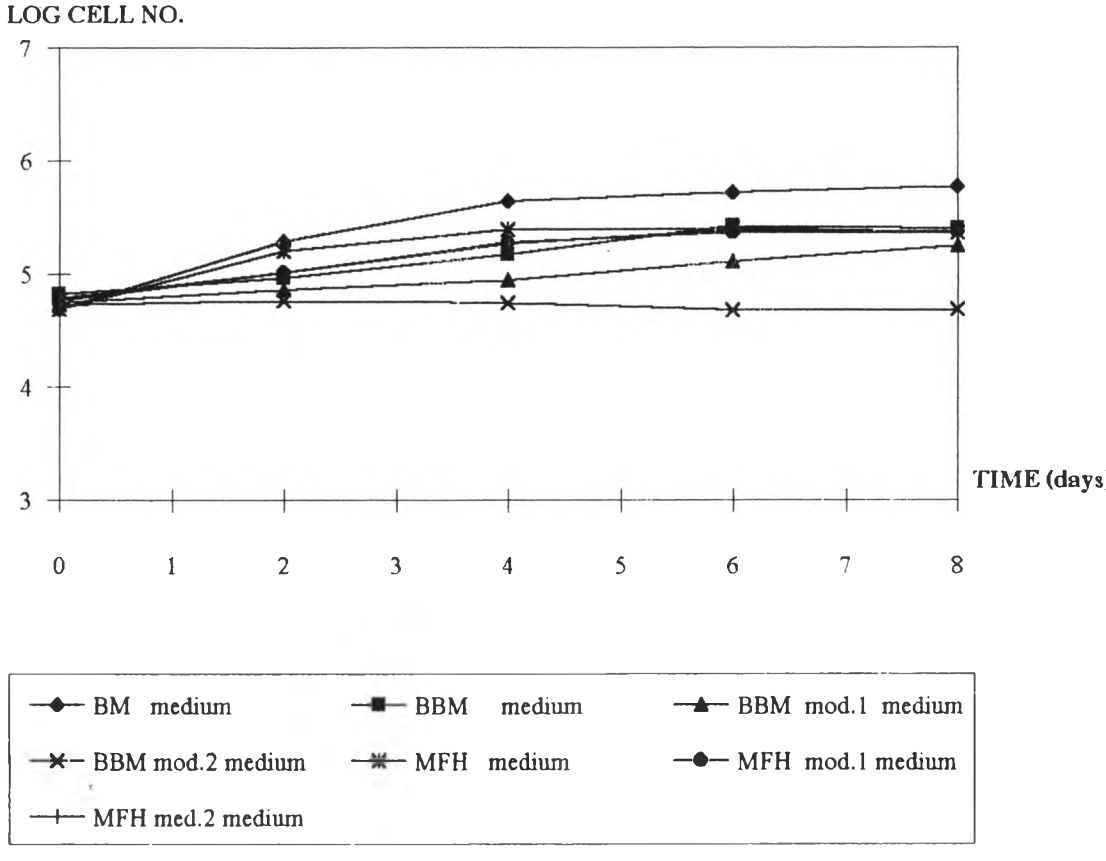


Figure 10 Growth of *H. pluvialis* in three types of media containing various concentrations of nitrogen source

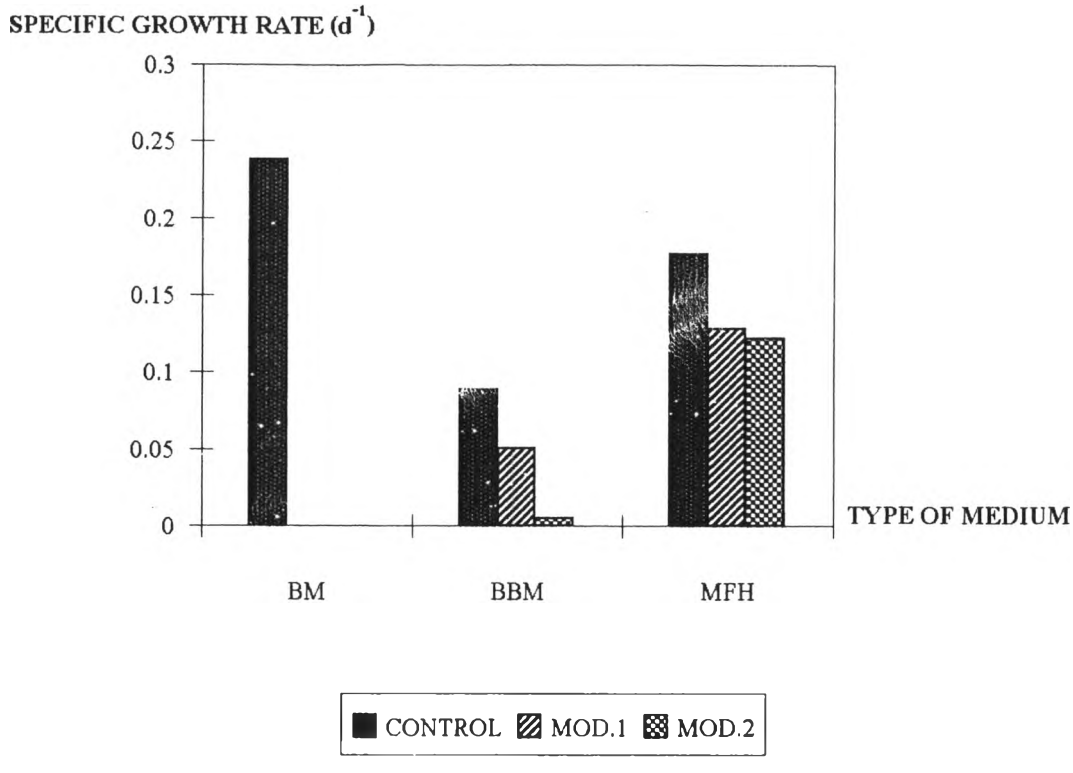


Figure 11 Specific growth rate of *H. pluvialis* in three types of media containing various concentrations of nitrogen source

2. Effect of Environmental Factors on Growth of *H. pluvialis* NIES 144 in The Basal Medium

2.1 Light intensity

H. pluvialis NIES 144 was cultured in The Basal Medium with initial cell number 5 x 10^4 cells per ml at 21 - 23°C. The light intensity was changed from 20 to 40 and 60 μ mol $m^{-2}s^{-1}$ respectively. Fig. 12 showed that the growth of *H. pluvialis* NIES 144 was slightly different when grown in the range of the light intensity between 20 to 60 μ mol $m^{-2}s^{-1}$. So, in the later experiment, the light intensity for growth of *H. pluvialis* NIES 144 was fixed at 20 μ mol $m^{-2}s^{-1}$, 12 h - dark and 12 h - light.

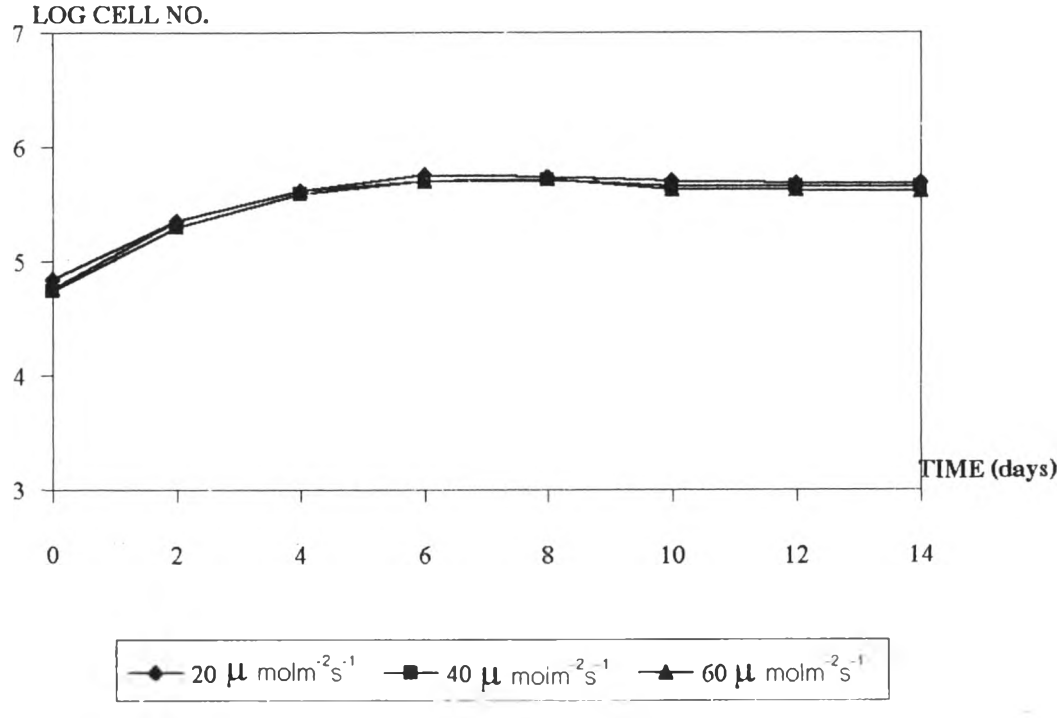


Figure 12 Growth of *H. pluvialis* exposed to 20, 40, and 60 μ mol $m^{-2}s^{-1}$

2.2 Content of carbon and nitrogen source

Cells were grown in The Basal medium with initial cell number 5 x 10^4 cells per ml at 21 - 23^{O}C under 20 μ mol $m^{-2}s^{-1}$; 12 h - dark ; 12 h - light illumination. The concentrations of CH_3COONa were changed from 0.8 to 2.0 g / l and the concentrations of yeast extract were changed from 2.0 to 3.0 g / l. Fig.13 and 14 showed that there were slight differences on growth when grown in the medium containing 0.8 to 2.0 g / l of CH_3COONa whereas slightly higher growth rate occurred in the medium containing yeast extract 2.0 to 3.0 g / l.

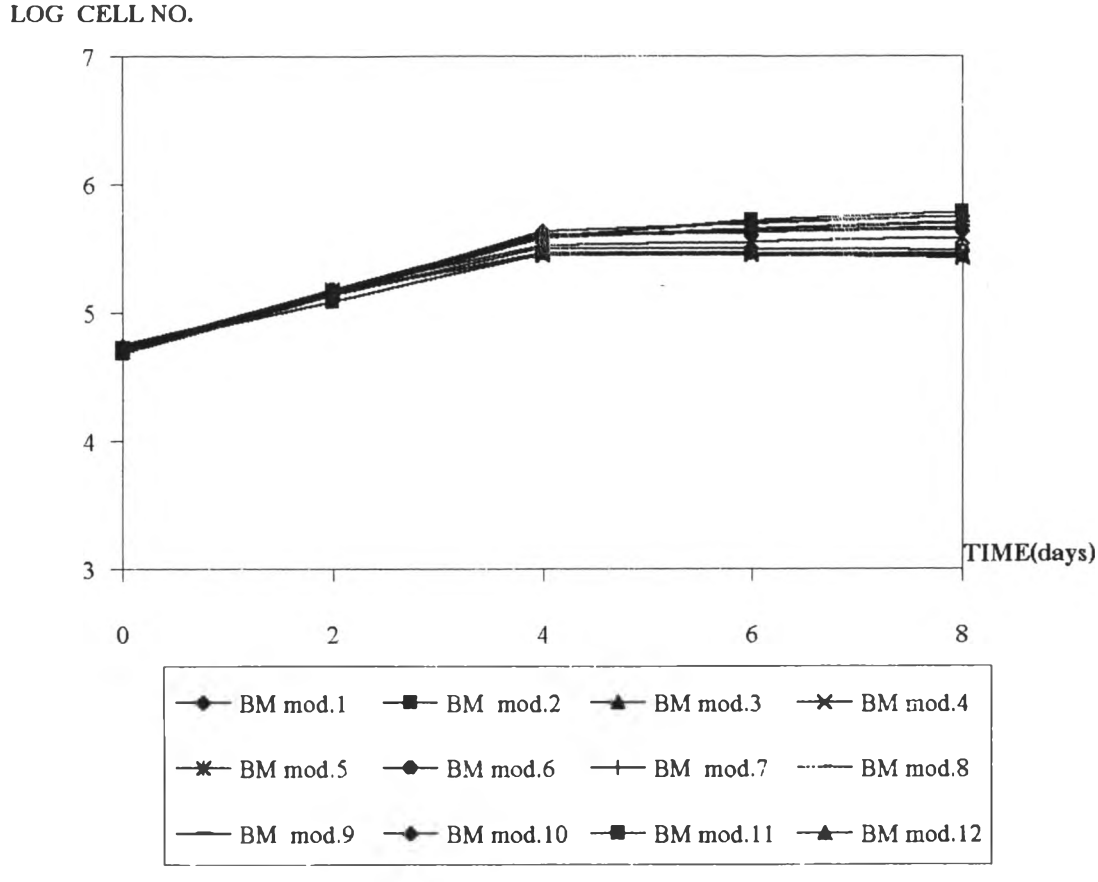


Figure 13 Growth of *H. pluvialis* in The Basal medium containing various concentrations of sodium acetate and yeast extract

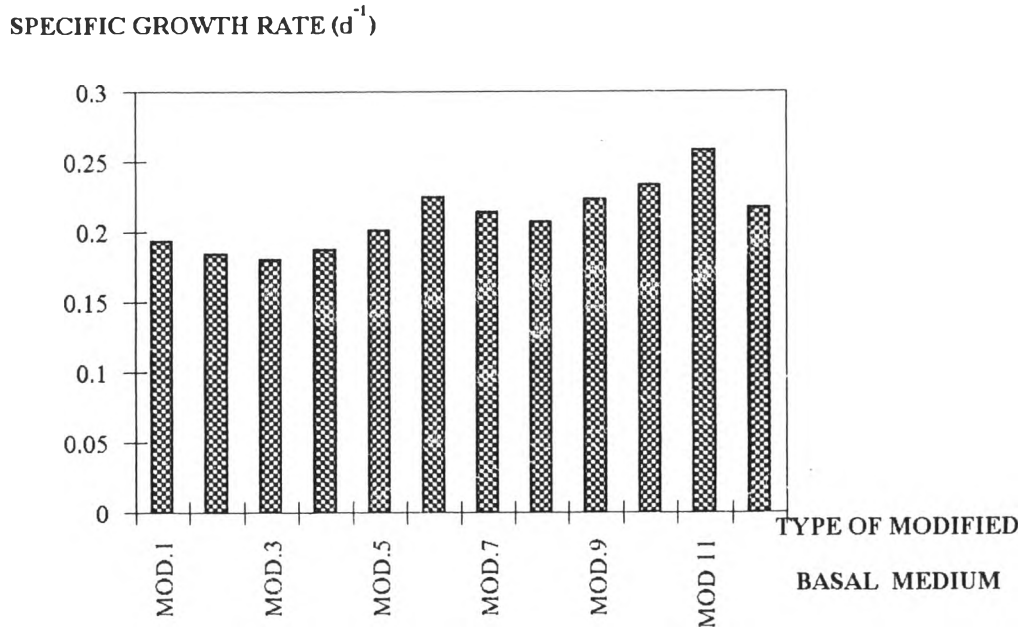


Figure 14 Specific growth rate of *H. pluvialis* in The Basal medium containing various concentrations of sodium acetate and yeast extract

2.3 Initial cell number

H. pluvialis NIES 144 was grown in The Basal Medium with initial cell number 5 x 10^4 cells / ml at 21 - 23 °C under 20 μ mol $m^{-2}s^{-1}$; 12 h - dark ; 12 h - light illumination. The initial cell numbers were changed between 10 to 200 x 10^4 cells / ml. The specific growth rates were slightly increased when grown with initial cell number 10 and 15 x 10^4 cells / ml, the specific growth rates were slightly decreased when grown with initial cell number 20 x 10^4 cells / ml. However, under the condition, the growth reached the stationary phase in day 4. Moreover, it was noticeable that at the stationary phase the cell number were slightly different(Fig. 15 and 16).

So, in the later experiments, the optimum condition for growth of *H. pluvialis* NIES 144 in The Basal Medium was 22°C under 20 μ mol $m^{-2}s^{-1}$; 12 h - dark ; 12 h - light illumination, initial cell number 20 x 10^4 cells / ml.

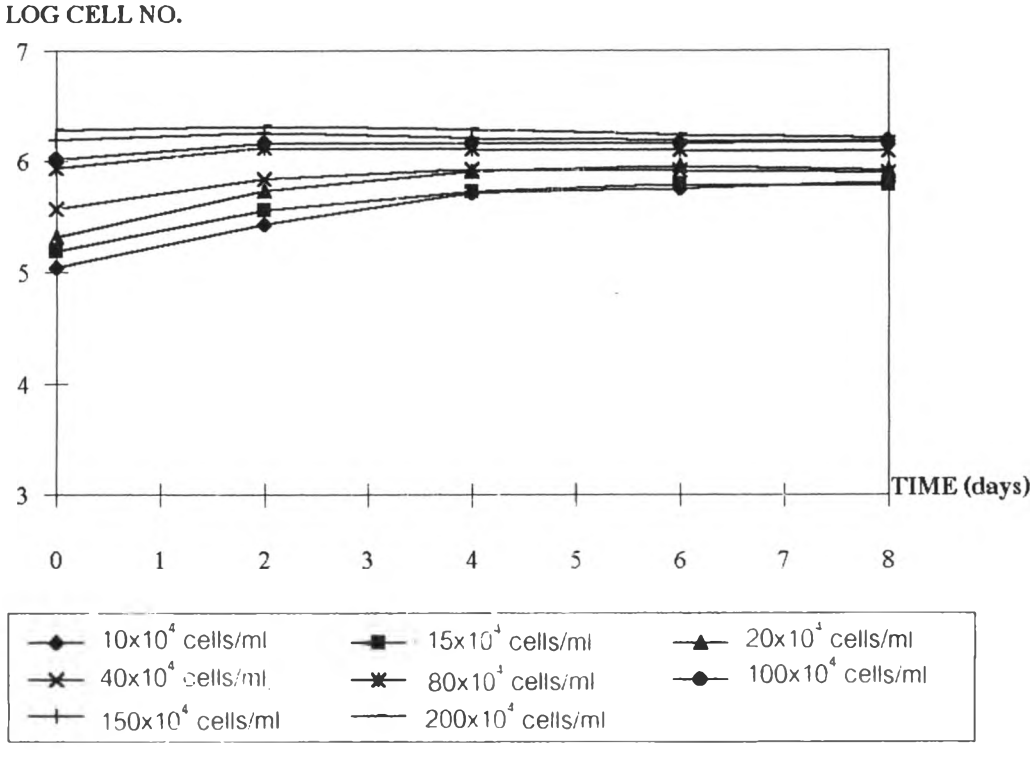


Figure 15 Growth of *H. pluvialis* in The Basal medium containing various initial cell numbers

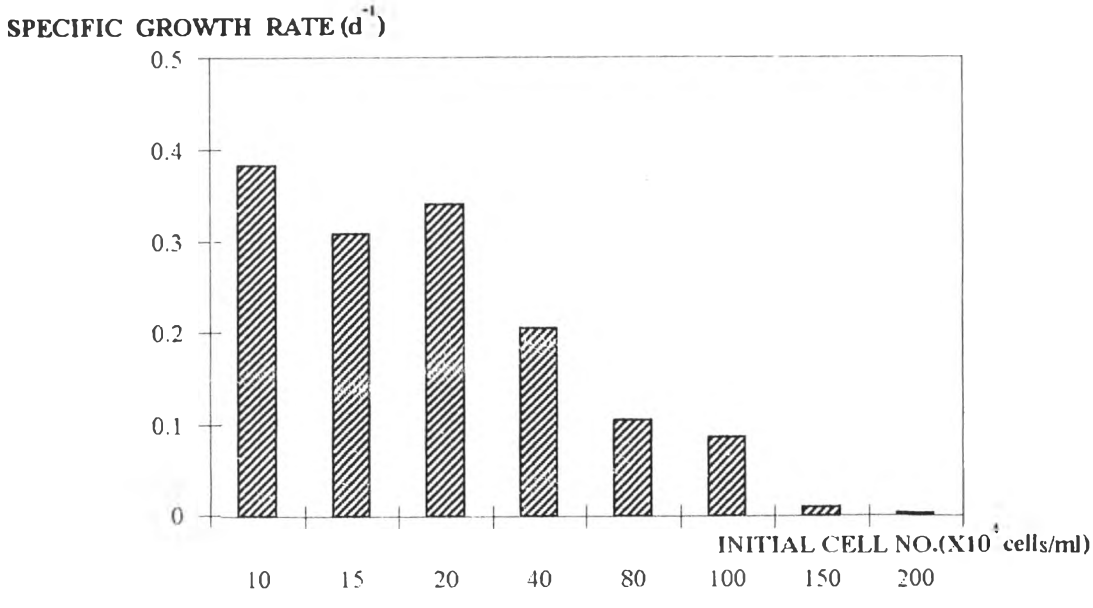


Figure 16 Specific growth rate of *H. pluvialis* in The Basal medium containing various initial cell numbers

3. Effect of Environmental Factors on Astaxanthin Content in *H. pluvialis* NIES 144

3.1 Effect of light intensity

After 4 - day, *H. pluvialis* NIES 144 cultures were exposed to various light intensities, i.e. 20, 50, 100, 150, and 200 μ mol $m^{-2}s^{-1}$ respectively. The illumination period was switched from 12 h - light , 12 h - dark to continuous illumination and the culture was incubated at 21 - 23^{O}C

At optimal light intensity for growth, 20 μ mol $m^{-2}s^{-1}$, the cell number was constant at 55 x 10^5 cells / ml. Astaxanthin and chlorophyll contents were 8.5 and 10.22 pg / cell. After 8 - day cultivation, light intensity could affect the accumulation of astaxanthin. The cell numbers were gradually decreased (Fig.17). The astaxanthin contents were increased when exposed to 20, 50, and 100 μ mol $m^{-2}s^{-1}$ and slightly decreased at 150, and 200 μ mol $m^{-2}s^{-1}$. Concomitantly chlorophyll contents were slightly decreased when exposed to 20, 50, and 100 μ mol $m^{-2}s^{-1}$ and sharply decreased at 150 and 200 μ mol $m^{-2}s^{-1}$. Maximal astaxanthin contents were 12 pg /cell when exposed to 50 and 100 μ mol $m^{-2}s^{-1}$ (Fig.18,19).

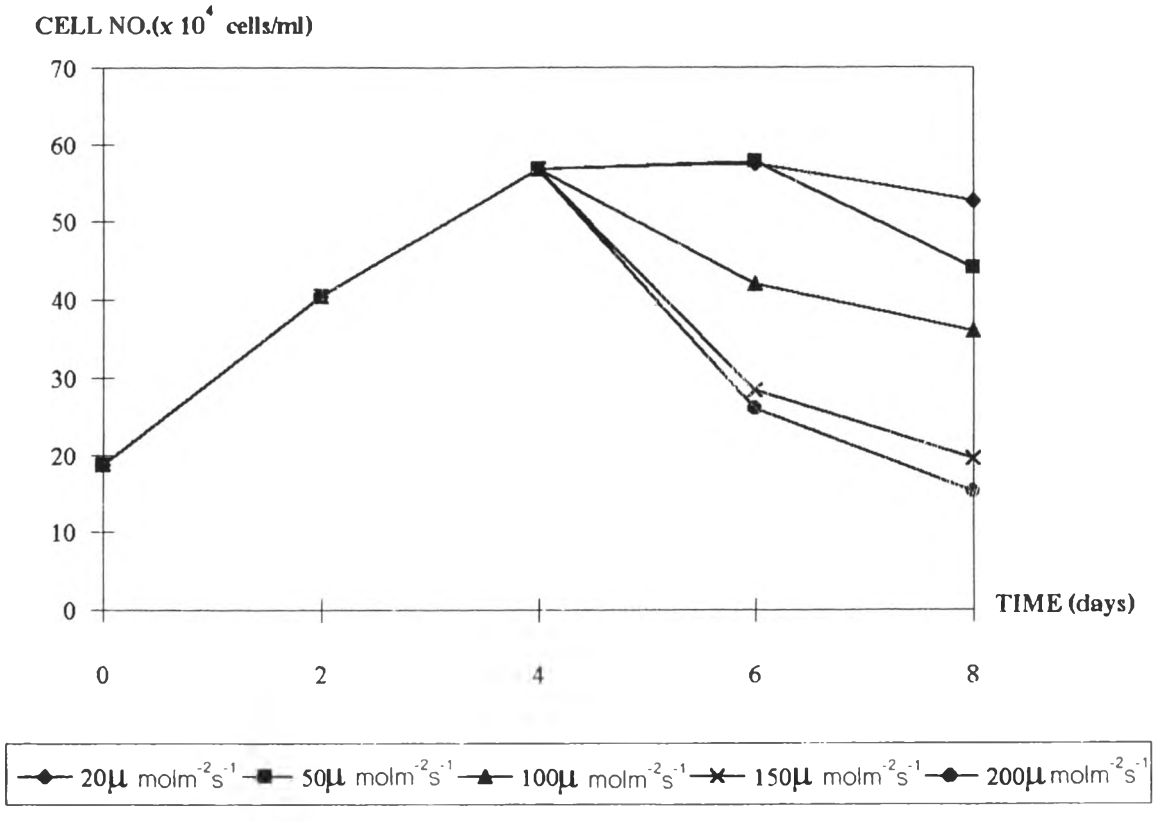


Figure 17 Growth of *H. pluvialis* in The Basal medium under various light intensities

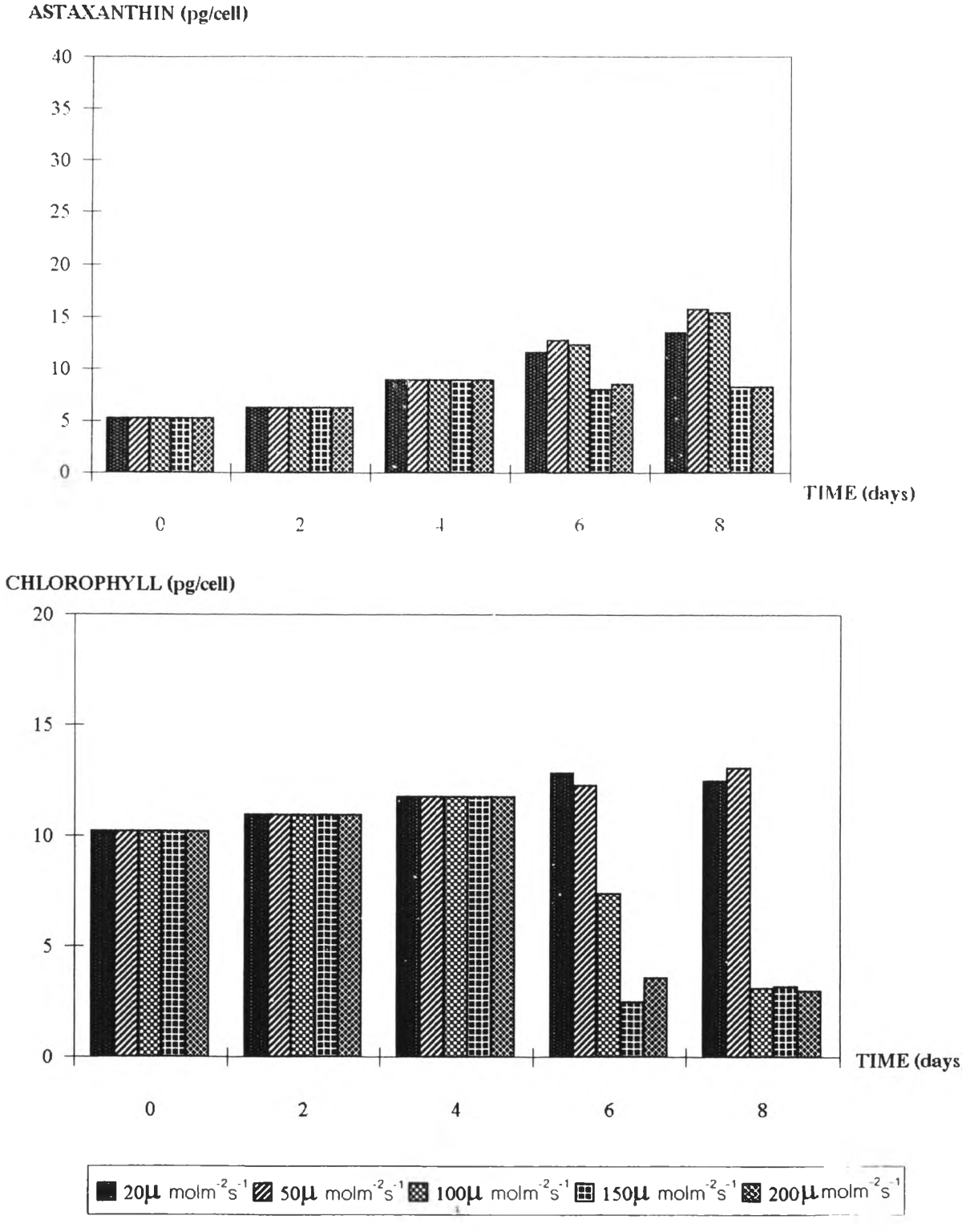


Figure 18 Effect of light intensities on the contents of astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation

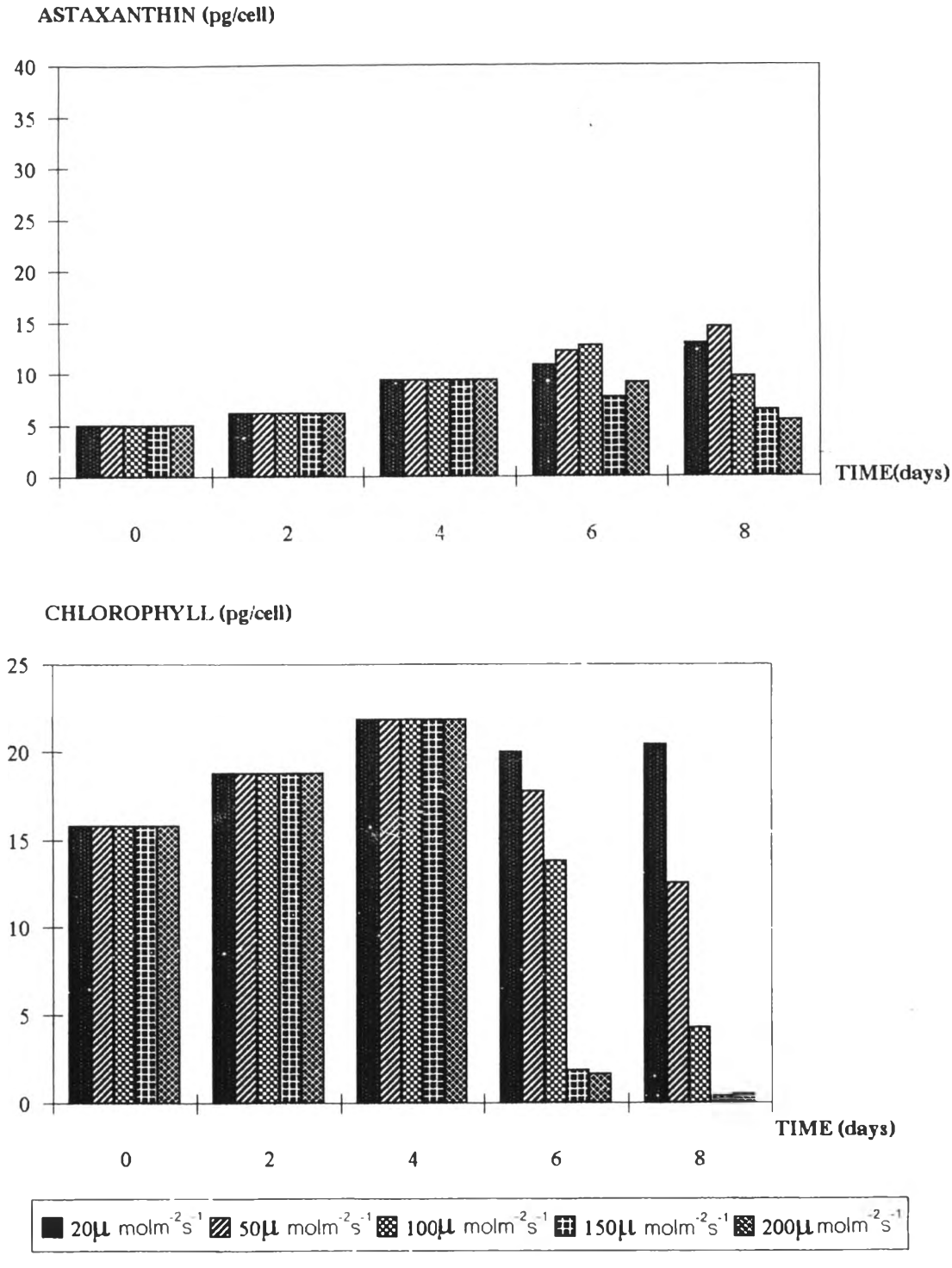
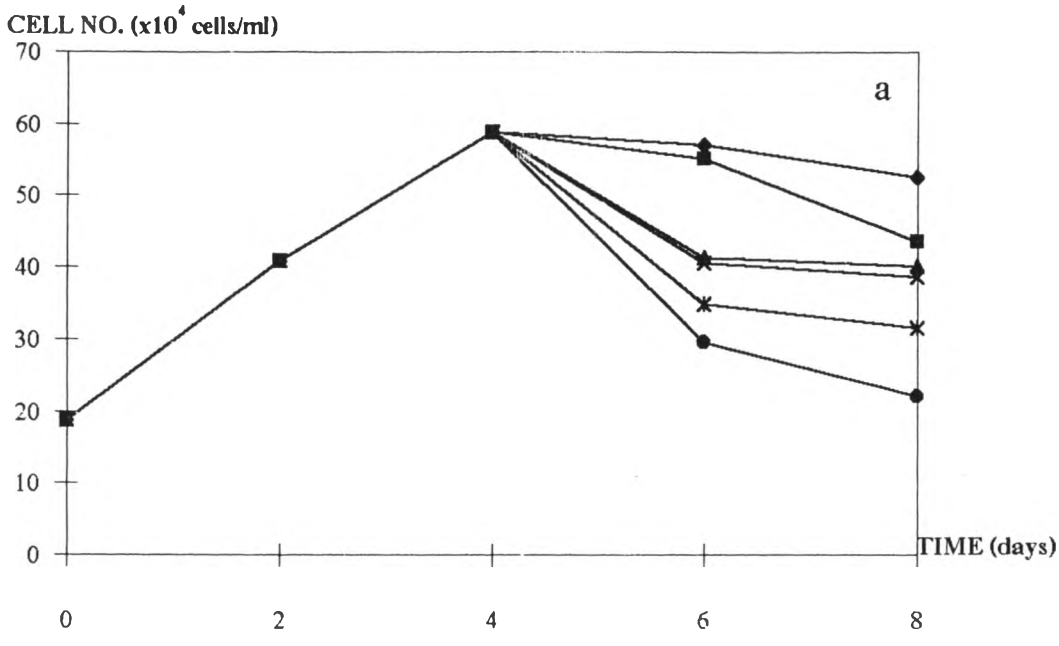


Figure 19 Effect of light intensities on the contents of astaxanthin and chlorophyll by 90% acetone extraction after 8 - day cultivation

3.2 Effect of sodium chloride

The 4 - day culture in The Basal Medium at late vegetative growth phase was supplemented with 0, 0.2, 0.4, 0.8, 1.2, and 1.6 % w /v NaCl respectively. After the addition, the light intensity was increased from 20 to 50, 100, 150, and 200 μ mol $m^{-2}s^{-1}$and incubated at 21-23^{O}C. Fig. 20 (a) to (c) showed the growth of *H. pluvialis* NIES 144 which was sharply decreased when added with 0.4 to 1.6 % (w/v) NaCl and exposed to high light intensity. The growth was inhibited in the presence of NaCl. Astaxanthin was lightly increased in the presence of 0.2 % (w/v) NaCl whereas it was sharply decreased at NaCl concentration higher than 0.2 % (w/v). The content of astaxanthin was 16 pg./cell after 8 days in comparison to 9 pg./ cell obtained under the optimal condition of growth. (Fig.21) On the other hand, chlorophyll contents at low concentration of NaCl (0 to 0.2 %) were not different, but sharply decreased at high concentration of NaCl.

The linear models procedure and Duncan's multiple range test showed that, light and sodium chloride affected astaxanthin content. Addition with 0.2 % NaCl and illumination with 50 to 100 μ mol $m^{-2}s^{-1}$ gave a high astaxanthin content as shown in appendix 5 .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TIME (days)
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2
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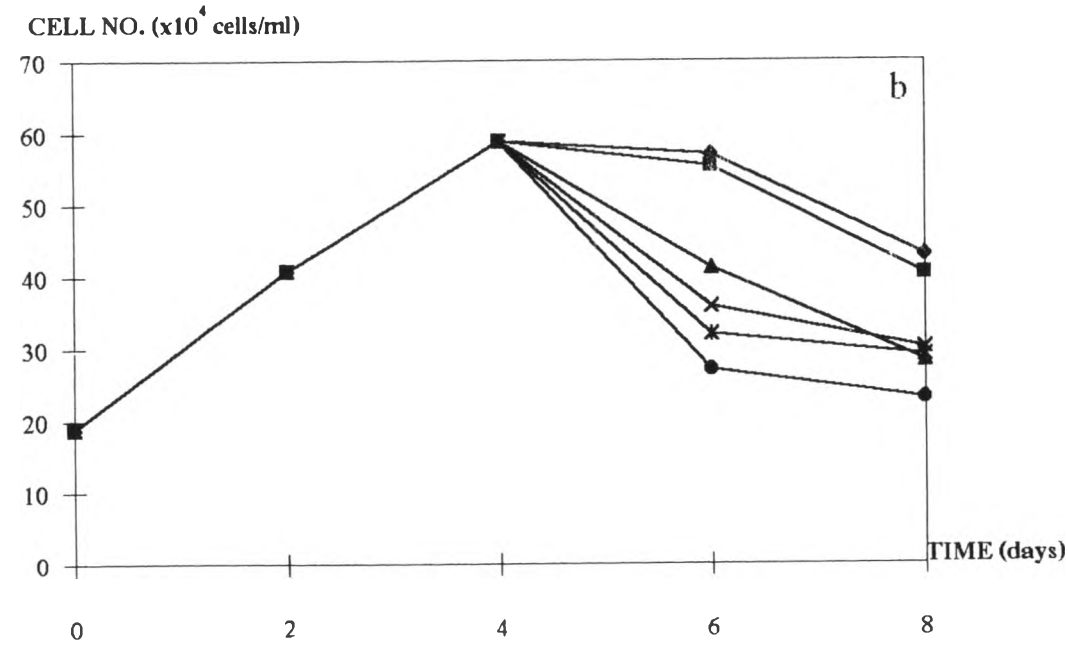
6
8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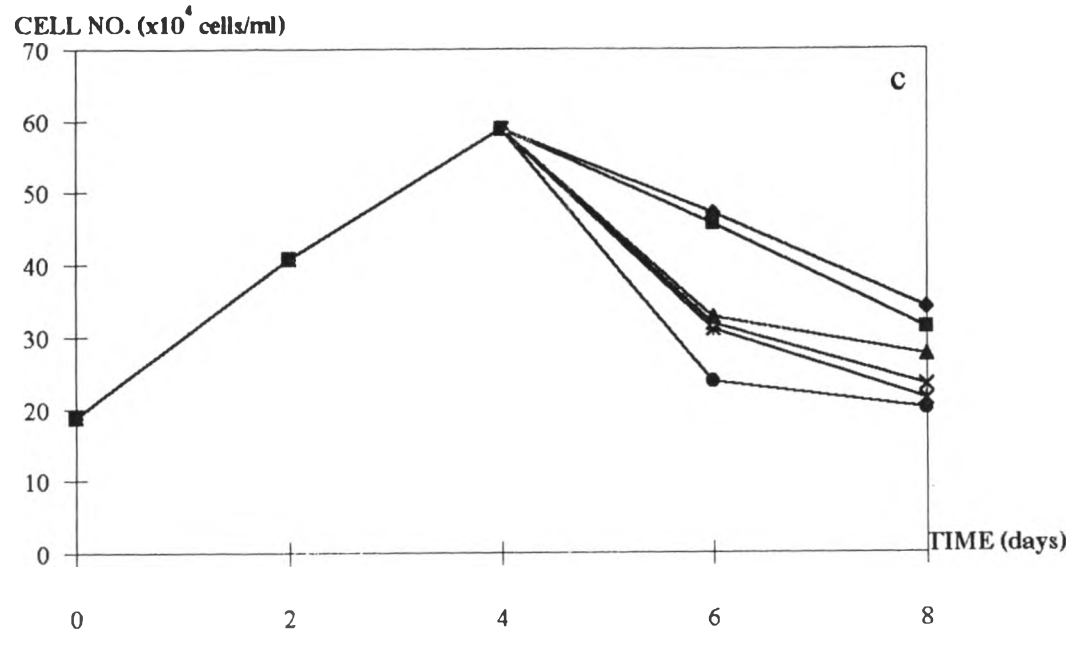
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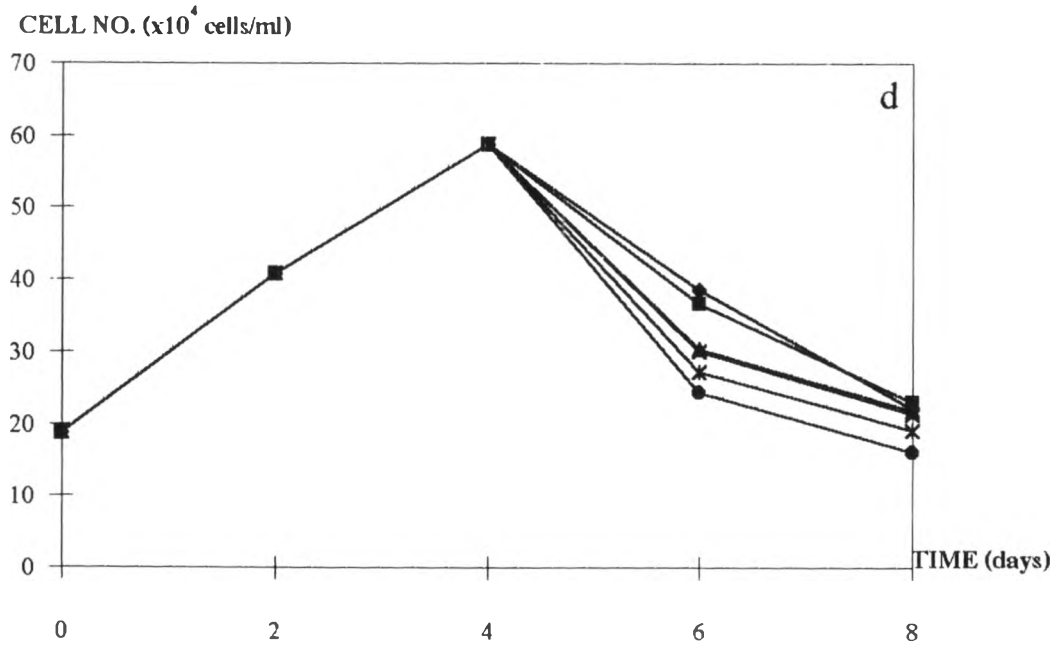
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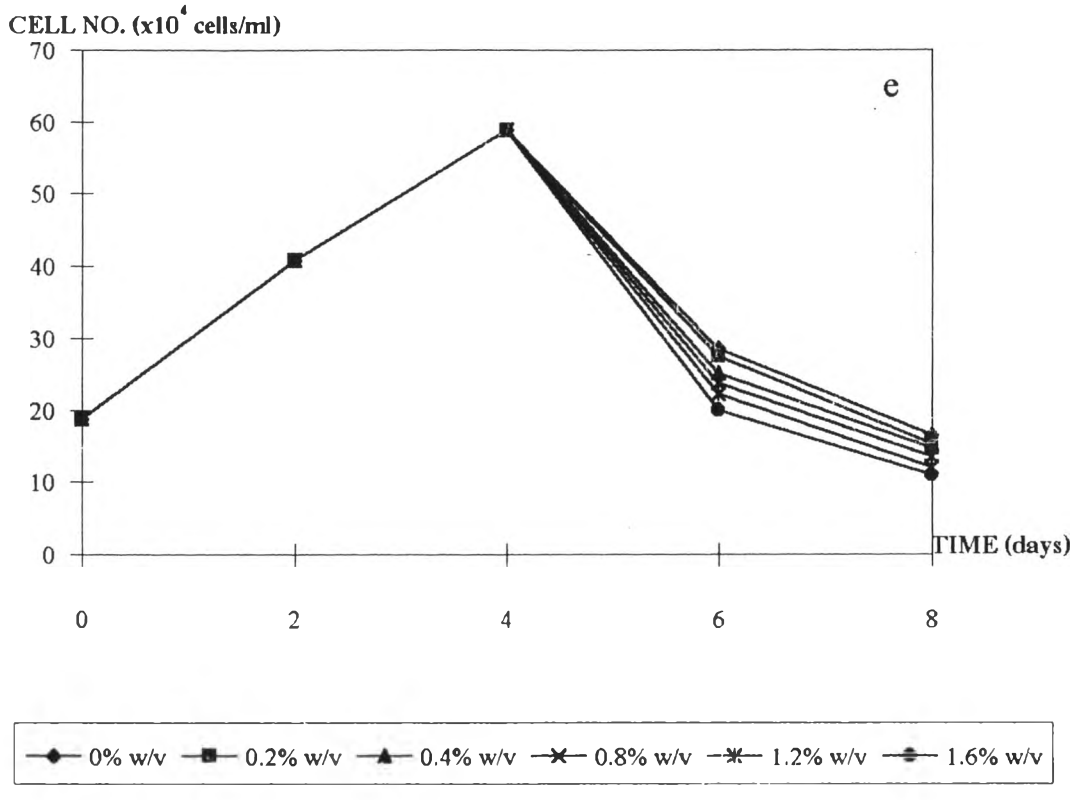


Figure 20 Growth of *H. pluvialis* in The Basal Medium containing different sodium chloride content ratios at 20 (a), 50 (b), 100 (c), 150 (d), and 200 (e) μ mol $m^{-2}s^{-1}$

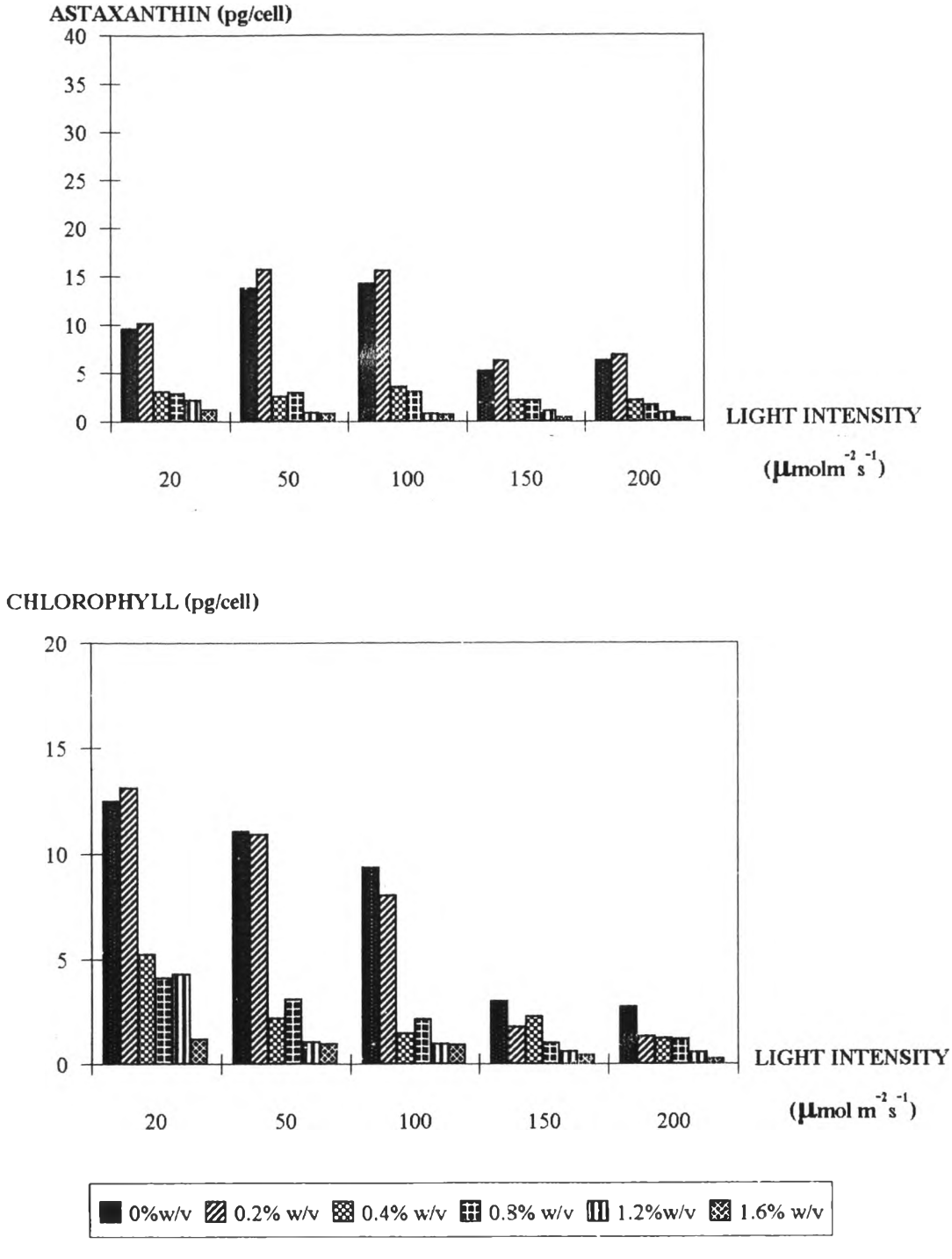


Figure 21 Effect of various NaCl concentration on the contents of astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation

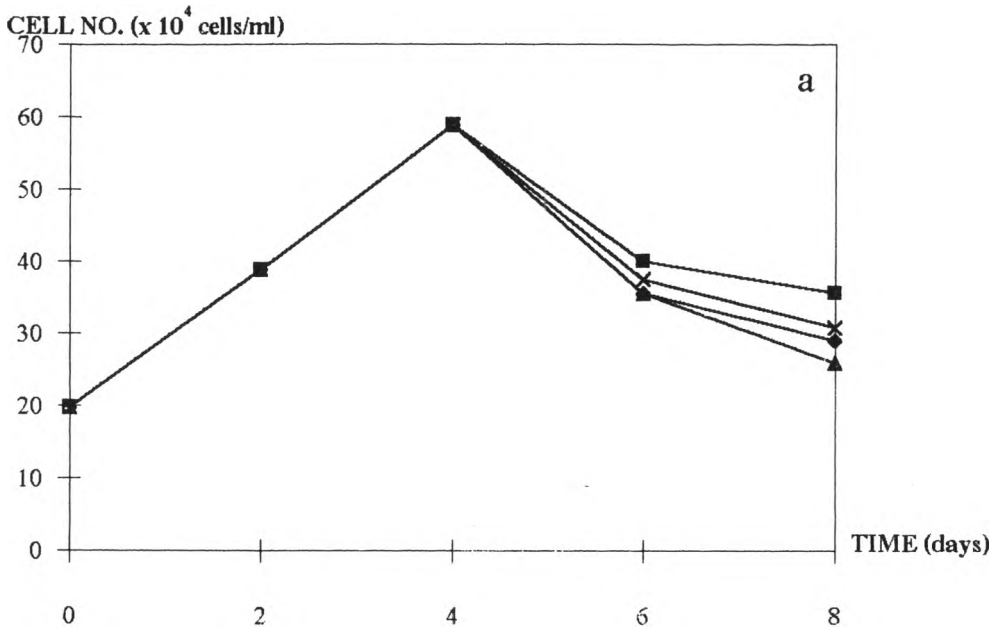
3.3 Effect of carbon and nitrogen

3.3.1 Effect of C/N content

The 4 - day culture on The Basal Medium was supplemented with sodium acetate and sodium nitrate so as to adjust the C / N content of the medium. The acetate concentration supplemented to the culture was fixed at 43.8 mM, while sodium nitrate concentrations were adjusted to 0, 21.9, and 43.8 mM. The cultures were cultivated at 21 - 23^{O}C under 20, 50, 100, 150, and 200 μ mol $m^{-2}s^{-1}$ continuous illumination. The algal cell number was decreased as shown in Fig. 22.

Astaxanthin accumulation was induced by the high C/N content as shown in Fig.23. Under the high C/N content (C/N=43.8:0) the astaxanthin content was increased reaching 28 pg. / cell after 4 day supplementation under 50, 100, and 150 μ mol $m^{-2}s^{-1}$ continuous illumination whereas under the low C/N content astaxanthin accumulation was not different from that without addition of carbon and / or nitrogen. In the case of chlorophyll, under high C/N content and / or high light intensity, the chlorophyll content was drastically decreased. Consequently, the ratio of C/N =43.8:0 was used for the later experiments.

The linear models procedure and Duncan's multiple range test showed that, light intensity did not affect astaxanthin content whereas C/N content did. Supplementation with 43.8 mM sodium acetate and without sodium nitrate gave high content of astaxanthin as shown in appendix 6.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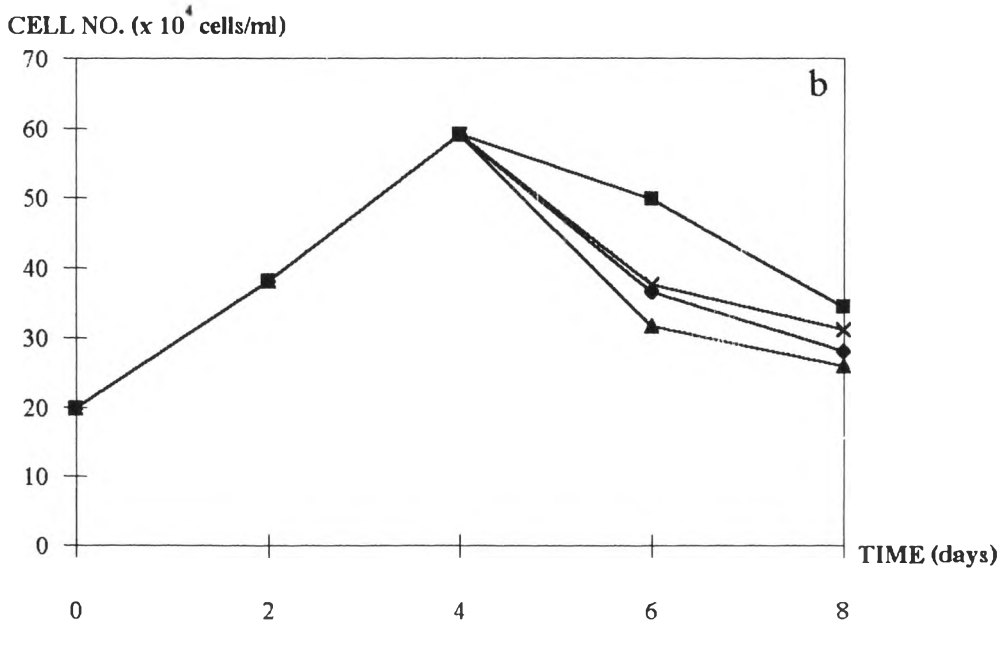
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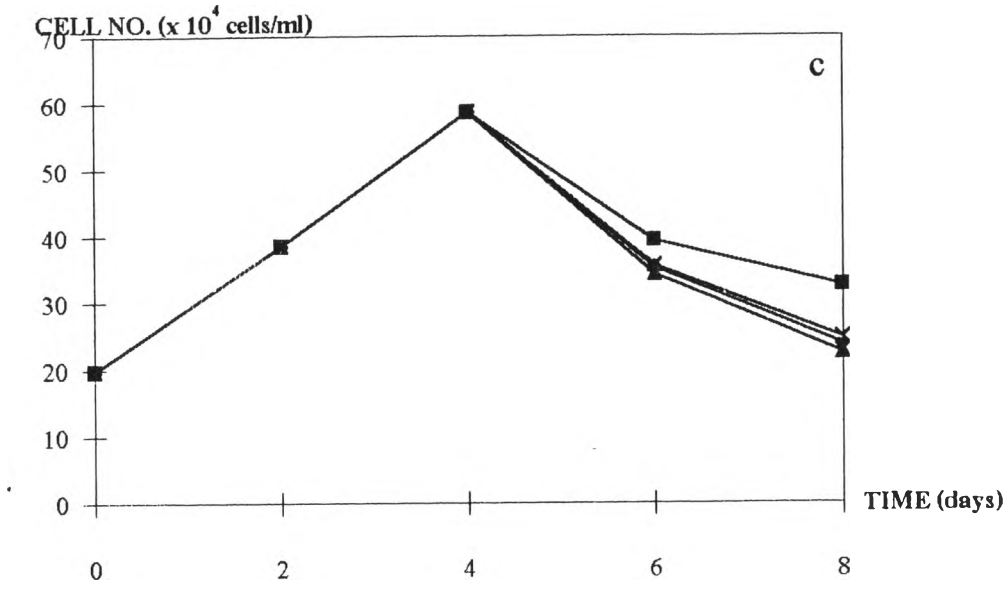
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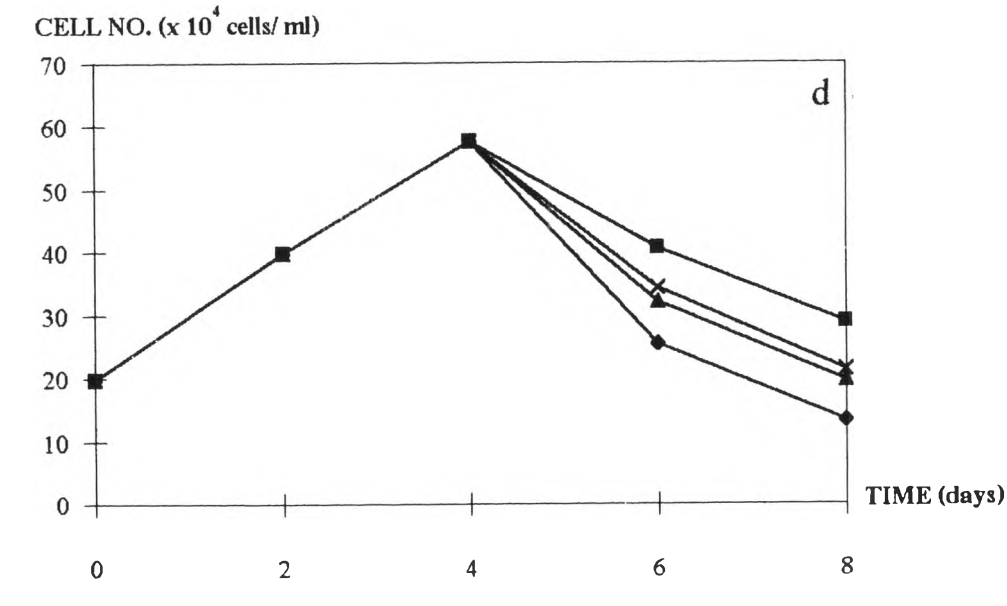
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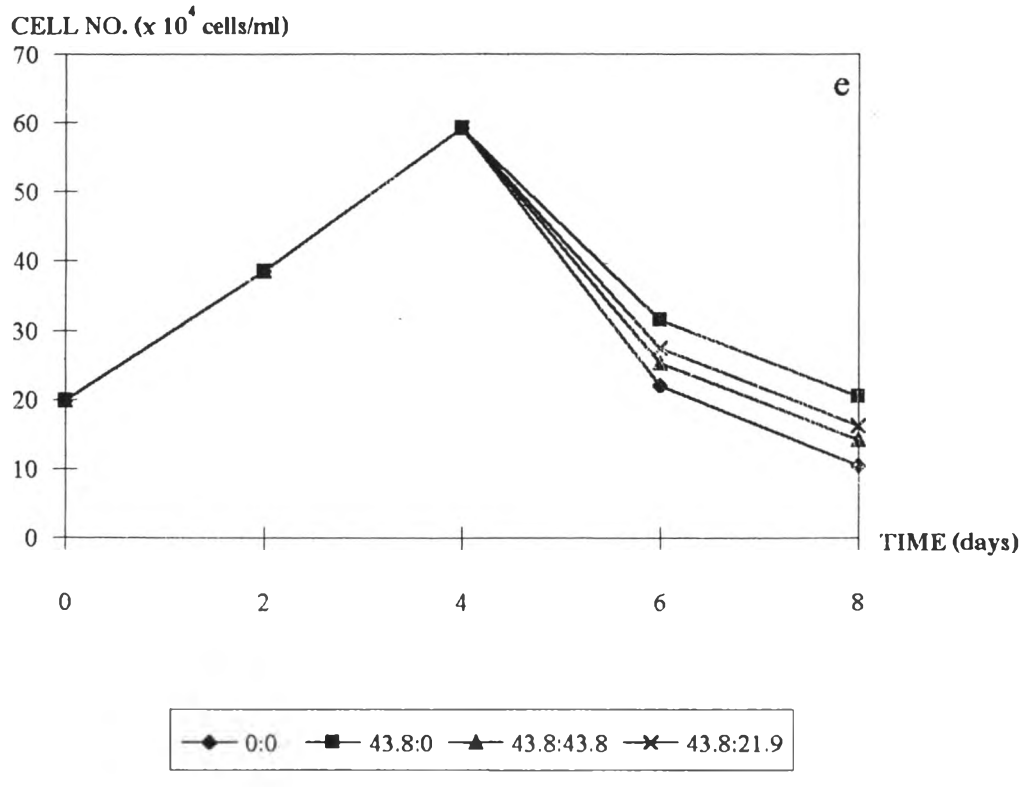


Figure 22 Growth of *H. pluvialis* in The Basal Medium when supplemented various C/N content at 20 (a), 50 (b), 100 (c), 150 (d), and 200 (e) μ mol $m^{-2}s^{-1}$

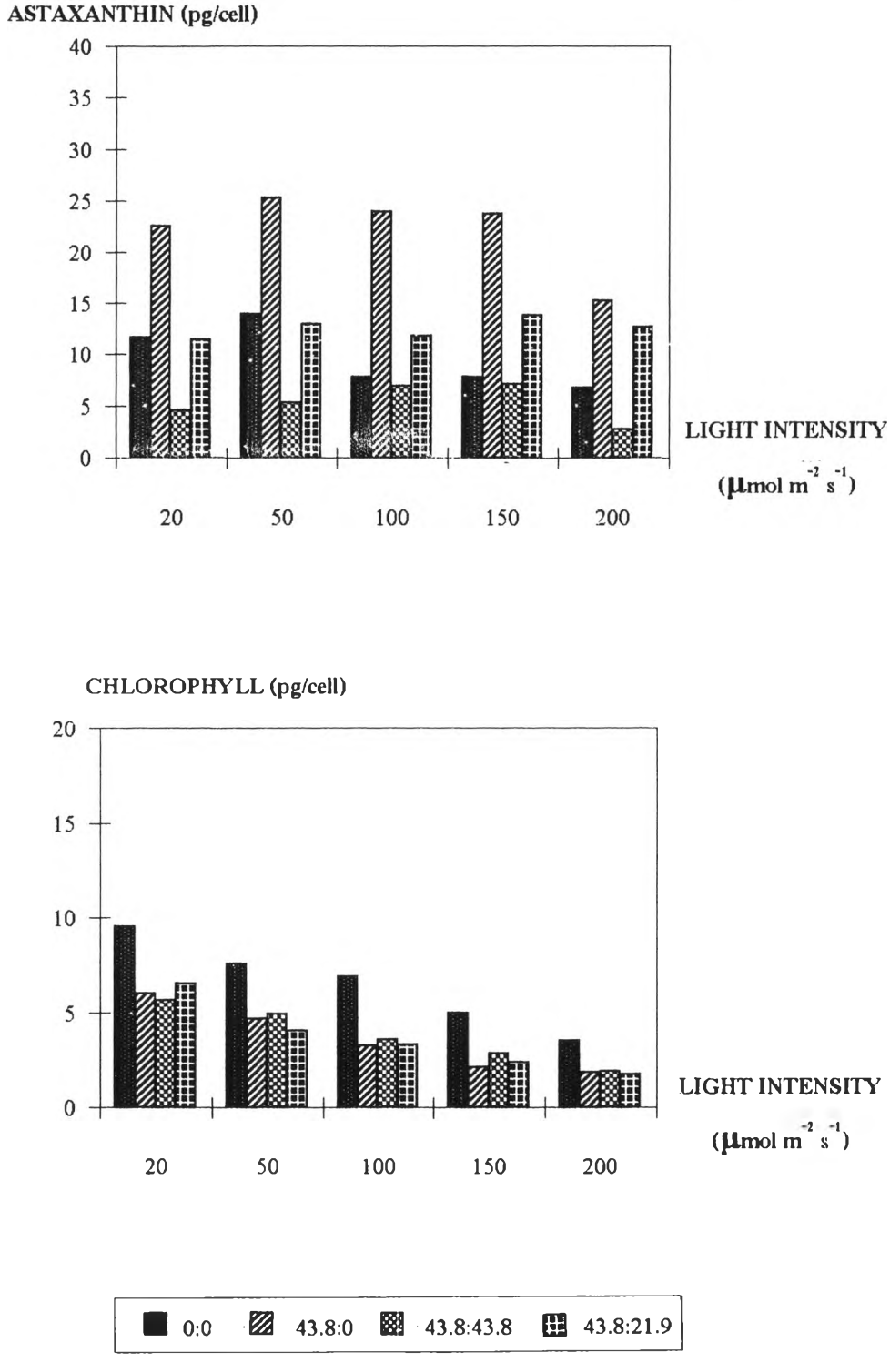


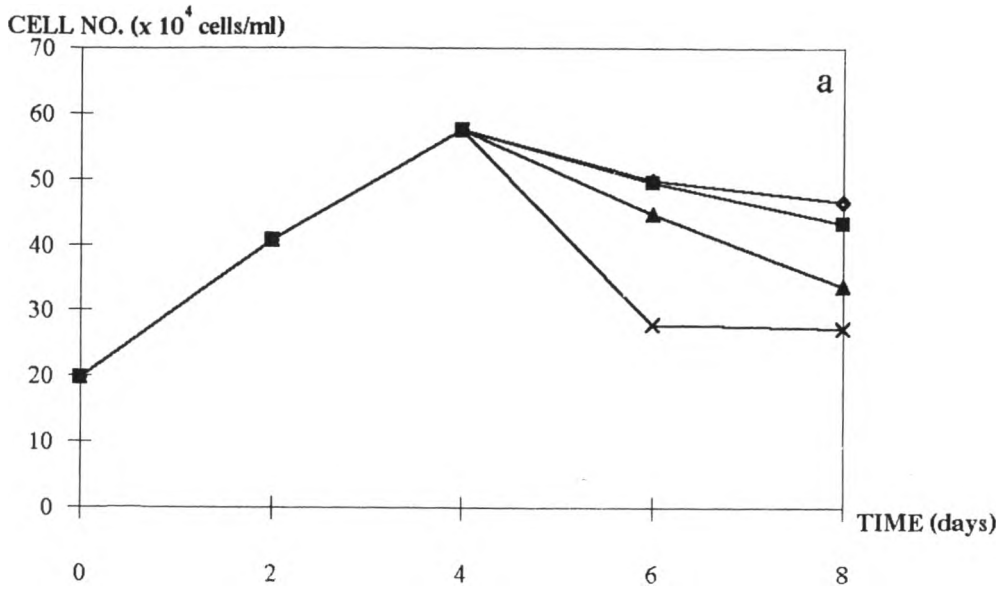
Figure 23 Effect of C/N content on the contents of astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation

3.3.2 Effect of sodium acetate (pH 7)

The 4 - day culture on The Basal Medium was used to test the effect of sodium acetate. The cultures were cultivated at 21 - 23^{O}C under the same condition as section 3.3. As shown in Fig.24, the cell number was slightly decrease when supplemented with 0 and 21.9 mM and gradually decreased when supplemented with 43.8 and 87.6 mM of sodium acetate under all light intensity regimes.

The Basal medium supplemented with 21.9 mM of sodium acetate accumulated astaxanthin as high as 30 pg./cell under 50, 100, and 150 μ mol $m^{-2}s^{-1}$continuous light illumination. On the other hand, chlorophyll content was not affected by sodium acetate at low light intensity at gradually decreased at high light intensity (Fig. 25).

The linear models procedure and Duncan's multiple range test showed that, light intensity did not affect astaxanthin content whereas sodium acetate did. Supplementation with 21.9 mM sodium acetate gave high content of astaxanthin as shown in appendix 7 .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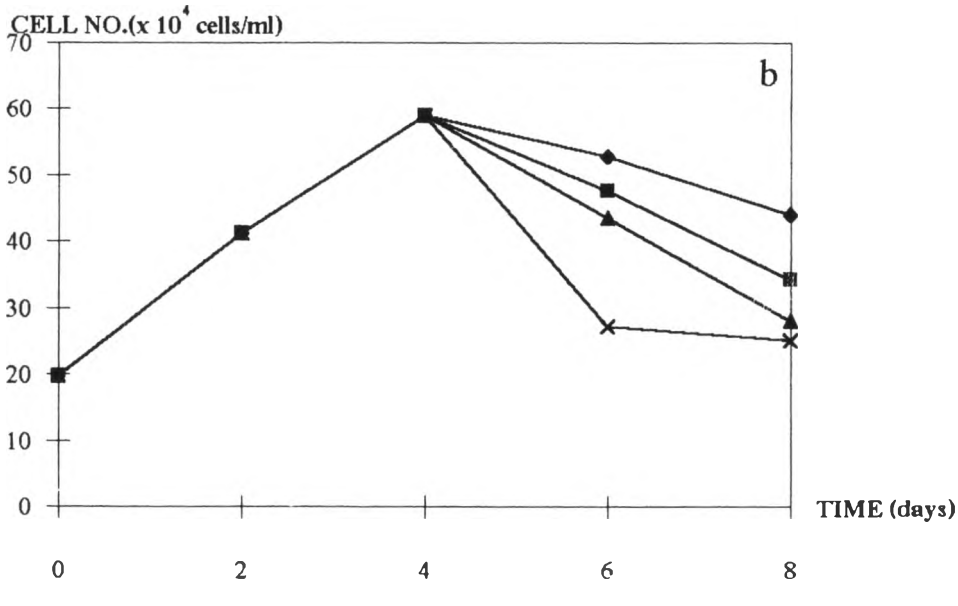
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b
TIME (days)
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2
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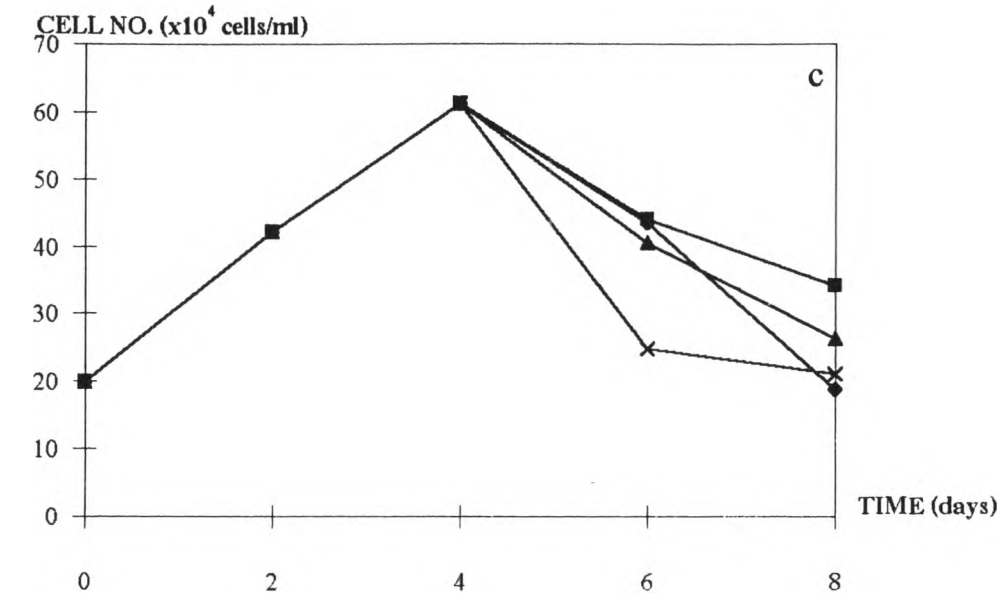
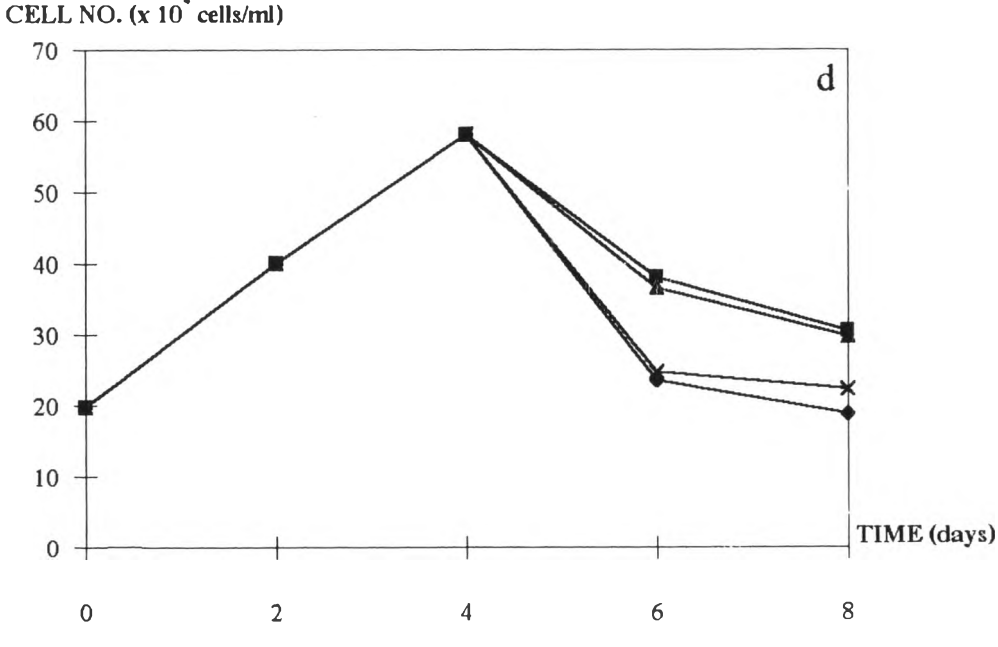
6
8


CELL NO. (x10⁴ cells/ml)
70
60
50
40
30
20
10
0
c
TIME (days)
0
2
4
6
8


CELL NO. (x 10⁴ cells/ml)
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60
50
40
30
20
10
0
d
TIME (days)
0
2
4
6
8

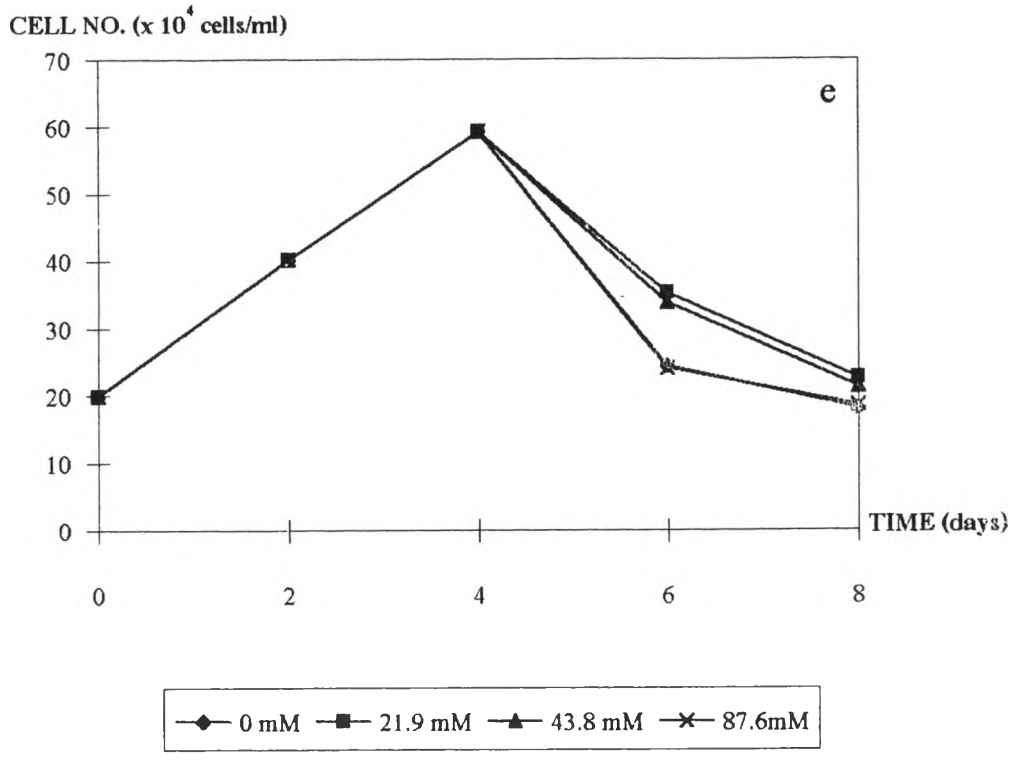


Figure 24 Growth of *H. pluvialis* in The Basal Medium when supplemented various concentration of sodium acetate at 20 (a), 50 (b), 100 (c), 150 (d), and 200 (e) μ mol $m^{-2}s^{-1}$

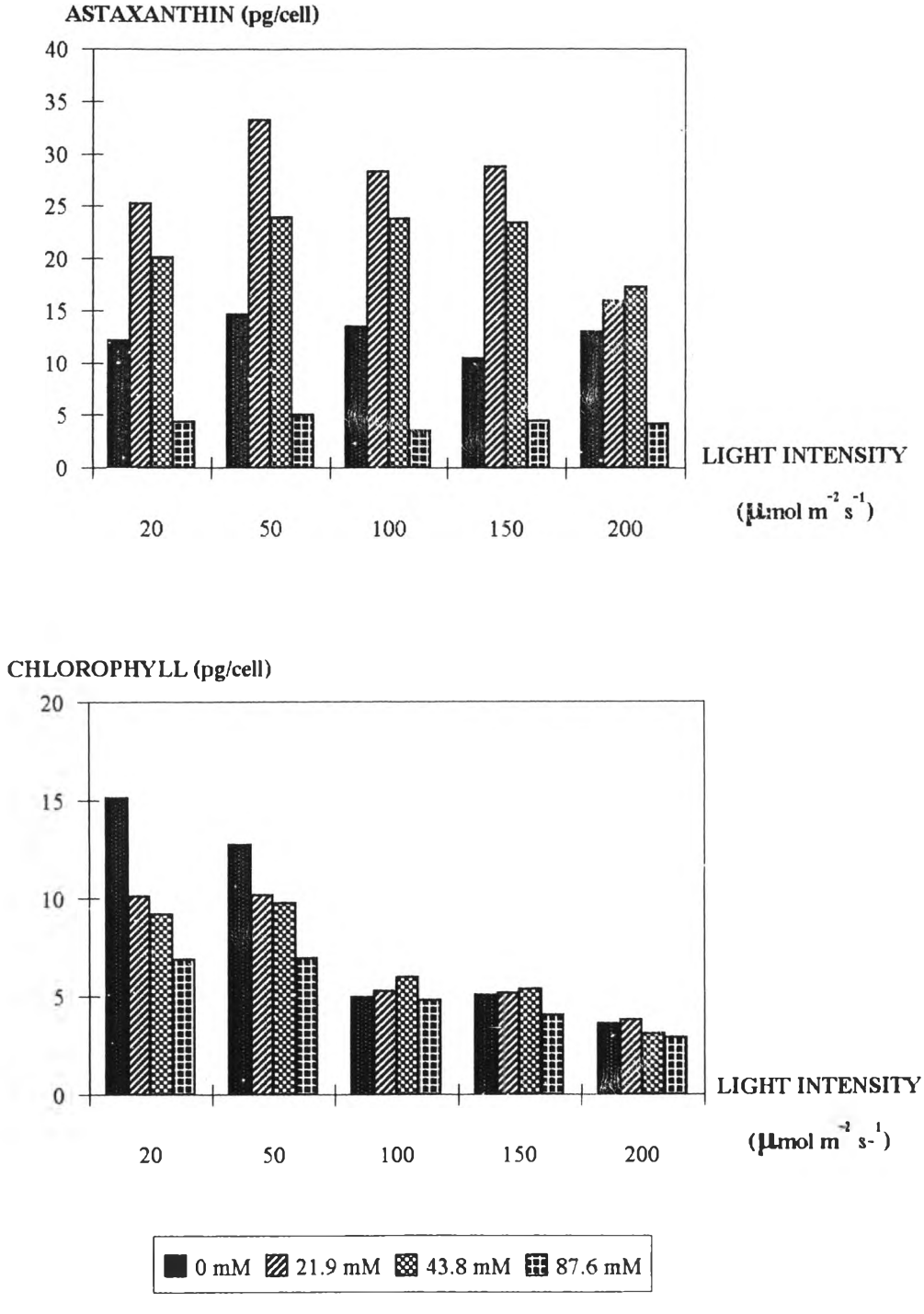


Figure 25 Effect of sodium acetate concentration on the contents of astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation

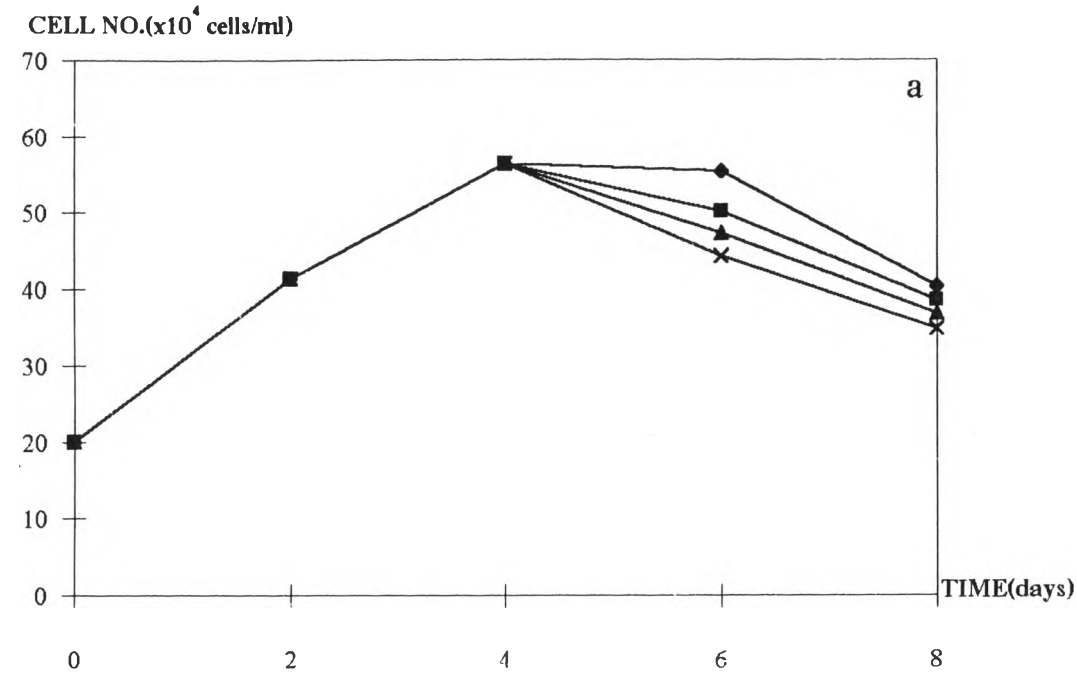
3.4 Effect of temperature.

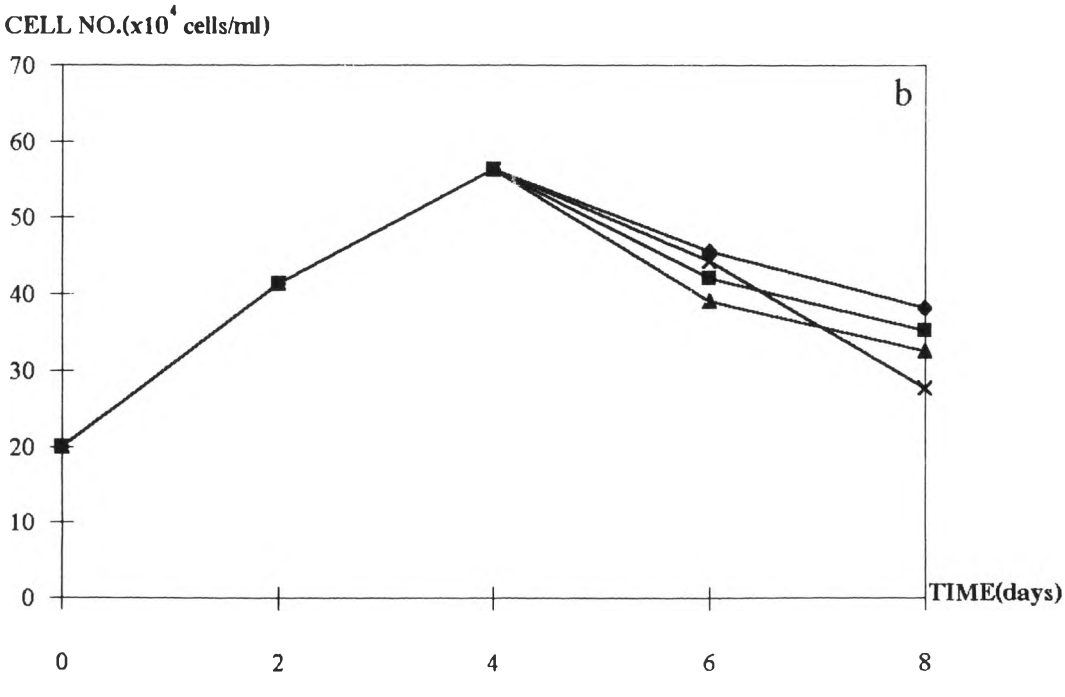
3.4.1 Effect of temperature and light intensity, supplemented with sodium chloride

The 4 - day of *H. pluvialis* NIES 144 on The Basal Medium was added with 0.2 % w/v NaCl and then cultivated at 22, 25, 30, and 35^{O}C under 50, 100, and 140 μ mol $m^{-2}s^{-1}$continuous illumination. The cell number was gradually decreased as shown in Fig. 26.

For the astaxanthin content, it was gradually decreased when the cultures were cultivated at high temperature and high light intensity. There were no differences in astaxanthin content when cultured on similar condition at temperature 22 and 25^{O}C but slight decrease occurred when cultivated higher than 30^{O}C. Furthermore, chlorophyll contents were decreased when the cells were exposed to high temperature and high light intensity (Fig. 27).

The linear models procedure and Duncan's multiple range test showed that, light intensity did not affect astaxanthin content whereas temperature did. Optimum temperature was 22 ^{O}C as shown in appendix 8.


CELL NO.(x10⁴ cells/ml)
70
60
50
40
30
20
10
0
a
TIME(days)
0
2
4
6
8


CELL NO.(x10⁴ cells/ml)
70
60
50
40
30
20
10
0
b
TIME(days)
0
2
4
6
8

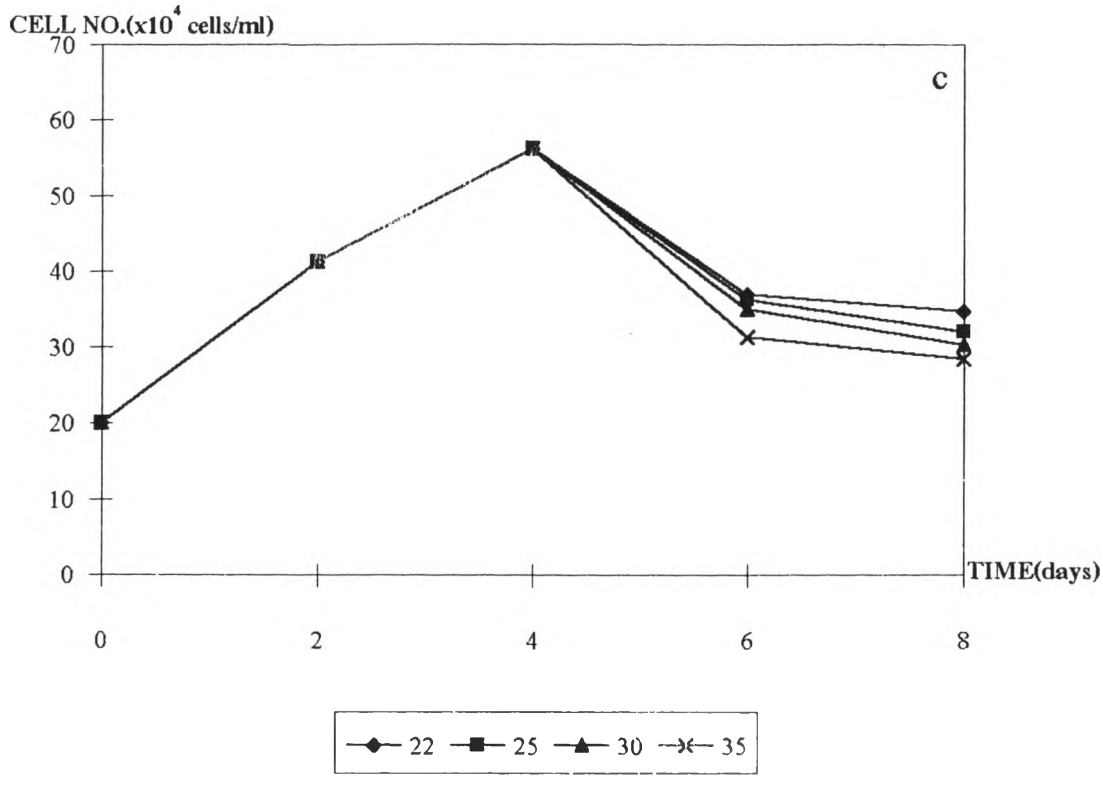


Figure 26 Growth of *H. pluvialis* in The Basal Medium on 0.2 % (w/v) NaCl supplemented culture at various temperatures under 50(a), 100(b), and 140(c) μ mol $m^{-2}s^{-1}$

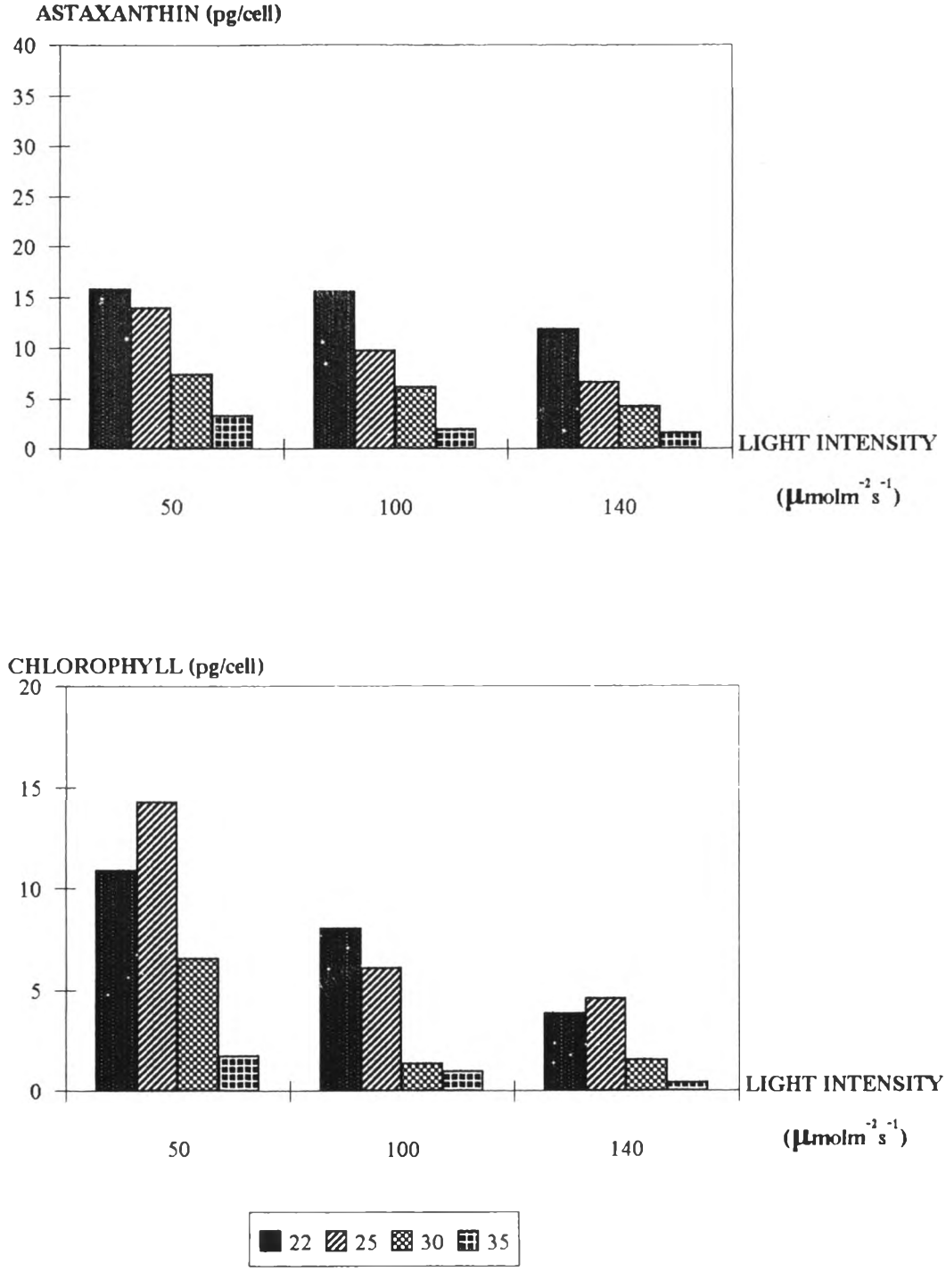


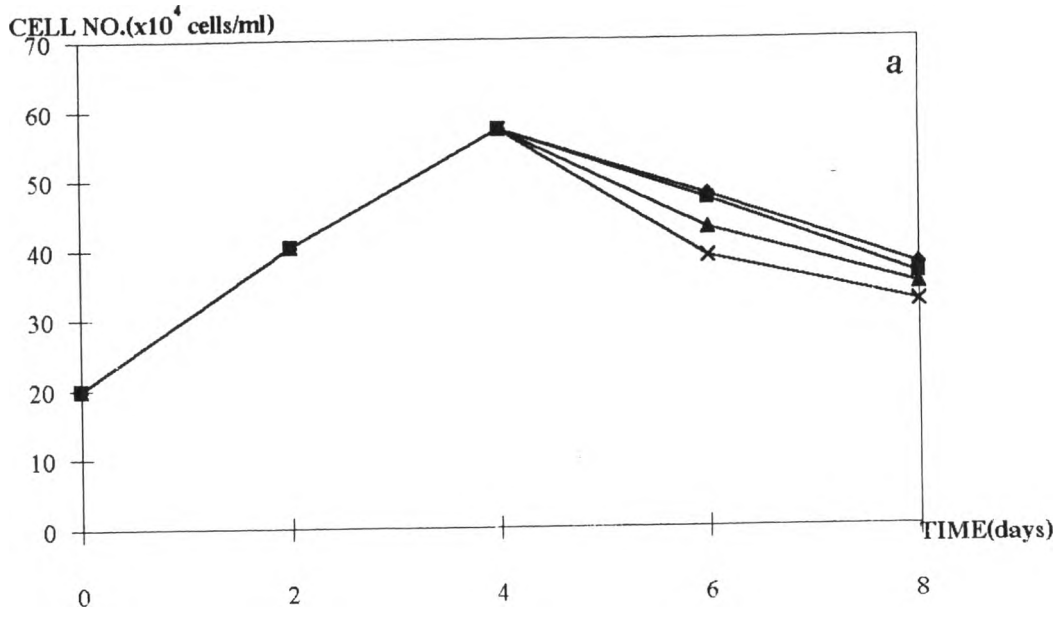
Figure 27 Effect of temperature on 0.2 % (w/v) NaCl supplemented culture on astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation

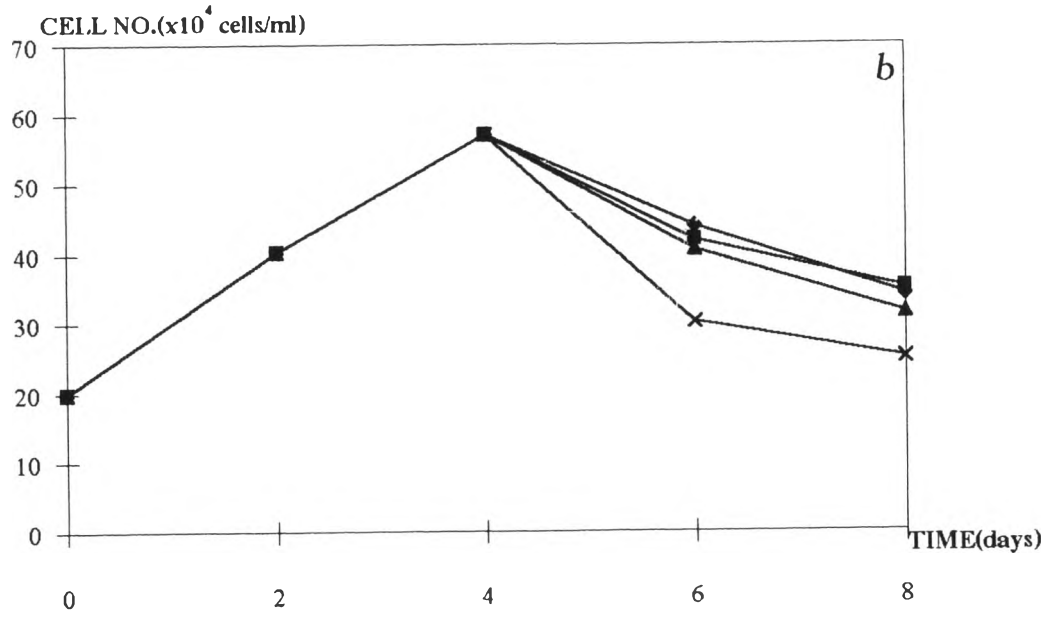
3.4.2 Effect of temperature and light intensity, supplemented with sodium acetate

The 4 - day culture of *H. pluvialis* NIES 144 on The Basal Medium was supplemented with 21.9 mM CH_3COONa and then was cultivated at 22, 25, 30, and 35^{O}C under 50, 100, and 400 μ mol $m^{-2}s^{-1}$continuous illumination. The cell number was decreased as shown in Fig. 28

After 8 days of cultivation, astaxanthin from the cultures cultivated at 22^{O}C under 50, 100, and 140 μ mol $m^{-2}s^{-1}$reached 30 pg./cell whereas those cultivated at 30 and 35^{O}C were decreased. Furthermore, the chlorophyll contents were slightly different (Fig. 29).

The linear models procedure and Duncan's multiple range test showed that, light intensity did not affect astaxanthin content whereas temperature did. Optimum temperature was 22 ^{O}C as shown in appendix 9.


CELL NO.(x10⁴ cells/ml)
70
60
50
40
30
20
10
0
a
TIME(days)
0
2
4
6
8


CELL NO.(x10⁴ cells/ml)
70
60
50
40
30
20
10
0
b
TIME(days)
0
2
4
6
8

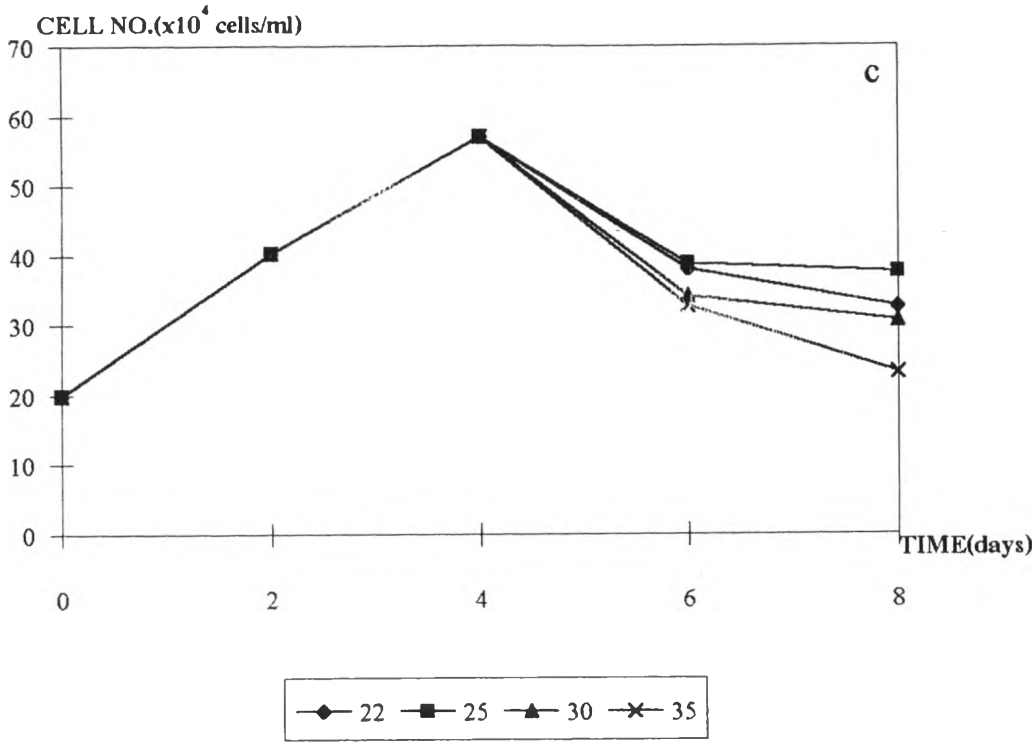


Figure 28 Growth of *H. pluvialis* in The Basal Medium on 21.9 mM supplemented culture of sodium acetate at various temperatures under 50 (a), 100 (b), and 140 (c) μ mol $m^{-2}s^{-1}$

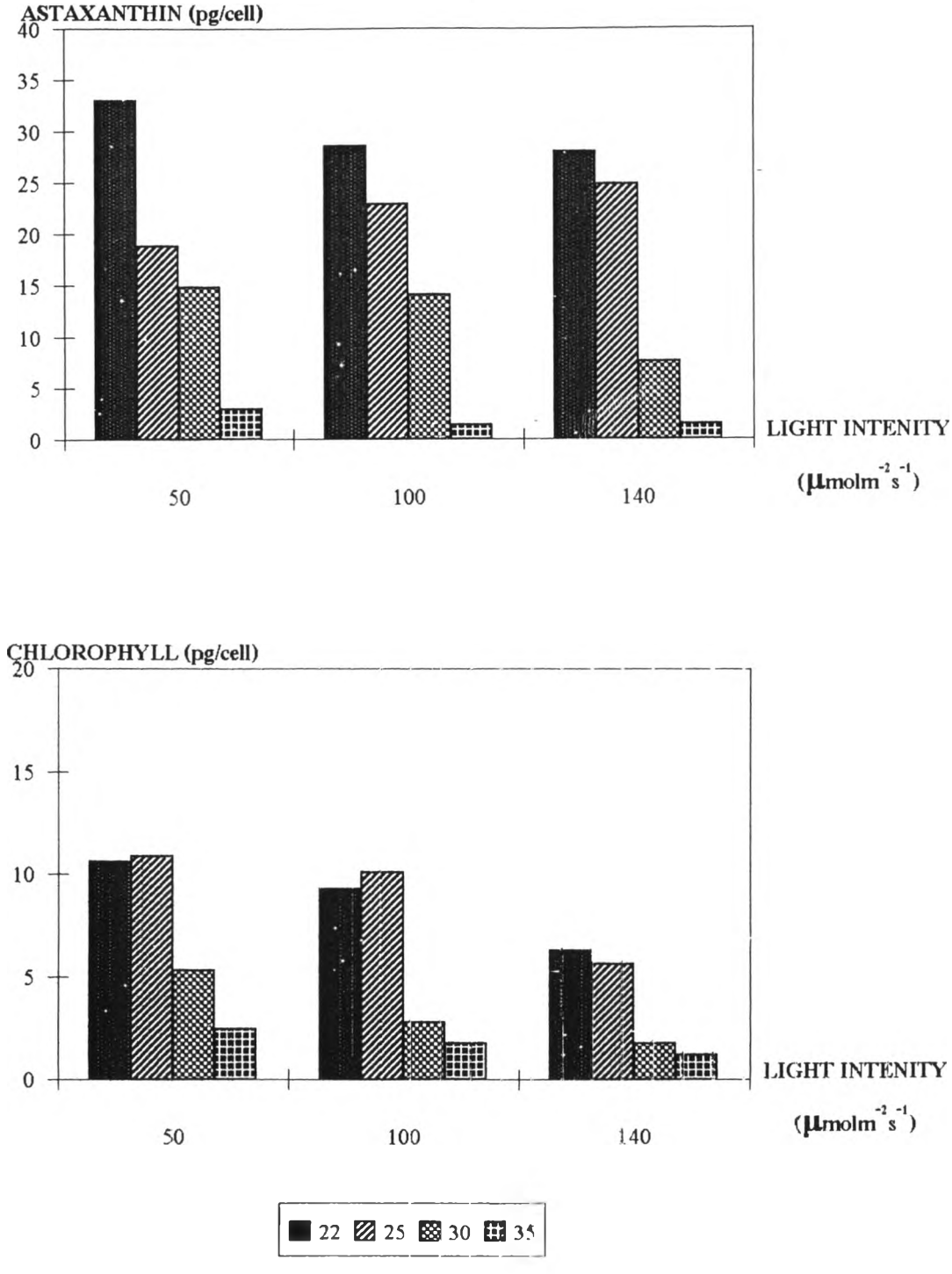


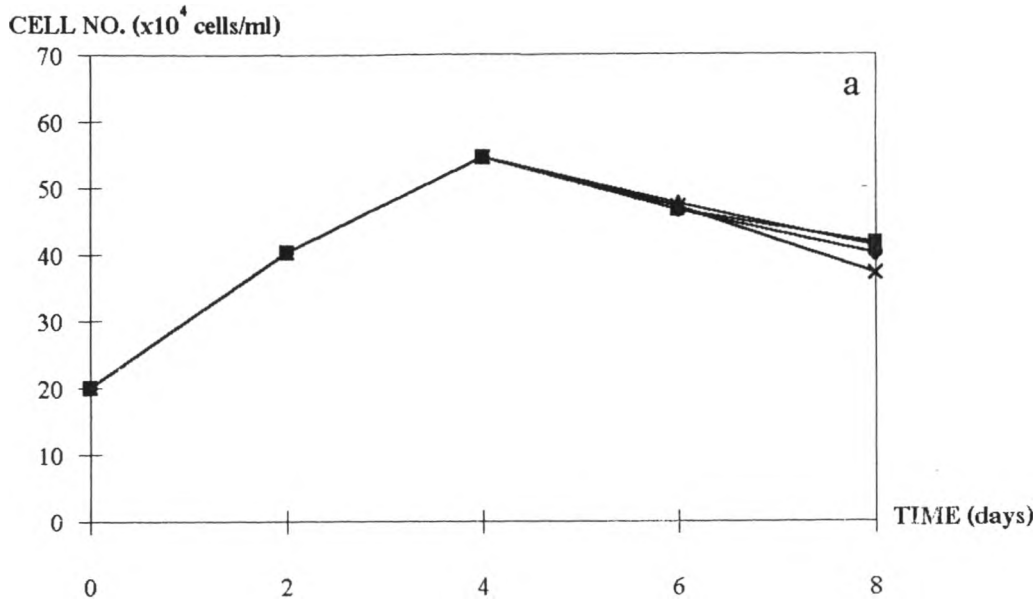
Figure 29 Effect of temperature on 21.9 mM sodium acetate supplemented culture on astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation

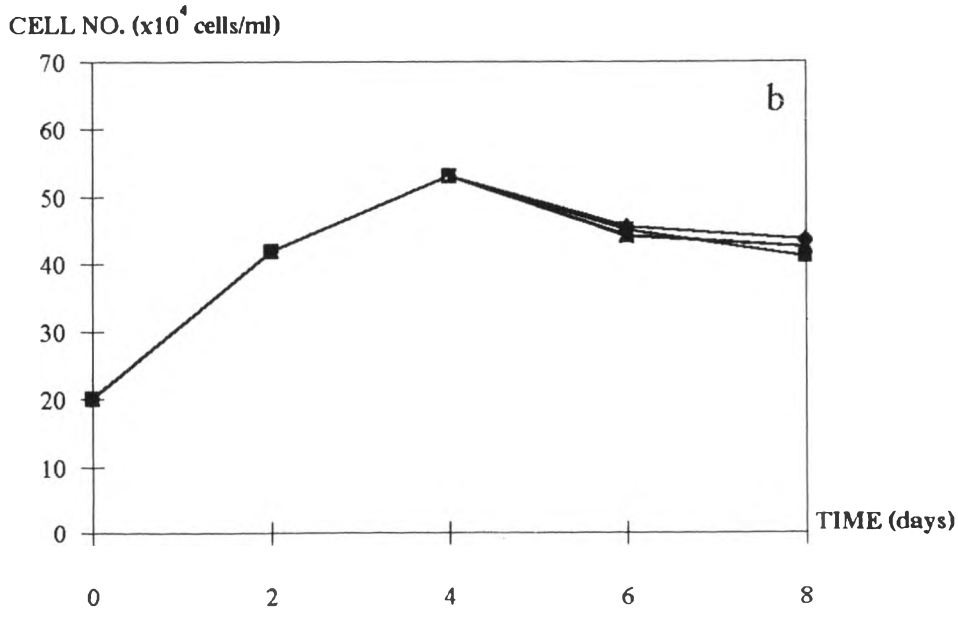
3.5 Effect of ferrous sulphate

The culture on The Basal Medium was initially supplemented with sodium acetate at 21.9 and 43.8 mM and cultivated under 20, 40 , and 60 μ mol $m^{-2}s^{-1}$ light illumination. Then 0, 225, 450, and 900 μ M of ferrous sulphate were further supplemented and incubated at 22°C under 140 μ mol $m^{-2}s^{-1}$. As shown in Fig.30 and 32, cell number was slightly decreased in both of the experiments.

For astaxanthin content, supplementation with ferrous sulphate did not affect astaxanthin accumulation. Astaxanthin contents reached 31 pg / cell. On the other hand, chlorophyll content was not different when initial light intensities were 20, 40, and 60 μ mol $m^{-2}s^{-1}$ (Fig.31 and 33).The wavelength were scanned from 300 to 800 nm as shown in fig 34.

The linear models procedure and Duncan's multiple range tested showed that light intensity did not affect astaxanthin content when supplemented with 21.9 and 43.8 mM sodium acetate. Ferrous sulphate affected astaxanthin content when supplemented with 21.9 mM sodium acetate but not with 43.8 mM sodium acetate. Addition of 225 μM ferrous sulphate gave high astaxanthin. In addition, initial light intensity from 20 - 60 μmol $m^{-2}s^{-1}$ did not affect astaxanthin content as shown in appendix 10.


CELL NO. (x10⁴ cells/ml)
70
60
50
40
30
20
10
0
a
TIME (days)
0
2
4
6
8


CELL NO. (x10⁴ cells/ml)
70
60
50
40
30
20
10
0
b
TIME (days)
0
2
4
6
8

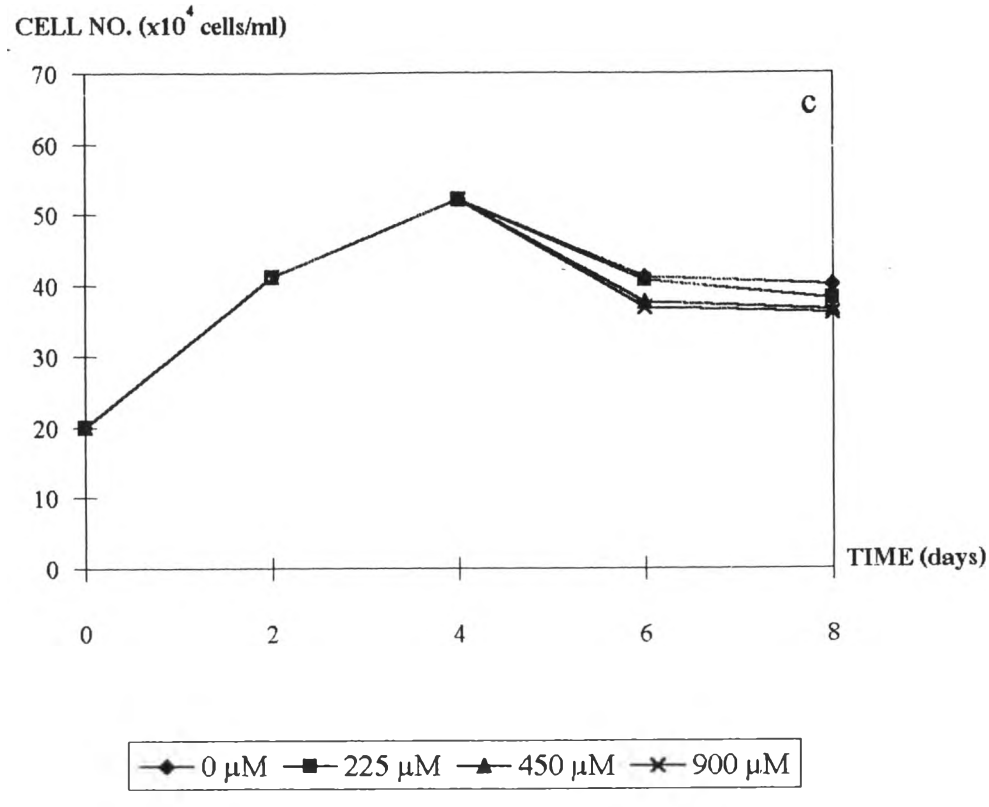


Figure 30 Growth of *H. pluvialis* in The Basal Medium initially cultivated at 20, 40, and 60 μ mol $m^{-2}s^{-1}$ and with 21.9 mM of sodium acetate at day 4 with various concentrations of ferrous sulphate under 140 μ mol $m^{-2}s^{-1}$ at 22°C

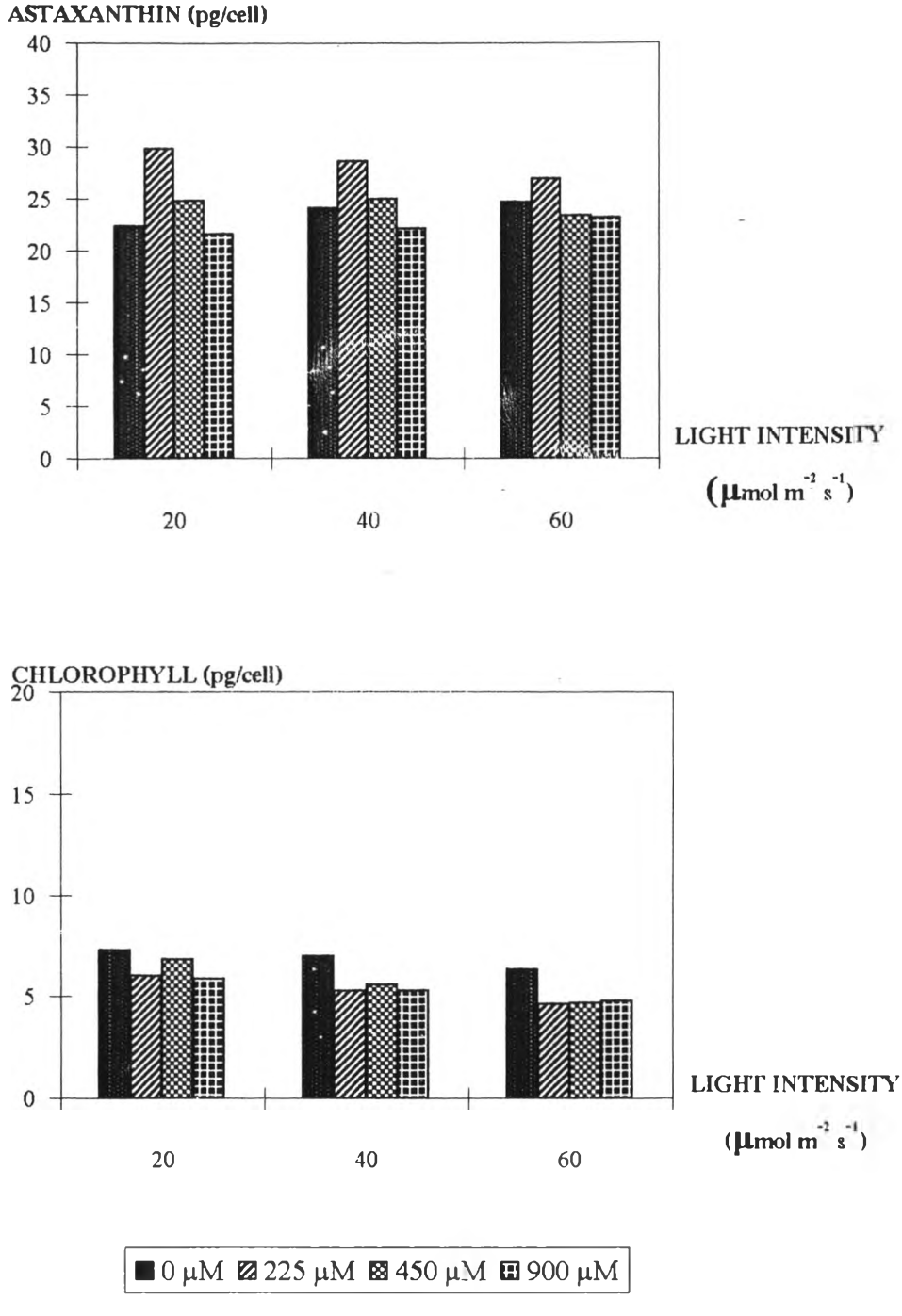
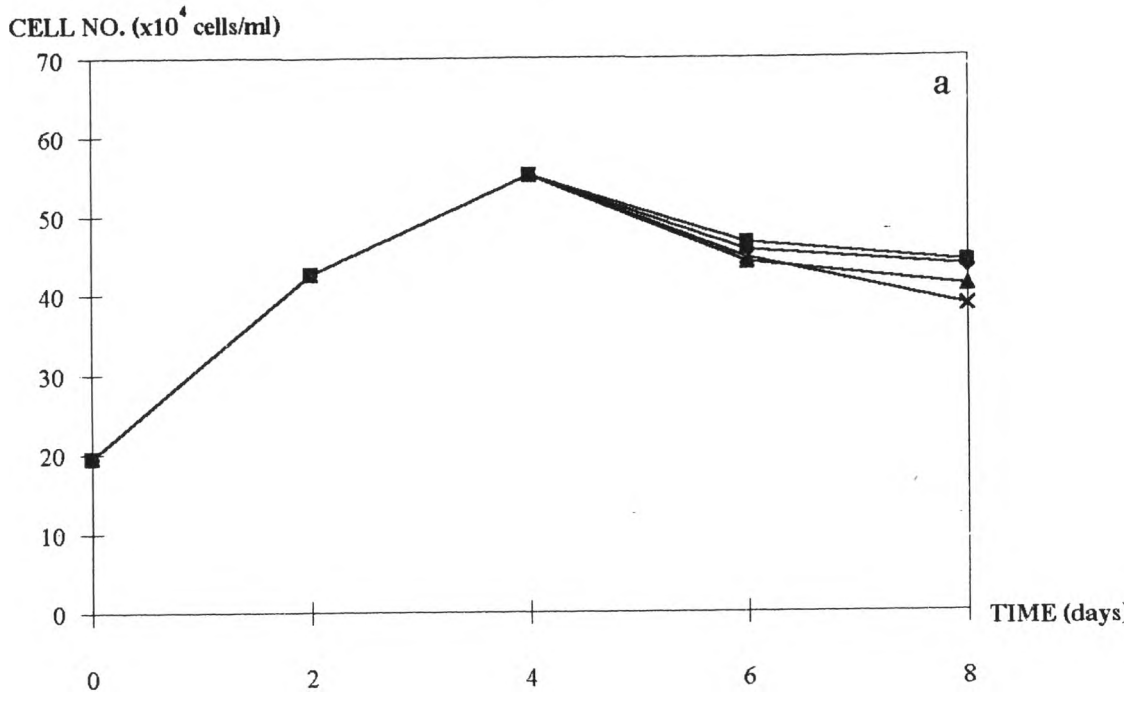
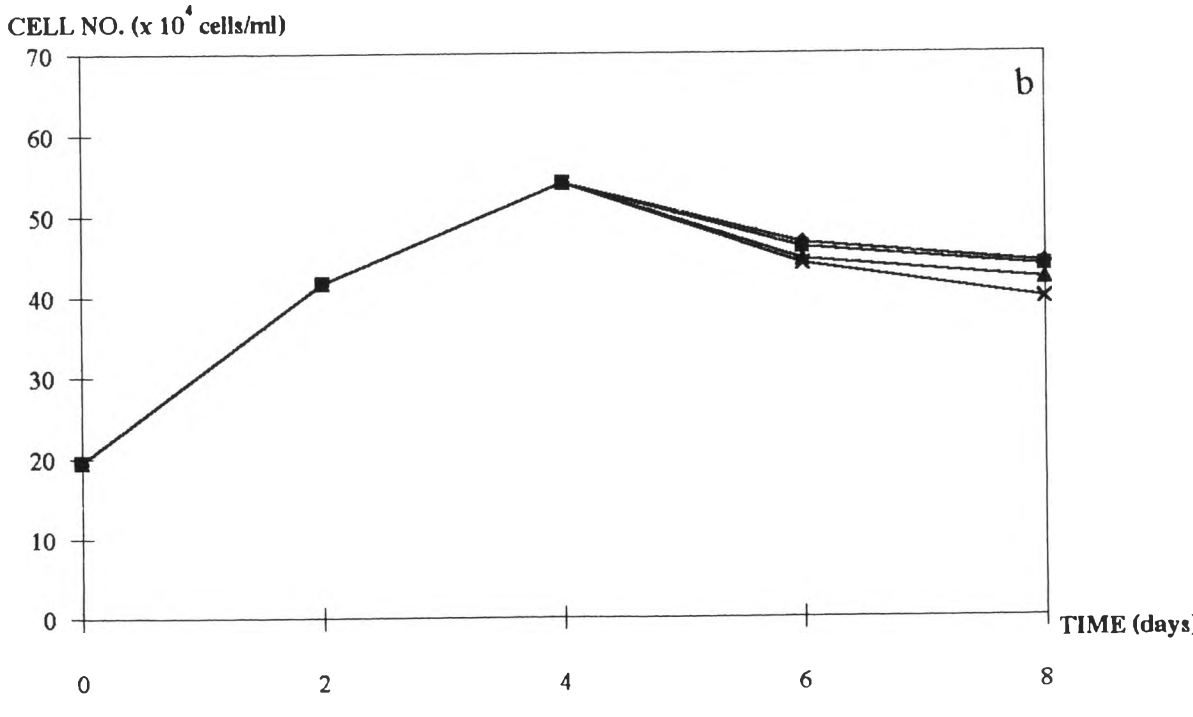


Figure 31 Effect of ferrous sulphate on 21.9 mM sodium acetate supplementation after cultivated under 20, 40, and 60 µ mol $m^{-2}s^{-1}$ at day 4 and following cultivated at 140 µ mol $m^{-2}s^{-1}$ for 4 days on astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation


CELL NO. (x10⁴ cells/ml)
70
60
50
40
30
20
10
0
a
TIME (days)
0
2
4
6
8


CELL NO. (x 10⁴ cells/ml)
70
60
50
40
30
20
10
0
b
TIME (days)
0
2
4
6
8

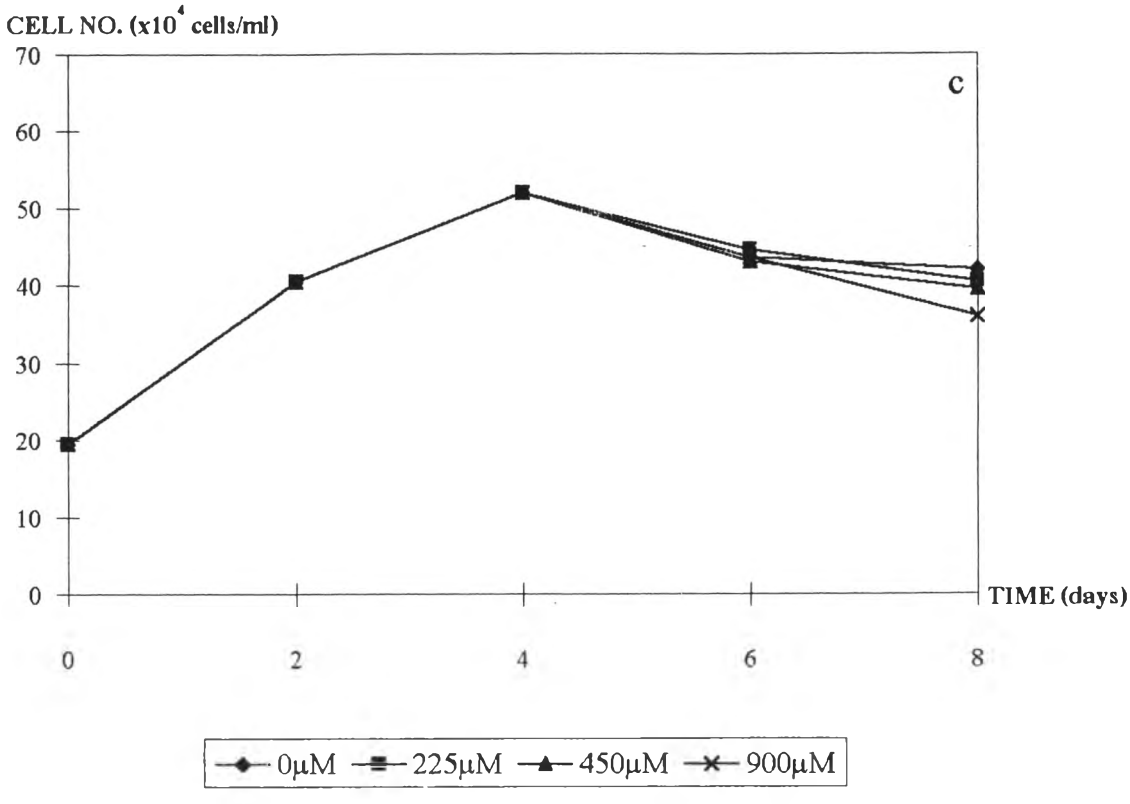


Figure 32 Growth of *H. pluvialis* in The Basal Medium initially cultivated at 20, 40, and 60 μ mol $m^{-2}s^{-1}$ and with 43.8mM of sodium acetate at day 4 with various concentrations of ferrous sulphate under 140 μ mol $m^{-2}s^{-1}$ at 22°C

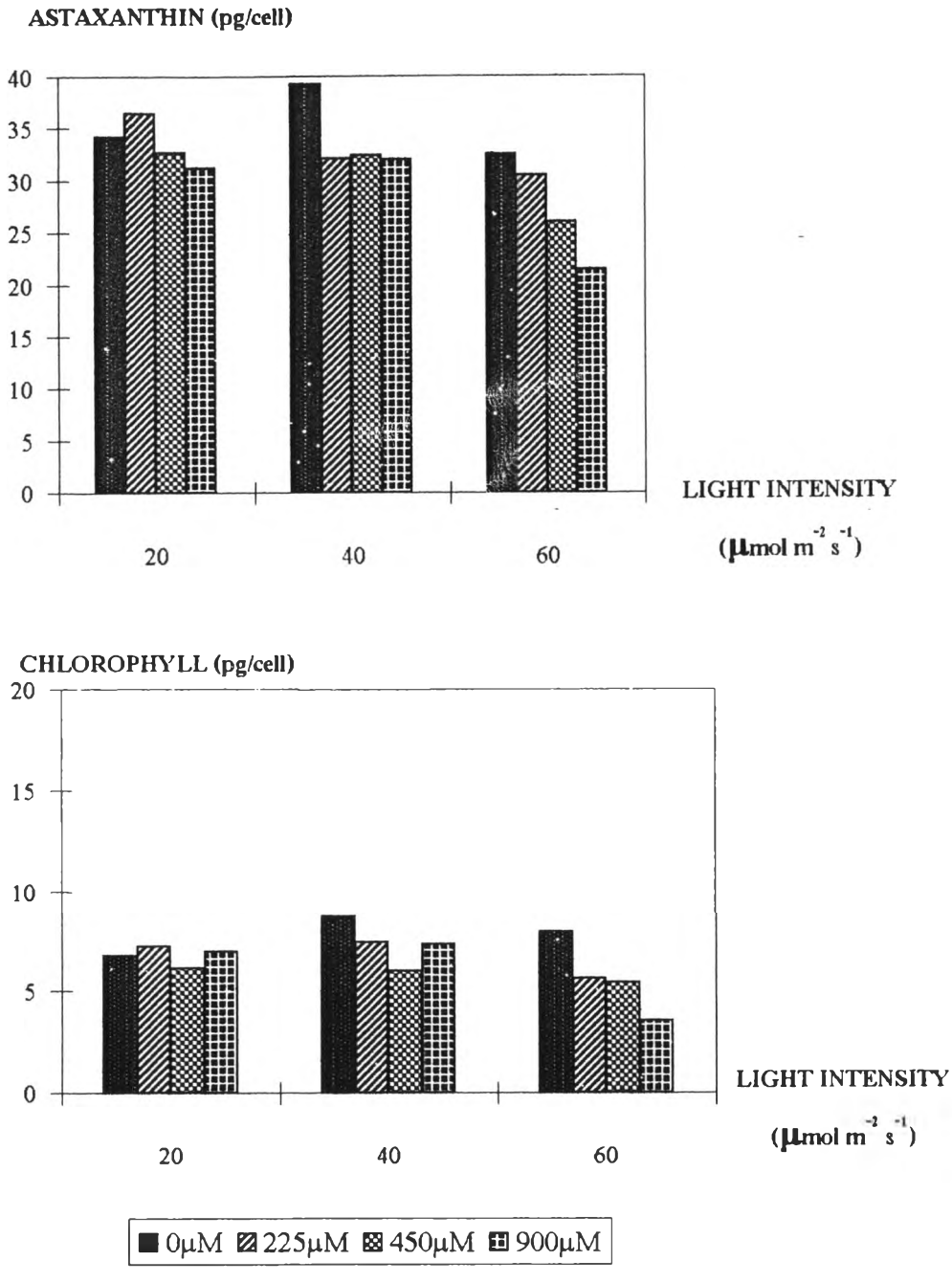


Figure 33 Effect of ferrous sulphate on 43.8 mM sodium acetate supplementation after cultivated under 20, 40, and 60 µ mol $m^{-2}s^{-1}$ at day 4 and following cultivated at 140µ mol $m^{-2}s^{-1}$ for 4 days on astaxanthin and chlorophyll by DMSO extraction after 8 - day cultivation

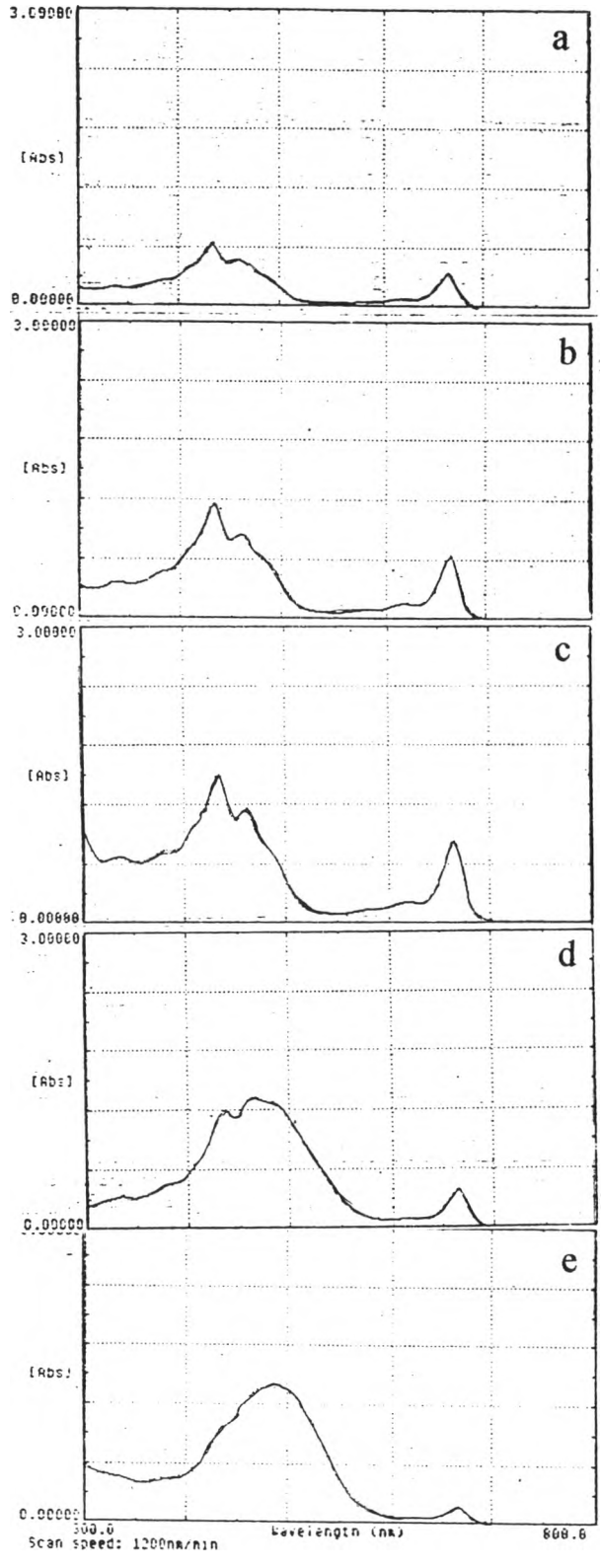


Figure 34 Absorbance of DMSO extraction of *H. pluvialis* on growth at day 0 (a), 2 (b), 4 (c) and after acetate supplementation at day 6 (d) and 8 (e)

4. Partial analysis of astaxanthin from *H. pluvialis* NIES 144 by HPLC method

0.01 g of lyophilized *H. pluvialis* NIES 144 cell was extracted with acetone. Chromatography was carried out by HPLC on a reverse phase C 18 column. Samples were injected into a 40 µl loop. The solvent system included acetonetrile : H_2O (9:1) solvent A, and 100 % ethyl acetate - solvent B. The pigments were separated by a step gradient between solvent A and B for 30 min as follows : 0 - 10 min, 0 - 60 % B ; 10 - 20 min, 60 - 100 % ; 20 - 30 min, 100 % B. Area under the peaks were integrated against peak of known quantities of standard astaxanthin.

As shown in Fig. 35, peaks were identified by typical retention time with standard astaxanthin at 12 min. The sharp peak of astaxanthin was observed. We calculated the astaxanthin and its ester contents by integrating peak 12 to peak 16. The value of 0.70 % (w/w) and 1.27 % (w/w) was found for green cell and red cyst cell of *H. pluvialis* NIES 144 respectively.

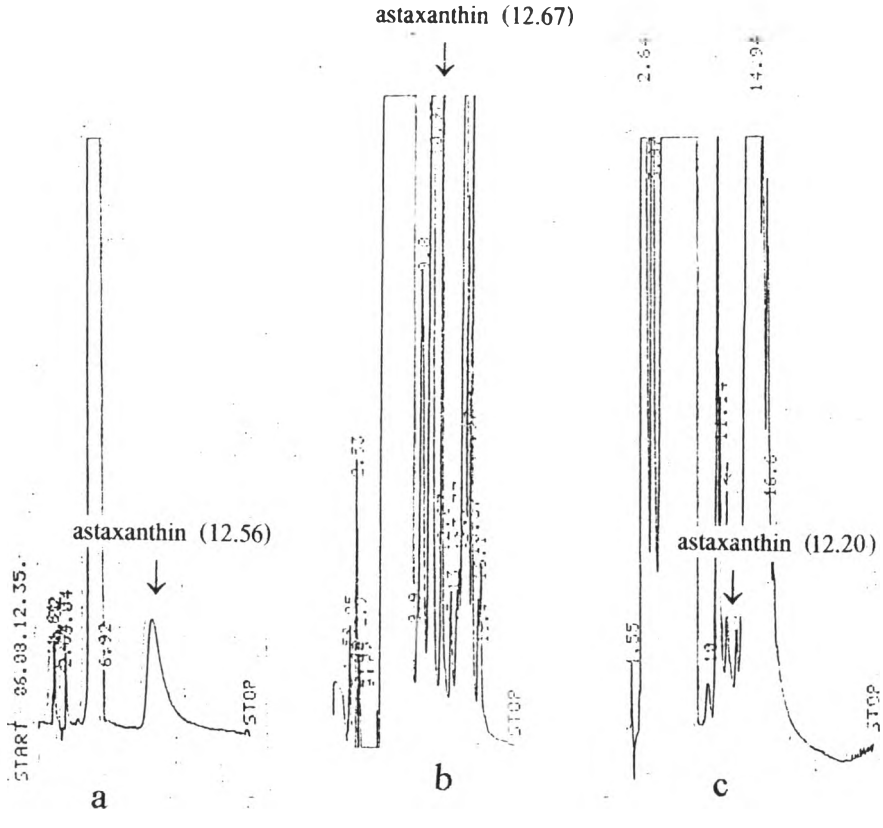


Figure 35 HPLC chromatogram of standard astaxanthin (a), astaxanthin and its esters in green vegetative (b) and red cyst cell (c) by acetone extraction using reverse phase C18 column with acetonitrile : H_2O and ethylacetate solvent system

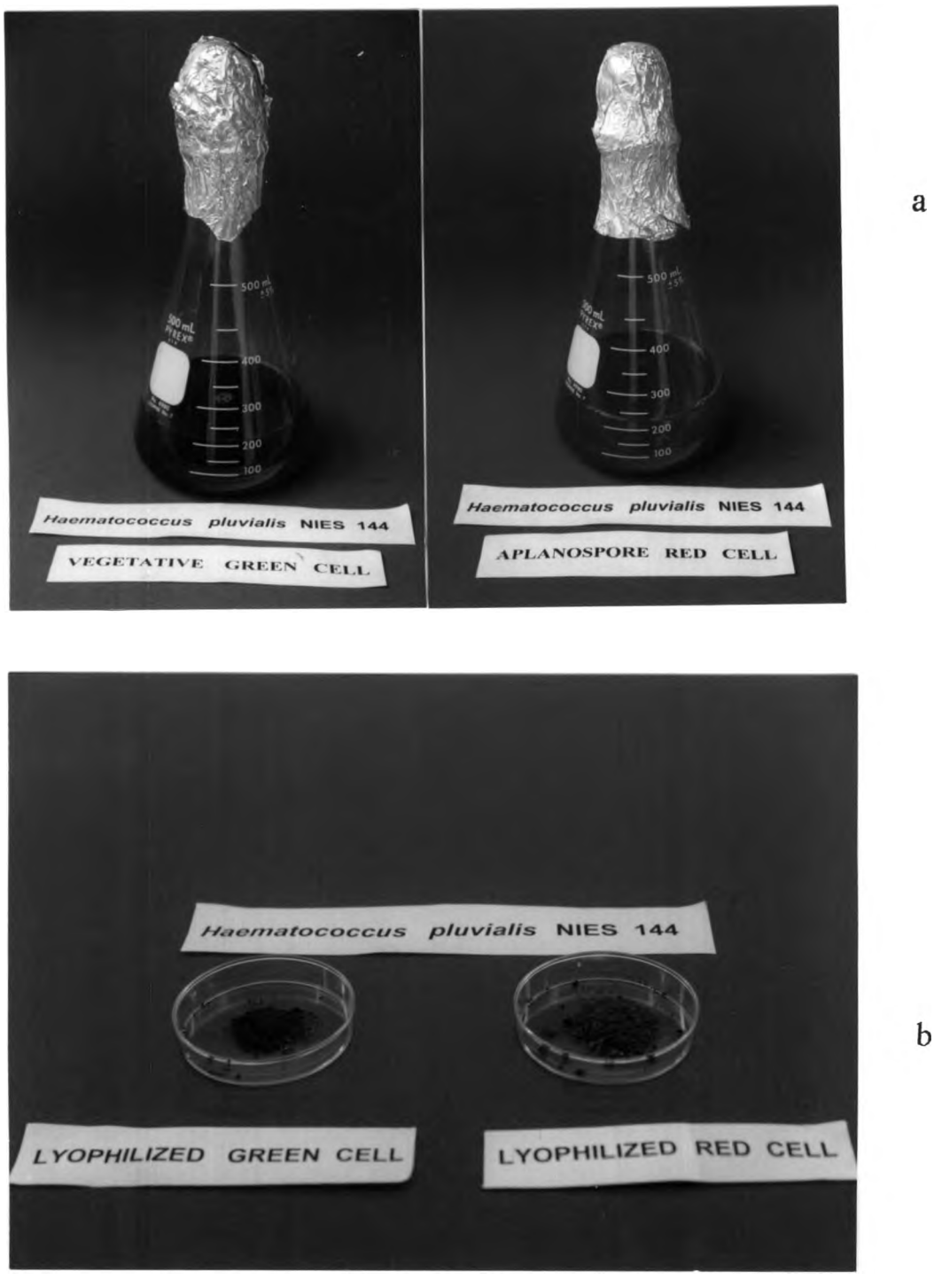


Figure 36 Cultures (a) and lyophilized products (b) of vegetative green cell and red cyst cell of *H. pluvialis* NIES 144