CHAPTER IV

DISCUSSION

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

H. pluvialis NIES 144 was grown in three types of media, namely The Basal Medium, medium for *H. lacustris* ATCC 30453 and The Basal Modified Medium which contained sodium acetate as the carbon source. From the result in Fig. 7, The Basal Medium was the best medium for growth. It used yeast extract as the nitrogen source. Kakizono et al, 1992 (a) reported that yeast extract was used as a nitrogen and vitamin source in the medium. In addition, Pringsheim (1996) reported that vitamins had the influenced on growth of *H. pluvialis*.

With regard to nutritional type. *H. pluvialis* can be autotrophic and heterotrophic. If it was autotrophic, it used inorganic compounds as a source of carbon and use light as a source of energy whereas it obtained carbon and energy form the organic carbon such as acetate for optimal growth. Kobayashi et al, 1992 (b) reported that *H. pluvialis* was able to grow heterotrophically as well as mixotrophically and the specific growth rate of the mixotrophic condition corresponded well to the sum of the specific growth rate of heterophic and autotrophic conditions. From the result in Fig.8 and 9, *H. pluvialis* could not use sodium carbonate as a carbon source in mixotrophic conditions so the specific growth rate was very low. Vimazal, 1995 reported that, acetate can be utilized for

heterotrophic growth in darkness by a wide range of algae. Acetate is generally oxidized metabolically through intermediates of the TCA cycle.

As a main constituent of protein and genetic material, nitrogen is an element of crucial importance for life. The normal requirement of nitrogen in culture of Chlorophyceae was found to be 6.5 - 8.3 % of the ash - free dry mass. Nitrogen is available to algae in three basic forms : free nitrogen gas, and as combined inorganic or organic compounds. In natural habitat the main sources of nitrogen are nitrate and ammonium salts, but in highly polluted waters the organic nitrogen compounds may become important and there may be a relationship between some of the products of animal excretion ; ammonia, urea, uric acid and amino acids. From the result in fig.11 *H. pluvialis* could grow by using urea, sodium nitrate and yeast extract as the nitrogen source. However, when the concentration of urea was too high, the cell would die because of the toxicity of NH_3 released as shown in the reaction (Syrett, 1981).

$$CO(NH_2)_2 + ATP + H_2O \rightarrow CO_2 + 2NH_3 + ADP + Pi$$

Effect of environmental factors on growth of *H. pluvialis* NIES 144 can grow well in the visible range of light between 20 to 60 μ mol $m^{-2}s^{-1}$ measured by lux meter which designed to measure illuminance in lux. But the measurements was appropriate for most photobiological events which depend on the number of quanta absorbed rather than on their energy content. Such quantum irradiance measurements was reported in SI units as micromole $meter^{-2}$ $second^{-1}$. Irradiance may also be measured and express energetically in units of watts $meter^{-2}$. For monochromatic light, irradiance and photon flux density may be readily interconverted as :

1 μ mol $m^{-2}s^{-1}$ = 1 μ E $m^{-2}s^{-1}$ = 6.02 x 10^{17} quanta $m^{-2}s^{-1}$ = (119.7 / λ) Wm^{-2} =50 lux (Vymzal , 1995)

According to the result from Fig. 13 and 14, *H. pluvialis* NIES 144 grown in the Basal Medium containing 0.8 - 0.2 g of sodium acetate and 1.0 - 3.0 g of yeast extract showed similar specific growth rate. Therefore in the later experiments the original Basal Medium was used (1.2 g of sodium acetate and 2.0 g of yeast extract). For the initial cell number from 10 to 200 x 10^4 cells per ml, the specific growth rates were not different and eventually yielded the same numbers of cells at day 8 (Fig.15 and 16). The cultures grown at lower initial cell numbers took 8 days to reach the stationary phase, whereas only 4 days required when cultured with 20 x 10^4 cells per ml.

A dense algal cell culture would be required to enhance the astaxanthin productivity. The two media designed in the present study, The Basal Medium was appropriate for the vegetative growth, and the modified medium was for the carotenoid synthesis. Thus we attempted to carry out a two - stage batch cultivation : for the first - stage, to attain a high cell concentration in the Basal Medium and for the second - stage, to enhance carotenoid formation with high cell concentration by the injection of another supplementation into the late of the first stage culture (Kobayashi et al , 1991).

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

In response to light intensity, *H. pluvialis* NIES 144 cultures at 4 - day were exposed to 20 to 200 μ mol $m^{-2}s^{-1}$. From the Fig. 17 there was a decrease in cell number and astaxanthin content is response to an increase of light intensity(fig.18,19). Richardson et al , 1983 reported that different microalgal classes have significantly different light requirements and there is some variability within each class. In general, growth appears to be light - saturated at lower irradiance and high light intensity often results in release of large proportions of photosynthate by algae, possibly due to membrane damage. Our results indicated that light intensity between 10 to 20 and 100 μ mol $m^{-2}s^{-1}$ were appropriated to produce high growth and high astaxanthin content.

Salinity is another important environmental parameter for physiological and biochemical changes in microalgae. From Fig. 20 and 21, the addition of 0.2 - 1.6 % (w/v) NaCl to the 4 - days cultures under low to high light intensity resulted in complete cessation of growth, Growth arrest is accompanied by a massive accumulation of astaxanthin. Addition of 0.2 % w/v NaCl gave higher content of astaxanthin than concentrations. Furthermore, the higher concentration of NaCl added in the media, the less of cell numbers were obtained. However, our results were in contrast with Boussiba and Vonshak (1991) in which they reported that an addition of 0.8 % NaCl to the growth medium caused the massive accumulation of astaxanthin reaching 47.2 pg / cell whereas in our experiment, astaxanthin was 15 pg / cell under 50 and 100 μ mol $m^{-2}s^{-1}$ after 4 days whereas we

used the Basal medium which contained the concentration of NaCl 0.3 % w/w of yeast extract. So, algae living in an environment with fluctuating salinities may have to adjust their physiological and biochemical process rapidly to be able to maintain their growth as constant as possible.

As sodium acetate and sodium nitrate were the main carbon and nitrogen source in the medium and under the high C/N balance, the astaxanthin content was increased with the cultivation time after the supplementation whereas under the low C/N balance, the astaxanthin content stayed at nearby the same level as with no addition. In the case of chlorophyll, under the high C/N balance, the chlorophyll content was drastically decreased. (Kakizono et al , 1992 ; b) As shown in Fig.23, astaxanthin content was higher when supplemented with the C/N content=43.8:0 than the C/N content = 0:0,43.8:43.8 and 43.8:21.9 under all light illuminations, and the optimal sodium acetate content which was supplemented for enhanced astaxanthin accumulation was 21.9 mM under continuously light intensity from 50 to 150 μ mol $m^{-2}s^{-1}$ (Fig.25)

Kobayashi et al, 1991 reported the effect of acetate supplementation on the astaxanthin accumulation of *H. pluvialis*. They concluded that the carotenoid synthesis rate was much enhanced in the modified medium. While the carotenoid biosynthesis was actively operated, the chlorophyll synthesis was apparently inhibited in the modified medium , resulting in the unvarying appearance of the red cells . The cell number did not increase in the modified medium compared to The Basal medium. Furthermore, approximately 10% of the population grew up in size which was two or three times larger than that of the basal medium. The rest of the population completely turned into colorless cells indicating drastic physiological change. In addition, Coleman, Rosen, and Schwatzbach,

(1988) reported that in unicellular green algae nitrogen starvation altered the partitioning of photosynthetically fixed carbon, resulting in an increased accumulation of carbohydrate and lipids which increased too 130 - 320 % of values observed for exponential phase cultures.

Temperature is one of the major factors controlling the rate of photosynthesis in all plants. It is probably the most widely measured environmental variable that affects algal growth. In general, growth rate increases exponentially with temperature up to an optimum temperature, than declines rapidly as temperature exceeds this optimum. There may be significantly different temperature optima for different species of the same genus. The temperature is an important environmental factor determining an algal growth and the geographical distribution of certain algae. The optimal temperature for growth in many marine and freshwater algae lie in the range 18-25°C . Resting stages of many algae posses a greater tolerance to extreme temperatures than do the vegetative stage (Spalding, 1989)

Fig. 26 and 28 showed the content of astaxanthin in *H. pluvialis* NIES 144 when added with 0.2% (w/v) of NaCl, supplemented with 21.9 mM sodium at day 4 and changed the temperature for culturing from 22°C to 25, 30, and 35°C under 50, 100, and 140 μ mol m^{-2} s^{-1} .

The results indicated that, astaxanthin content was not increased when temperature was changed from optimal temperature to elevated temperature after 4 day of supplementation. In contrast, the alga carried out vegetative cell growth at the elevated temperature over 30°C throughout the cultivation period for 8 days. Normal vegetative growth should be interrupted at a growth impermissible temperature and this would stimulate formation of active oxygen species, which eventually contributed to hyper-accumulation of astaxanthin (Tjahjono et al, 1994).

Sources of iron in water are municipal wastewater, sewage sludge discharge, continental and volcanic dust flux, base metal mining and natural weathering. The primary oxidation states of iron in water are Fe^{2+} (ferrous) and Fe^{3+} (ferric). In most surface water, Fe^{3+} predominates and when combined with its salts, it is practically insoluble. Forms of dissolved and particulate iron in water depend on pH, redox potential and the presence of complexion substances of both inorganic and organic origin (Vymazal, 1995).

Iron has been long known to be essential to algae. It is a key element in metabolism, being a constituent of numerous iron-containing enzyme, e.g. peroxidase, nitrate reductase, nitrogenase and catalase. It was suggested that the phytoplankton take up iron mainly as the ferrous ions (Fe^{2+}), but ferric ions (Fe^{3+}) may be taken up also (Ester, 1964).

In our experiments, the astaxanthin content of *H. pluvialis* NIES 144 was drastically enhanced in Fe^{2+}-rich modified medium, which led to purse the role of Fe^{2+} in algal carotenoid formation. In Kobayashi et al, (1993) study, the astaxanthin formation of *H. pluvialis* was drastically enhanced in ferrous ions (Fe^{2+}) rich modified medium. From several lines of evidence, it was shown that ferrous ions (Fe^{2+}) would possibly function as an HO generator via an iron-catalyzed Fenton reaction and that HO or other active oxygen species play essential roles in the enhanced carotenoid formation in the algal cyst cells.

Our experimental results showed that supplementation with sodium acetate and ferrous ion (from ferrous sulphate) enhanced the astaxanthin accumulation. There was no difference in content of astaxanthin when supplemented with various concentrations of ferrous at 4

day cultured under initial light intensity from 20 to 60 μ mol $m^{-2}s^{-1}$.(Fig.31 and 33) Furthermore, it was very likely that the morphological changes could be attributed to the high concentration of metal in the modified medium. In addition, only slight differences were detected in the chemical forms of astaxanthin (Kobayashi et al, 1991).From our results , we noticed that the stationary growth phase should be the suitable phase to enhance carotenoid formation because of the cell concentrations and astaxanthin contents were higher than those exposed to the logarithmic growth phase.

The Extraction of Astaxanthin and Its Analysis

Acetone and dimethysulfoxide were selected as the solvent for astaxanthin extraction. 90% acetone is the most commonly used solvent for the extraction of carotenoid containing tissue. From the experiment it took an hour for 90% acetone extraction and the method of homogenization was also used whereas dimethylsulfoxide extraction took only 10 min for complete extraction as evidenced by the white cell debris appearance. However, astaxanthin content from both extractions were not different whereas chlorophyll content by these 2 methods of these extraction was slightly different. Furthermore, appropriate standardization is essential for quantitative carotenoid measurement. Sample preparation usually involves multiple steps during which losses and errors can be introduced. The incorporation of a volume correction standard (frequency referred to as an internal standard, is highly recommended because it accounts for volume losses due to incomplete discovery, evaporation, and injection variability. Concentrations should be value assigned to calibration standard spectrophotometrically and then corrected for HPLC purity. (Craft, 1992)

The partial analysis of astaxanthin was carried out by HPLC on a reversed phase C 18 column. The solvent system included acetonitrile: H_2O (9:1) and 100% ethylacetate. The pigments were separated by a step gradient for 30 min. From Fig.35 , retention time of standard astaxanthin of 12.56 min with a broad peak was observed whereas retention time of astaxanthin extracted from red cyst cells and green cells were 12.2 and 12.02 respectively with the sharp peaks. In our experiment, we calculated the sum of peak 12 to peak 16 which were assumed to be astaxanthin and its esters and concluded that astaxanthin content in the red cyst cell and green vegetative cells were 1.27 and 0.7% of algal dry weight. The result indicated that astaxanthin contained in the red cyst cell or the green cell was not in the free form as synthetic astaxanthin. In addition Grung et at (1992) reported that most of the astaxanthin accumulated in the form of mono-ester (50%) and di-ester (45%) and very little was free form

High performance liquid chromatography (HPLC) is the method for the carotenoids. Carotenoid separation have been performed by both normal phase HPLC (NPLC) and reversed phase HPLC (RPLC). The most common column packing used for carotenoids separation is octyldesylsaline (ODS or C18). Reason for the popularity of C18 packing include compatibility with most solvents , usefulness for the entire polarity range of carotenoids ,and wide commercial availability. The advantages of gradient elution include a wider range of analysis , improved sensitivity , improved selectivity and elution of the strongly retained compounds whereas the disadvantages include increased complexity , requirements for additional equipment and column reequilibration after each analysis. However, no single HPLC separation is capable of separating all natural

elution include a wider range of analysis , improved sensitivity , improved selectivity and elution of the strongly retained compounds whereas the disadvantages include increased complexity , requirements for additional equipment and column reequilibration after each analysis. However, no single HPLC separation is capable of separating all natural carotenoids. Therefore, more one technique may be necessary to accomplish the degree of separation required.(Craft ,1992)

Sommer et al, 1992 concluded that extracts from the alga were not quantified using HPLC methods because of difficulties in analyzing esterified carotenoids which *H. pluvialis* contained in abundance. Saponification or hydrolysis methods used to convert carotenoids to their free form, before HPLC analysis.

In addition, free astaxanthin and it esters are very sensitive to oxidation, forming the biologically unimportant pigment, astacane. Dry cyst cells are mixed with liquid nitrogen and added the suitable antioxidant powder such as butylated hydroxytoluene , ethoxyquin and cryogenically ground at -170°C to preventing astaxanthin oxidation.