การเพาะเลี้ยงสาหร่าย *Haematococcus pluvialis* ในถังปฏิกรณ์ชีวภาพแบบอากาศยก

นางสาวกมลพรรณ แก้วปิ่นทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
สาขาวิชาวิศวกรรมเคมี ภาควิชาวิศวกรรมเคมี
คณะวิศวกรรมศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2547

ISBN 974-53-1162-6

CULTIVATION OF *Haematococcus pluvialis* IN AIRLIFT BIOREACTOR

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering in Chemical Engineering
Department of Chemical Engineering
Faculty of Engineering
Chulalongkorn University
Academic Year 2004
ISBN 974-53-1162-6

Thesis Title	CULTIVATION OF *Haematococcus pluvialis* IN AIRLIFT BIOREACTOR
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กมลพรรณ แก้วปิ่นทอง : การเพาะเลี้ยงสาหร่าย *Haematococcus pluvialis* ในถังปฏิกรณ์ชีวภาพแบบอากาศยก. (CULTIVATION OF *Haematococcus pluvialis* IN AIRLIFT BIOREACTOR) อ. ที่ปรึกษา : ผศ.ดร. อาทิวรรณ โชติพฤกษ์, อ.ที่ปรึกษาร่วม : รศ.ดร. ประเสริฐ ภวสันต์, 113 หน้า. ISBN 974-53-1162-6.

งานวิจัยนี้ได้ทำการศึกษาการเลี้ยงสาหร่าย *Haematococcus pluvialis* NIES-144 เพื่อผลิต astaxanthinจากการทดลองพบว่าสาหร่าย *H. pluvialis* เจริญเติบโตได้ดีในอาหารสูตร F1 (Fabregas et al., 1998) และพบว่าการเติมวิตามินบีลงไปในอาหารสูตร F1 จะทำให้สาหร่ายเจริญเติบโตได้ดียิ่งขึ้น การทดลองเลี้ยงสาหร่ายในถังปฏิกรณ์ชีวภาพพบว่าการเพาะเลี้ยงสาหร่าย *H. pluvialis* โดยใช้ถังปฏิกรณ์ชีวภาพแบบอากาศยกจะให้อัตราการเติบโตจำเพาะสูงกว่าเมื่อเลี้ยงในถังสัมผัสธรรมดาที่มีขนาดเท่ากัน และความเร็วของอากาศที่ให้กับระบบมีค่าเท่ากัน นอกจากนี้พบว่าการผสมก๊าซคาร์บอนไดออกไซด์ลงในอากาศจะทำให้สาหร่ายมีการเจริญเติบโตดีขึ้น จากการทดลองพบว่าการเลี้ยงสาหร่ายในสภาวะการดำเนินงานแบบกะโดยให้อากาศที่ผสมก๊าซคาร์บอนไดออกไซด์ 1 เปอร์เซ็นต์โดยปริมาตร ที่ความเร็ว 0.4 เซนติเมตรต่อวินาที และความเข้มแสง 20 ไมโครโมลโฟตอนต่อตารางเมตรต่อวินาที จะให้ความหนาแน่นเซลล์สูงสุด ส่วนการเพาะเลี้ยงสาหร่ายในสภาวะการดำเนินงานแบบกึ่งต่อเนื่องโดยใช้ถังปฏิกรณ์ชีวภาพแบบอากาศยกนั้นพบว่าสามารถผลิตเซลล์ได้อย่างต่อเนื่อง โดยทำการเก็บเกี่ยวเซลล์ทุก 4 วัน ได้ผลผลิตเซลล์สูงสุด 5.52×10^{4} เซลล์ต่อมิลลิตรต่อวัน และได้อัตราการเติบโตจำเพาะ 0.31 ต่อวัน ในส่วนของการผลิต astaxanthin จากสาหร่าย *H. pluvialis* พบว่าแสงและอาหารเป็นปัจจัยที่มีความสำคัญต่อการสร้าง astaxanthin ของสาหร่าย *H. pluvialis*

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต.....
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4570201921 : MAJOR CHEMICAL ENGINEERING
KEY WORD: *Haematococcus pluvialis* / AIRLIFT BIOREACTOR/ BUBBLE COLUMN/ ASTAXANTHIN /

KAMONPAN KAEWPINTONG : CULTIVATION OF *Haematococcus pluvialis* IN AIRLIFT BIOREACTOR. THESIS ADVISOR : ASST. PROF. ARTIWAN SHOTIPRUK, Ph.D., THESIS CO-ADVISOR : ASSOC. PROF. PRASERT PAVASANT, Ph.D. 113 pp. ISBN 974-53-1162-6

The production of *Haematococcus pluvialis* NIES-144 was investigated in this experiment. The F1 medium (Fabregas et al., 1998) with a vitamin B complex supplement at 12 μg/L was shown to yield the highest cell density of *H.pluvialis*. The cultivation of this alga in the 3L airlift bioreactor was proven to be superior to that in the bubble column of the same size operated at the same aeration rate. CO_2 was important for a better growth of the alga. The operation with a mixture of air and 1% CO_2 at the superficial gas velocity of 0.4 cm/s was found to give the highest cell growth. No appreciable effect of the ratio between the downcomer and riser cross sectional areas in the airlift system was observed in this work, however, the higher area ratio was selected as it required less power consumption in aeration. The light intensity for the highest growth rate was found to be at 20 μmol photon m^{-2} s^{-1}. A semi-continuous culture could be achieved where the harvest was performed at every 4 days. The specific growth rate and productivity in semi-continuous culture were 0.31 day^{-1} and 5.52 cell mL^{-1} day^{-1}, respectively. The potential of inducing astaxanthin from *H.pluvialis* was tested where light intensity and the nutrient composition were found to significantly affect the accumulation of astaxanthin.

Department Chemical Engineering Student's signature…….....
Field of study Chemical Engineering Advisor's signature…….....
Academic year 2004 CO-advisor's signature……

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Assistant Professor Artiwan Shotipruk, my advisor and Associate Professor Prasert Pavasant, my co-advisor, for their valuable suggestions, guidance and stimulation throughout my master program and especially helpful in the writing up of thesis work. Furthermore, I would like to express my sincere thanks to Dr. Sorawit Powtongsook. This thesis could not complete without the helpful suggestion, supports and encouragement from him. I would also like to thank Assistant Professor Supakanok Thongyai, chairman of thesis committee, Assistant Professor Seeroong Prichanont, member of thesis committee for their valuable comments and also for useful suggestions.

I would like to special thank Miss Maliwan who always consults me during my master program as well as Miss Paveena, Miss Suttikarn and Mr. Parinya for help and sharing the time during my master program. Moreover, special thanks are also express to all friends and members in Marine Biotechnology Research Unit (at Chulalongkorn University) National Center of Genertic Engineering and Biotechnology and the Biochemical Engineering Laboratories for their assistance and friendship.

I would like to thank the Marine Biotechnology Research Unit (at Chulalongkorn University) National Center of Genetic Engineering and Biotechnology for allowing me to setup the experimental apparatus and using the accessories. Moreover, this research received the grant support from Thailand Research Fund (TRF).

Finally, I would like to express love and thanks to my warmhearted family, for side by side walked me, supports, inspiration, patience and just being.

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CHAPTER I

Introduction

1.1 Motivation

Microalgae are a potential source for various kinds of commercially valuable chemicals. Among these algal products, astaxanthin attracts a great commercial interest due primarily to its versatile applications and high price (approximately 2,500-3,000 US$/kg) (Lorenz and Cyseski, 2000). Astaxanthin is a red pigment employed as a pigmentation inducer in aquaculture (Lorenz and Cyseski, 2000). It has also been shown to possess a higher antioxidation property than other carotenoids that may play an important role in cancer protection (Kobayashi et al., 1991). Currently, astaxanthin can be produced from both chemical (synthesis) and (biological natural) pathways. The synthesis of astaxanthin, however, is a complicate process which leads to its high price. Chemical synthesized astaxanthin was also reported to potentially contain unnatural configurational and carotenoid-like compounds which might not be as effective as that produced naturally. All these factors have contributed to the interest in using natural source of astaxanthin (Johnson and An, 1991; Gong and Chen, 1997).

In nature, although some kinds of plant, bacteria and yeast are known to synthesize astaxanthin (Kobayashi et al., 1991; Gong and Chen, 1997), the green microalga, *Haematococcus pluvialis* is reported to be capable of accumulating the most superior amount of astaxanthin (Harker et al., 1996). Thus, *H. pluvialis* is considered as an important natural source for the mass production of astaxanthin. *H. pluvialis* accumulates astaxanthin in the transition between green vegetative cells and cyst (aplanospore) which is a resting stage of cells as a result of stress conditions such as high salt concentration, high light intensity and depletion of nitrogen or phosphorous (Cordero et al., 1996; Fabregas et al., 1998; Orosa et al., 2005). This simply means that the achievement of the mass production of

astaxanthin from *H. pluvialis* is the establishment of conditions for a sustained production of vegetative cells.

Generally, the culture system of microalgae can be performed in open and close systems. Open systems are the oldest and simplest form of culturing ponds. They have a number of advantages including minimal costs of construction and operation. However, this mode of cultivation possesses a number of drawbacks including low cell densities, potential contamination by other microorganisms and the inability to control the culture environment such as light irradiation and temperature. Since *H. pluvialis* is susceptible to easy contamination, slow growth and preference for low growth temperature (Harker et al., 1996), outdoor cultivation practice has not been made possible. This leads to a need in developing closed bioreactor culture systems. Although, the bioreactor cultures require several additional steps in operation, e.g. medium sterilization, aseptic operation, they offer a number of advantages including a better control of culture environment, protection from ambient contamination and achievement of high cell density (Chen, 1997).

In recent years, various types of bioreactor systems have been introduced for the cultivation of algae. Most bioreactors are designed with either a mechanical agitated such as stirred tank bioreactors or pneumatically agitating such as bubble columns and airlift bioreactors in order to provide mixing and thus maintaining a nearly homogeneous culture. Among these, a stirred tank bioreactor could provide the highest levels mass transfer and mixing. However, mechanical movement is prone to producing high shear which is not suitable for shear sensitive cell. In practice, pneumatically agitated are usually recommended for slow growth, shear sensitive cells where mass transfer and mixing are not rate limiting steps.

Airlift bioreactors are being applied to a variety of biotechnological processes. The main advantages of airlift bioreactors include simple construction, well-defined fluid flow pattern and relatively high gas-liquid mass transfer rate. In addition the mixing in airlift bioreactor could be obtained with out causing too much shear force in liquid phase, which could inhibit the growth of algae.

This work focused on the cultivation of *H. pluvialis* for the production of astaxanthin in airlift bioreactors. Due to the different culture requirements of the alga during the various stages of algal life cycle, a two-stage culture system was employed i.e. the first stage is to provide suitable condition for growth of green motile cells, and the second is for the production of astaxanthin in cyst.

1.2 Objectives

The objectives of this thesis were:

1.2.1 To determine the optimal growth condition in flask (medium and vitamin B)

1.2.2 To determine the optimal growth condition in bioreactor. These conditions included (i) ratio between the downcomer and riser cross sectional area (A_d/A_r), (ii) superficial gas velocity and light intensity

1.2.3 To primarily test for astaxanthin production from *H. pluvialis* in airlift photobioreactor

1.3 Scopes of work

1.3.1 The determination of suitable nutrient conditions was performed in 250 mL flask.

1.3.2 The reactor employed in this work was a small lab-scale 3.6 L airlift contactor with the dimension as stated in Table 3.3.

1.3.3 The ratio between the downcomer and riser cross sectional area (A_d/A_r) were fixed at 0.9 and 3.2 due to the availability of the commercial column size.

1.3.4 The superficial gas velocity was approximately controlled in a range of 0.4-3 cm s^{-1} during the growth stage.

1.3.5 The light intensities were approximately controlled in a range 12-60 μmol photon m^{-2} s^{-1} during the growth stage.

1.3.6 The light intensities were approximately controlled in a range of 20-1500 μmol photon m^{-2} s^{-1} during the astaxanthin accumulation stage.

CHAPTER II

Backgrounds and Literature Review

2.1 *Haematococcus pluvialis*

2.1.1 Biological information

Haematococcus pluvialis is a freshwater unicellular alga. *H. pluvialis* reproduces asexually by division from a single cell into two and/or four motile cells (Droop, 1995). Smith (1964) defined the taxonomy of *H. pluvialis* as follows:

Division	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	Chlamydomonadaceae
Genus	*Haematococcus*
Species	*Haematococcus pluvialis*

The cell morphology of *H. pluvialis* falls into one of the two forms: vegetative cell (Figure 2.1) and cyst (Figure 2.2). Under suitable growth conditions, most cells remain in vegetative form that produces chlorophyll *a* and *b* and primary carotenoid, especially β- carotene and lutein (Rockette, 1970). These cells are green with spherical or ellipsoid shapes a diameter is approximately 10-20 μm. The cells are enclosed by cell walls and are motile with two flagellate. Under stress conditions, such as depletion of nutrient, e.g., phosphate or nitrate, and/or light induction, the cells convert to a cyst form which is still spherical in shape. They lose flagella and their motility and form new thick cell wall. The volume of alga increases dramatically from 10-20 μm to 40-50 μm in diameter. Moreover,

these cells produce secondary carotenoids such as echinenone, canthaxanthin and astaxanthin following a decrease in chlorophyll and primary carotenoids (Droop, 1954; Lee et al., 1991). Growth rate of *H. pluvialis* in this stage decreases, cells begin the massive accumulation of astaxanthin. Astaxanthin deposition is first noted around nucleus and proceeds radically until the entire protoplast is red (Lee et al, 1991). Fully encysted cells contain up to 5% dry weight astaxanthin (Renstom et al., 1981; Bubrick, 1991).

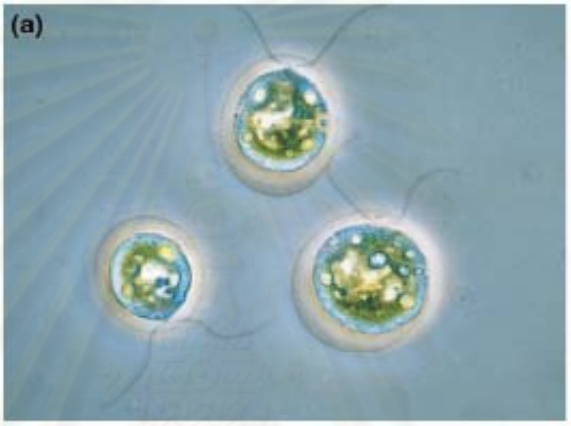


Figure 2.1 Vegetative cells of *Haematococcus pluvialis*
(Lorenz and Cysewski, 2000)

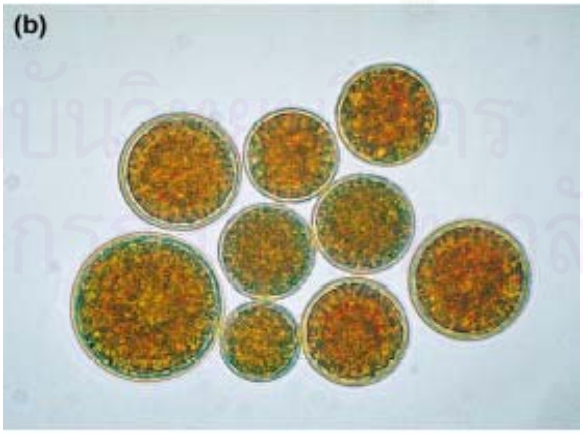


Figure 2.2 Accumulation of astaxanthin in cysts of *Haematococcus pluvialis*
(Lorenz and Cysewski, 2000)

2.1.2 Life Cycle of *Haematococcus pluvialis*

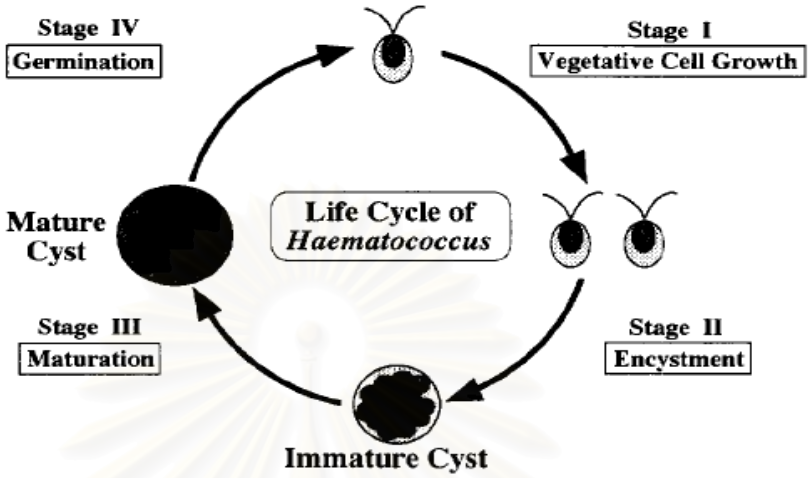


Figure 2.3 Life Cycle of *Haematococcus pluvialis*
(Kobayashi et al., 1992)

Life cycle of *H. pluvialis* is divided into four stages (Figure 2.3) as described below:

(i) Vegetative cell growth

At this stage, cells remain in a vegetative form. Ellipsoidal vegetative cells are capable of swimming with two flagella and are capable of increasing in number. These cells contain high level of chlorophyll and protein but very low carotenoid contents.

(ii) Encystment

At this stage, vegetative cells transform into immature cyst which have brown spherical shape. During the encystment stage, chlorophyll and protein decrease whereas the level of carotenoid biosynthesis and protein production increases.

(iii) Maturation

At this stage, immature cyst transform into mature, immotile cyst. During maturation, carotenoid, chlorophyll and protein syntheses declines.

(iv) Germination

At this stage, chlorophyll and protein synthesis, and carotenoid degradation occur.

2.2 Astaxanthin

2.2.1 Chemical Properties of Astaxanthin

Carotenoids are exogenously derived from isoprenoid compounds which are responsible for pigmentation in nature and have essential biological functions in animals. They also impart attractive pigmentation to many farmed animals. Usually carotenoids constitute large fraction of expenses to farmer (John and An, 1991).

Astaxanthin is a principle carotenoid pigment in *H. pluvialis*. Since most animals lack an ability to synthesize astaxanthin from other carotenoids and most of them acquire it in their food (Fross et al., 1987; Bjerkenget al., 1990), the supplement of astaxanthin is often regarded as necessary especially for the large scale production of the farm animal. Moreover, astaxanthin is also the preferred pigment in aquaculture since it is deposited more efficiently than other carotenoid. There is also an interest in the use of this pigment as a colorant for egg yolk in the poultry and in the aquaculture where it is used as a feed supplement in the production of salmon, trout and shrimp (Fan et al., 1992). Due to the antioxidative activity of astaxanthin, it also could be applied in clinical applications (Tjahjono et al., 1994).

Astaxanthin or 3,3'-dihydroxy-β,β'-carotene-4,4'-dione is an oxycarotenoid with a molecular formula $C_{40}H_{52}O_4$ and a molecular weight of 596.86. Its melting point is approximately 224° C. It is insoluble in aqueous solution and most organic solvents but can be dissolved at room temperature in non-polar solvents such as acetone, dimethyl-sulfoxide.

There are two different pathways for astaxanthin biosynthesis: the first one starts with the oxydation of β-carotenoid of β-carotene with echinenone, canthaxanthin and adonirubin talking part as intermediates, and the second one starts with β-carotene hydroxylation with β-cryptoxanthin, zeaxanthin and adonixanthin as intermediates (Schoefs et al., 2001) (Figure 2.4).

The astaxanthin molecule has two asymmetric carbons located at the 3 and 3' positions of benzenoid rings on either end of molecule. There have three configurations of astaxanthin; free astaxanthin, astaxanthin monoesters and astaxanthin diesters (Figure 2.5).

2.2.2 Sources of Astaxanthin

There have been two major sources of astaxanthin; chemical (synthetic) and biological (natural) source.

2.2.1.1 Synthetic source

Synthetic astaxanthin is presently the principle source in aquaculture, more than 95% of this market consume synthetically astaxanthin used in feed to produce coloration (Lorenz and Cysewski, 2000). However, synthetic astaxanthin is expensive, unnatural configurational and involves potentially harmful process (Parker, 1992).

2.2.1.2 Natural sources

A. Crustaceans byproduct

Crustacean wastes have been used as natural pigment sources for trout and salmon but they have relatively low contents of astaxanthin and high levels of moisture, ash and other nutrients, which cause several practical problems in feed formation that limits their usefulness in animal feed (Bubrick, 1991).

B. Yeast

Yeast under genus *Phaffia* is characterized by the synthesis of astaxanthin. However, it contents of astaxanthin in wide strain is only 200 to 300 μg/g yeast (0.02-0.03%). The content of astaxanthin depends on strain and method of culture (John and An, 1999).

C. Algae

Astaxanthin can be produced from other strains of algae such as *Ankistrodesmus branuii*, *Chlorella zofingiensis* and *Dunaliella salina* (Borowitzka, 1989), *Euglena rubida* (Czeczuga, 1974). However, the quantitity of astaxanthin generated from these microorganism were relatively low and not suitable for mass production. The green algae, *H. pluvialis* have received much attention due to its capacity to accumulate high amount of astaxanthin (Vanshak, 1990).

D. Other microorganisms

Some bacteria such as *Mycobacterium lacticola* and *Brevibacterium sp*. and fungi in genus *Peniophora* were also reported to be able to accumulate astaxanthin (Borowitzka et al., 1989). Carotenoid level of these microorganisms is low and growth is slow (Droop, 1985). Sources of astaxanthin produced by microorganisms are shown in Table 2.1

2.3 Production of astaxanthin by *H. pluvialis*

The procedure in the production of astaxanthin begins with the cultivation of *H. pluvialis* biomass under condition conductive to growth, followed by the stimulation of astaxanthin by changing the cells environment to stress promoting conditions. These two steps are controlled by two different sets of conditions as detailed below.

2.3.1 Environmental Growth Factors for *H. pluvialis*

Several physiological parameters such as light and temperature, as well as nutritional and other environmental factors could potentially affect the growth of *H. pluvialis*. Each factor is described below.

2.3.1.1 Light

Light is an essential factor for the growth of varieties of organisms. According to the literature the optimal light intensities for the growth of *H. pluvialis* ranges between 2 and 24 klux (Fan et al., 1994; Harker et al., 1996).

2.3.1.2 Temperature

Temperature is one of the major factors controlling the rate of photosynthesis in all plants. In general, growth rate increases with temperature up to an optimum temperature then declines rapidly as the temperature exceeds this optimum. The optimum temperature for growth in *H. pluvialis* lies in the range of 15 and 27°C (Borowitzka et al., 1989; Harker et al., 1996; Kobayashi et al., 1997).

2.3.1.3 pH

The optimum pH range for growth in *H. pluvialis* is between 6.5 and 8 (Hata et al., 2001).

2.3.1.4 Nutrients

Essential elements are usually divided into macronutrients and micronutrients. Macronitrients are required in relatively high quantity. Examples of macronutrients are carbon, nitrogen, phosphorous, calcium, etc. In *H. pluvialis*, growth rate was rapid at high nitrogen and phosphate concentrations (Borowitza, 1991; Boussiba et al., 1992). Proctor (1957) found that N-nitrate was more preferable to N-ammonium for the growth of *H. pluvialis*, Micronutrients are required in relatively low quantities, within the concentration range of $mg \cdot L^{-1}$ to $\mu g \cdot L^{-1}$. Examples of micronutrients needed for algal growth are iron, boron, manganese, copper and vitamins.

2.3.2 Effect of Environment Factors on Astaxanthin accumulation in *H. pluvialis*

Astaxanthin production in *H. pluvialis* is regulated by several environmental parameters, including light, aeration, nutrient and other factors as follows (Johnson and An, 1991);

2.3.2.1 Light

Light is important for the regulation of carotenogenesis in a wide variety of organisms. Light is known to stimulate astaxanthin formation in *H. pluvialis* (Boussiba and Vonshak, 1991; Kakizono et al., 1992). Goodwin and Jamikorn (1954) showed that low carotenoid content was obtained if *H. pluvialis* cultures were placed in the dark. Optimum light intensities for astaxanthin accumulation of *H. pluvialis* ranged between 75 and 100 klux (Kobayashi et al., 1991; Fan et al., 1994). Kobayashi et al (1992) showed that under higher light intensity than 50 $\mu mol\ m^{-2}s^{-1}$, algal morphology changed from vegetative to cyst. This occurred simultaneously with the with carotenoids formation. Note that carotenoids formation was more efficiently enhanced under blue light than under red light.

2.3.2.2 Temperature

Astaxanthin accumulation, gradually increased when the cultures were cultivated at high temperature. Optimal temperature for astaxanthin synthesis was reported to be higher than 30°C (Tjahjono et al., 1994; Fan et al., 1994).

2.3.2.3 Nutritional factors

The nutritional factors inducing astaxanthin syntheses in green algae are described below.

A. Nitrogen and Carbon

In *H. pluvialis* cultures, nitrogen limitation is a key factor for the accumulation of astaxanthin (Kobayashi et al., 1991). Acetate and glycine was demonstrated to stimulate astaxanthin formation in *H. pluvialis*.

B. Phosphorous

Phosphorous is one of the important nutrients for astaxanthin synthesis. Astaxanthin accumulation could be induced by altering phosphate content. Under the phosphate deficiency condition, *H. pluvialis* could accumulate high amount of astaxanthin (Harker et al., 1976; Boussiba and Vonshak, 1991).

C. Iron

The astaxanthin formation of *H. pluvialis* was enhanced in Fe^{2+} rich medium (Kobayashi, Kakizono and Nagai, 1992).

2.3.2.4 Salt stress

The accumulation of astaxanthin in cyst under salt stress condition was reported both in the dark (Koyabashi et al., 1997) and in the light (Spencer, 1989;

Borowitzka et al., 1991; Boussiba and Vonshak, 1991; Cordero et al., 1996). In addition, Sarada et al. (1992) found that the age of the culture was crucial in triggering astaxanthin production in the salt stress induced culture. Cordero et al. (1996) demonstrated that *H. pluvialis* could accumulate high amount of astaxanthin when they were subject to the solution with 0.2% sodium chloride.

2.4 Culture System

Several types of culture systems were reported for the growth of microalgae. The selection of culture systems depends on several factors, e.g. biology of the algae, energy, the cost of land, nutrient and the type of final product (Borowitzka, 1992). In brief, the culture systems can be categorized into two groups: (i) open and (ii) close systems.

2.4.1 Open system

Open systems are the oldest and simplest from of culture systems for algal cultivation. In this system, algae are cultivated under conditions identical to the external environment. Since microalgae are very efficient in converting solar energy, many attempts have been made to cultivate them in this simple manner such as shallow open ponds. However, pure culture cultivation of microalgae has found only limited success.

Open systems for mass cultivation of microalgae have been succeeded for only a few species. These algae are usually achieved by maintaining extreme culture environments such as high salinity, high alkalinity and high nutritional. To date, only *Dunaliella*, *Spirulina* and *Chlorella* have been successfully mass cultured and marketed commercially (Lee, 2001). However, Cynotech Corporation has reported commercial production of astaxanthin by *H. pluvialis* for reddening stage in open cultivation ponds, but detail has not been disclosed.

A few achievements in the cultivation of astaxanthin in open systems were reported. This included the work of Harker et al. (1996) who studied the

cultivation of *H. pluvialis* in 250 L open raceway pond and found that the culture was subject to high level of cyanobacteria contamination and predation by protozoa, which resulted in poor alga growth. In addition, the cultivation of *H. pluvialis* in 25,000L raceway pond was experimented by Olaizola (2000) who achieved an average growth rate of approx. 0.14 day^{-1}. This level was still far lower than those obtained in the closed system as elucidated in the next section.

2.4.2 Closed system

Not all alga species are suitable for the culture in an open system. The assumption that high cell concentration is necessary to achieve higher biomass productivity, and the need to maintain monoculture for microalgae that grow in mild culture conditions have led to the development of enclosed photobioreactors.

A bioreactor is the component of biochemical processes. The main function of bioreactor is to provide control environment to obtain the optimal growth and product formation. Table 2.2 summarizes advantages and disadvantages of culture system.

For microbial culture, many bioreactor systems are available but they may be classified into two types; (i) mechanically agitated systems such as stirred tank reactor, and (ii) pneumatically agitated systems such as bubble column and airlift reactor. Table 2.3 summarizes advantages and disadvantages of each type bioreactors.

2.4.2.1 Stirred tank reactor

Stirred tank reactors refer to agitated mechanically reactor. A reactor of this type consists of a vessel where mixing and bubble dispersion is achieved by mechanical agitation. The agitator or agitators provide adequate heat and mass transfer, mixing, for several purposes, e.g. the uniformity of suspension. (Figure 2.6)

The stirred tank reactor has been one of the most employed close systems. This system provides most major requirements for culturing microorganism. For instance, Glaue and Maxey (1994) obtained the culturing of *Nitzschia alba* in stirred tank reactor with high cell density of 45 g L^{-1} and volumetric productivity of 20 g L^{-1} day^{-1}. Atkinson and Mavituna (1983) cultivated *Chlorella* in stirred tank reactor with the yield of 91-353 g L^{-1} day^{-1}. However, Harker et al. (1993) reported that the shear effects of agitation could adversely influence the morphology of *H. pluvialis* leading to a low growth culture.

2.4.2.2 Pneumatic reactor

Pneumatic reactors acquired the mixing from the aeration alone which helps lessen the shear effect caused by mechanical agitation. Examples of this type of reactors are bubble column and airlift bioreactor (Figure 2.7-2.8).

Bubble columns are simple to construct and operates. They are generally cylindrical vessels with the height greater than the diameter. In bubble columns, aeration and mixing are achieved by gas sparging at the bottom into the liquid contained by the column. The net liquid flow may be either co-current or counter-current to the gas flow direction, or the not flow (Figure 2.7).

Little was available on the growth of *H.pluvialis* in bubble columns. The only work was reported by Choi et al, (2003) who cultivated the *H. pluvialis* in the 2 L bubble column. The maximum cell density was obtained at the light intensity of 30 µmol photon m^{-2} s^{-1} which achieved the cell density of 5.2×10^{5} cell mL^{-1} in 9 days. This was equivalent to the growth rate of 0.23 day^{-1}.

Similar to bubble columns, mixing in airlift reactors can be accomplished without mechanical agitation. Airlift reactors are often chosen for cultivation of plant and animal cells because shear stress level is lower than that in stirred tank reactors. Airlift reactors typically consist of a liquid pool divided into two zones, a riser and a downcomer. Gas is sparged only into a part of the vessel cross section, the riser. Gas disengages at the top of the vessel leaving heavier bubble free liquid

to recirculate through the downcomer. Liquid circulates in airlift reactors as a result of the density difference between the riser and the downcomer.

Airlift reactors can be classified into two types. The first is the internal loop airlift reactor (Figure 2.8(a)-(c)) whose the riser and downcomer are separated by draft tube or the plate. The second is the external or outer loop airlift reactor (Figure 2.8(d)) whose riser and downcomer are two vertical tubes that are connected by short horizontal pipes at the top and the bottom.

Airlift bioreactors have been employed for the production of astaxanthin from *H. pluvialis* by Harker et al, (1996). The mass cultivation of *H. pluvialis* in the 30 L airlift bioreactor was reported with the specific growth rate of approx. 0.1 day^{-1}. However, this work concentrated more on the astaxanthin induction by the presence of NaCl and therefore the condition of the reaction might not be suitable for the growth of the alga.

A summary of the growth of *H. pluvialis* is given in Tables 2.4-2.6 where details of the cultivation such as environmental conditions, nutrients, and growth data are provided. It can be seen that most of the cultivation could only achieve a low level of growth rate, i.e. in the range of 0.09 to 0.32 day^{-1}. The highest growth rate was obtained from the work of Kobayashi et al. (1992) who could achieve 0.58 day^{-1} but this was performed in a small flask where the environmental conditions could be well controlled.

Table 2.1 Biological sources of astaxanthin (Simpson et al., 1981; Borowitzka, 1989; Harker et al., 1996)

Source	Astaxanthin(μg/g cell)	Astaxanthin (%)
Bacteria *Brevibacterium*	30	
Mycobacterium	30	
Yeast *Phaffia rhodozyma*	200-300	0.02-0.03
Algae *Chlamydomonas*	<50	
Euglena rubida	<50	
Haematococcus	7,000-55,000	0.2-2

Table 2.2 Advantages and Disadvantages of open and closed algal cultivation

Parameter	Open pond	Closed systems
contamination risk	extremely high	low
space required	high	low
water losses	extremely high	almost none
CO_2 losses	high	almost none
weather dependence	absolute	insignificant,because closed configurations allow production also during bad weather
species	are restricted to a few algal varieties	may be cultivated
biomass concentration	low	high
efficiency of treatment process	low	high

Table 2.3 Comparison of properties of different large scale algal culture system

Reactor type	Mixing	Light utilization efficiency	Temperature control	Gas transfer	Hydronynamic stress on algae	Species control	Scale up
unstirred shallow ponds	very poor	poor	none	poor	very low	difficult	very difficult
tank	poor	very poor	none	poor	very low	difficult	very difficult
circular stirred ponds	fair	fair good	none	poor	low	difficult	very difficult
stirred tank reactor	largely uniform	fair good	excellent	low high	high	easy	difficult
airlift reactor	generally uniform	good	excellent	high	low	easy	difficult
tubular reactor	uniform	excellent	excellent	low-high	low-high	easy	easy
flat plate reactor	uniform	excellent	excellent	high	low-high	easy	difficult

Table 2.4 Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Fabregas et al. (2001)	tube	70	OHM	2 step													
				1.mixotrophic (semicontinuous)	2 (12D:12L)	cool white fluorescent lamp	15 + CO_2		7.2	25	5.72					0.381	
				2.mixotrophic (batch)	12				-7.8		6.25		49			0.446	3.267
Fabregas et al. (1998)	tube	70	1.OHM	mixotrophic	2 (12L:12D)	daylight fluorescent lamp	15+ CO_2		8	25	3.77					0.775	
			2.BBM								1.20					0.240	
			3.CHU								0.58					0.116	
Tripathi et al. (1999)	flask		KM1	heterotrophic						25 + 1	4.35					0.870	
Hata et al. (2001)	flask	500	Basal	heterotrophic (fed-batch)				40	8	25	10	7				0.667	
	flask	500	Basal + 10 mM SA	2 step													
				1.heterotrophic	0			40	7.5-8	25	10.0	3.50				0.667	
				2.autotrophic	47.5			100	7.5-8	30	6.2	6.10	144	123		0.295	5.142
Grunewald et al. (1997)	Flask	100	2 step														
			1.Bold's basal	autotrophic (batch)	1	white light				20+2							
			2.Bold's basal (No nitate)		7.5						2.90			13.34		0.414	

* 1. SA = Sodium Acetate

Table 2.4 (cont) Effect ofmedium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5} cell/mL.day)	productivity of astaxanthin (mg/L.day)
Sarada et al. (2001)	flask		Basal + SA +NaCl	mixotrophic	1.5					25±1			10.93	12.25	6.2		0.683
Tjahjono et al. (1994)	flask	200	2 step														
			1.Basal+45mMSA	mixotrophic	1.5 (12D:12L)	cool white fluorescent			6.8	20	5.13			15.0		1.28	
			2.Basal+45mMSA +450mM Fe^{2+}		8.6						5.22			20.6		1.30	
Tripathi et al. (1999)	flask		1.BBM	autotrophic	1.5					25±1	1.50					0.150	
			2.Z8								0.80					0.080	
	flask		1.MM2	mixotrophic	1.5					25±1	3.25					0.325	
			2.KM2								4.20					0.420	
Kobayashi et al. (2001)	flask	200	1.Basal + 45 mM SA	mixotrophic	1.25 (12L:12D)	white fluorescent				20							
			2.Basal + 45 mM SA		6	lamp							$4.5x10^{-3}$ pg/um^{3}				
	flask	200	1.Basal + 45 mM SA	mixotrophic	1.25 (12L:12D)	white fluorescent				20							
			2.Basal + 45 mM SA + 450 mM $FeSO_4$		6	lamp							$6.5x10^{-3}$ pg/um^{3}				

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Kobayashi et al. (1991)	flask	200	1.Basal + SA	mixotrophic	8.6				6.8	20		0.87		5.5			
			2.Basal + SA Fe^{2+} 150 um									1.10		13.5			
			3.Basal + SA Fe^{2+}300 um									1.25		20.0			
			4.Basal + SA Fe^{2+} 450 um									1.60		23.7			
			5.Basal + SA Fe^{2+} 600 um									0.90		3.7			
	flask	200	1.Modified	mixotrophic	4.5				7	20		0.652		2.692			
			2.Modifiedl + 20 mM SA									0.723		15.000			
			3.Modifiedl + 40 mM SA									0.476		12.923			
			2.Modifiedl + 60 mM SA									0..324		8.462			
Tripathi et al. (2002)	flask	100	1.KM1	mixotrophic	1.5					25±1	4.10					0.820	
			2.KM1 + feruric acid								4.05					0.810	

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Kobayashi et al. (1991)	flask	200	1.Modified	mixotrophic	4.5				7	20		0.276		2.692			
			2.Modified + 10 mM Pyruvate									0.478		3.846			
			3.Modified + 20 mM Pyruvate									0.770		9.231			
			4.Modified + 30 mM Pyruvate									0.893		20.385			
			5.Modified + 40 mM Pyruvate									0.693		17.692			
	flask	200	1.Modified + SA	mixotrophic	4.5				7	20		0.770		16.538			
			2.Modified + SA +3 mM Pyruvate									0.770		15.769			
			3.Modified + SA +6 mM Pyruvate									0.831		17.692			
			4.Modified + SA +9 mM Pyruvate									0.785		20.769			
			5.Modified + SA +12 mM Pyruvate									0.770		21.538			

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Kobayashi et al. (1991)	flask	200	1.Modified	mixotrophic	4.5				7	20		0.75		14.8	3.5		
			2.Modified + 3mM Mevalonate									0.88		18.2	5.6		
			3.Modified + 3mM Isopentyl alcohol									0.83		15.3	4.4		
			4.Modified + 12 mM Pyruvate									0.77		20.6	0.8		
			5.Modified + 3 mM Malonate									0.77		17.7	2.3		
			6.Modified + 3 mM L-Leucine									0.57		11.2	2.4		
			7.Modified +6 mM Dimethyllacrylate									0.70		16.9	3.0		
Kobayashi et al. (1998)	flask	200	Basal	mixotrophic	1.5 (12L:12D)	fluorescent			6.8	20	7.00		8.40	10	17	0.875	1.050
	flask	200	Modified	mixotrophic	4.5	fluorescent			7	20	3.10		13.35	15	6.5	0.388	1.669

Table 2.4 (cont) Effect of medium ต่อ cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Sarada et al. (2002)	flask		1.Basal	mixotrophic	1.5					25±1			2.32	2.05	6.50		0.18
			2.Basal + Ca_2NO_3								6.2		2.52	2.23	6.50		0.19
			3.Basal + KNO_3								1.65		1.25	1.88	4.97		0.10
			4.Basal +NH_4NO_3										1.16	1.34	3.58		0.09
			5.Basal + $NaNO_3$										3.21	4.19	12.40		0.25
	flask		1.Basal + NaCl + SA	mixotrophic	1.5					25±1			1.72	2.24	1.34		0.07
			2.Basal + NaCl + SA + Ca_2NO_3										2.24	4.66	4.03		0.10
			3.Basal + NaCl + SA + KNO_3										1.21	1.55	0.22		0.05
			4..Basal + NaCl + SA+NH_4NO_3										1.03	1.03	0.22		0.04
			5.Basal + NaCl + SA +$NaNO_3$										1.72	2.59	1.56		0.07

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Harker et al. (1996)	stirred tank	250	1.Bold'S basal+ 18uMFeSO$_4$.7H$_2$O	autotrophic	1.75	cool white fluorescent		80		22	2.25		5.13			0.075	0.76
			2.Bold'S basal+ 36uMFeSO$_4$.7H$_2$O								1.98		3.03			0.066	1.03
			3.Bold'S basal+ 72uMFeSO$_4$.7H$_2$O								1.65		0.68			0.055	1.47
	stirred tank	250	1.Bold'S basal+ 0.85mM K$_2$HPO$_4$	autotrophic	1.75	cool white fluorescent		80		22	1.83		32.0			0.061	1.06
			2.Bold'S basal+ 1.7mM K$_2$HPO$_5$								2.70		19.8			0.09	0.66
			3.Bold'S basal+ 3.4mM K$_2$HPO$_6$								2.94		19.5			0.098	0.65
	stirred tank	250	1.Bold's basal	autotrophic	1.75	cool white fluorescent		80		22	2.81		36.8			0.093	1.23
			2.Bold's basal+ 40 mM KCl								0.41		16.1			0.013	0.54
			3.Bold's basa+l 70 mM KCl								0.23		5.8			0.01	0.19
			2.Bold's basa+l 100 mM KCl								0.18		1.8			0.006	0.06

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell andproductivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number ($x10^{-5}$cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell ($x10^{-5}$ cell/mL.day)	productivity of astaxanthin (mg/L.day)
Harker et al. (1996)	stirred tank	250	1.Bold's basal	autotrophic	1.75	cool white fluorescent		80		22	0.33		11.7			0.011	0.39
			2.Bold's basal+ 0.75 mM $NaNO_3$								0.81		10.8			0.027	0.36
			3.Bold's basal+ 1.5 mM $NaNO_3$								0.90		16.4			0.030	0.57
			4.Bold's basal+ 3.0 mM $NaNO_3$								2.55		11.8			0.085	0.39
			5.Bold's basal+ 6.0 mM $NaNO_4$								2.4		9.7			0.080	0.32
	stirred tank	250	1.Bold's basal	autotrophic	1.75	cool white fluorescent		80		22	2.90		43.5			0.09	1.45
			2.Bold's basal+ 40 mM NaCl								1.20		51.4			0.04	1.71
			3.Bold's basal+ 70 mM NaCl								1.10		44.0			0.03	1.47
			4.Bold's basal+ 100 mM NaCl								0.36		20.1			0.01	0.67

Table 2.4 (cont) Effect of medium ต่อ cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Chen et al. (1997)	stirred tank	3710		mixotrophic (batch)	8.5	fluorescent lamp (18W)	100	400	7	25	0.7	1.52				0.088	
	stirred tank	3710		heterotrophic (batch)			100	400	7	25	1.45	0.50				0.181	
Zhang et al. (1999)	stirred tank	3700	Hong Kong + SA[1]	mixotrophic (fed-batch)			100	350	7	30			64.40				3.22
	stirred tank	3700	Hong Kong	mixotrophic (batch)	2.25 - 9		100	350	7				53.43				2.67
Schoefs et al. (2001)	Air-lift	2500	Bristol + 9 mM $NaNo_3$	2 step													
				1.autotrophic	2.5	2 lamp (250 W)				20±1							
				1.autotrophic	55	Polychromatic light							24.49				
Hagen et al. (2000)			2 step														
			1.control	autotrophic	1.25						2.75						
			2.control (No nitrate)		7.5												
Harker et al. (1996)	Air-lift	30000	2 step														
			1.Bold's basal	autotrophic	2.5	4 fluorescent	90-180		7-	14 -	2.50	1.57					
			2.Bold's basal + NaCl	(batch)		tube	+ CO_2		8.25	27				40.50		0.114	

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Cordero et al. (1996)			1.Control	mixotrophic	1.3 (12D:12L)	white fluorescent	360 + CO_2		7.5	25			6.7				1.34
			2.Control + 0.025g/L SA										13.6				2.72
			3.Control + 0.05g/L SA										13.3				2.66
			4.Control + 0.1g/L SA										6.4				1.28
			1.Control + 0.1% NaCl	mixotrophic	1.3 (12D:12L)	white fluorescent	360 + CO_2		7.5	25			5.7				1.14
			2.Control + 0.2% NaCl										18.6				3.72
			3.Control + 0.4% NaCl										7.0				1.40

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Cordero et al. (1996)			1.Control + 0.1% NaCl + 0.025 g/L SA	mixotrophic	1.3 (12D:12L)	white fluorescent	360 + CO_2		7.5	25			9.7				1.94
			2.Control + 0.1% NaCl + 0.05 g/L SA										10.4				2.08
			3.Control + 0.1% NaCl + 0.1 g/L SA										15				3.00
			1.Control + 0.2% NaCl + 0.025 g/L SA	mixotrophic	1.3 (12D:12L)	white fluorescent	360 + CO_2		7.5	25			9.0				1.80
			2.Control + 0.2% NaCl + 0.05 g/L SA										6.3				1.26
			3.Control + 0.2% NaCl + 0.1 g/L SA										11.7				2.34

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Cordero et al. (1996)			1.Control + 0.4% NaCl + 0.025 g/L SA	mixotrophic	1.3 (12D:12L)	white fluorescent	360 + CO_2		7.5	25			7.3				1.46
			2.Control + 0.4% NaCl + 0.05 g/L SA										7.6				1.52
			3.Control + 0.4% NaCl + 0.1 g/L SA										3.0				0.60
Chumpolkulwong, et al. (1997)			1.Basal + SA	mixotrophic	7					20	5.50					0.55	
			2.Basal + SA + 1.3 mM Compactin								4.50					0.45	
			3.Basal + SA + 1.7 mM Compactin								2.50					0.25	
			4.Basal + SA + 2.0 mM Compactin								0.43					0.04	

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Fabregas et al. (1998)		70	1.Control	mixotrophic	2 (12D:12L)	cool white fluorescent lamp	15 + CO_2		7.2 - 7.8	25	6.25		0		17.9	0.446	0
			2.Control (No KNO_3)								1.83		23.5		5.5	0.131	1.679
			3.Control (No $MgSO_4$)								2.65		6.7		4.2	0.189	0.479
			4.Control (No KNO_3+$MgSO_4$)								1.58		15.6		4.6	0.113	1.114
			1.Control	mixotrophic	11.5 (12D:12L)	cool white fluorescent lamp	15 + CO_2		7.2 - 7.8	25	4.75		37.5		10.9	0.337	2.679
			2.Control (No KNO_3)								1.15		49.5		4.8	0.082	3.536
			3.Control (No $MgSO_4$)								3.25		25.7		3.1	0.232	1.836
			4.Control (No KNO_3+$MgSO_4$)								1.28		28.8		3.8	0.091	2.057

Table 2.4 (cont) Effect of medium on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Monstant et al. (2001)			2 step 1.BG-11 2.BG-11 (No nitrate)	mixotrophic	5				6.8 - 7.5	25		1.33mg/cell 8.1mg/cell		6.12pg/cell 241pg/cell	29.14pg/cell 47.25pg/cell		
Chumpolkulwong et al. (1997)			Modified	mixotrophic	7					20	2.808		13.46			0.468	1.346

Table 2.5 Effect of light intensity on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5}cell/mL.day)	productivity of astaxanthin (mg/L.day)
Fabregas et al. (2000)	tube	70	Initial	mixotrophic	0.4	daylight fluorescent lamp	15+ CO_2		8		1.5					0.3	
					2						1.46					0.29	
					3.9						1.06					0.21	
					8.85						0.87					0.17	
Harger et al. (1996)	flask	250	Bold's basal	autotrophic	2						0.81		2.7			0.027	0.09
					37						1.38		9.9			0.046	0.33
					89						0.27		1.8			0.009	0.06
Zhang et al. (1999)	stirred tank	3700	Hong Kong	mixotrophic (batch)	45		100 $+CO_2$	350		30			29.3				1.47
					90								42.0				2.10
					180								52.6				2.63
					360								63.3				3.17
	stirred tank	3700	Hong Kong	mixotrophic (fed-batch)	45		100 $+CO_2$	350	7	30			45.4				2.27
					90								60.0				3.00
					180								74.5				3.72
					360								82.7				4.16

Table 2.6 Effect of pH on cells density, dry cell weight, astaxanthin concentration, carotenoid concentration, chlorophyll concentration, productivity of cell and productivity of astaxanthin under different condition

reference	reactor	vol of reactor (mL)	medium	condition	light intensity (klux.)	light source	aeration rate (L/hr)	agitation rate (rpm)	pH	Temp. (°C)	cell number (x10^{-5}cell/mL)	dry cell weight (g/L)	astaxanthin conc. (mg/L)	carotenoid conc. (mg/L)	chlorophyll conc. (mg/L)	productivity of cell (x10^{-5} cell/mL.day)	productivity of astaxanthin (mg/L.day)
Sarada et al. (2001)	flask		Basal	mixotrophic	1.5				6	25±1			0.08	0.24	0.29		0.006
									7				2.12	2.40	5.32		0.163
									8				1.64	2.16	4.45		0.126
									9				1.00	1.40	1.95		0.077
	flask		Basal+NaCl+ SA	mixotrophic	1.5				6	25±1			1.20	1.42	0.19		0.052
									7				5.26	6.36	1.45		0.229
									8				3.07	3.72	1.25		0.133
									9				1.09	1.20	0.58		0.047

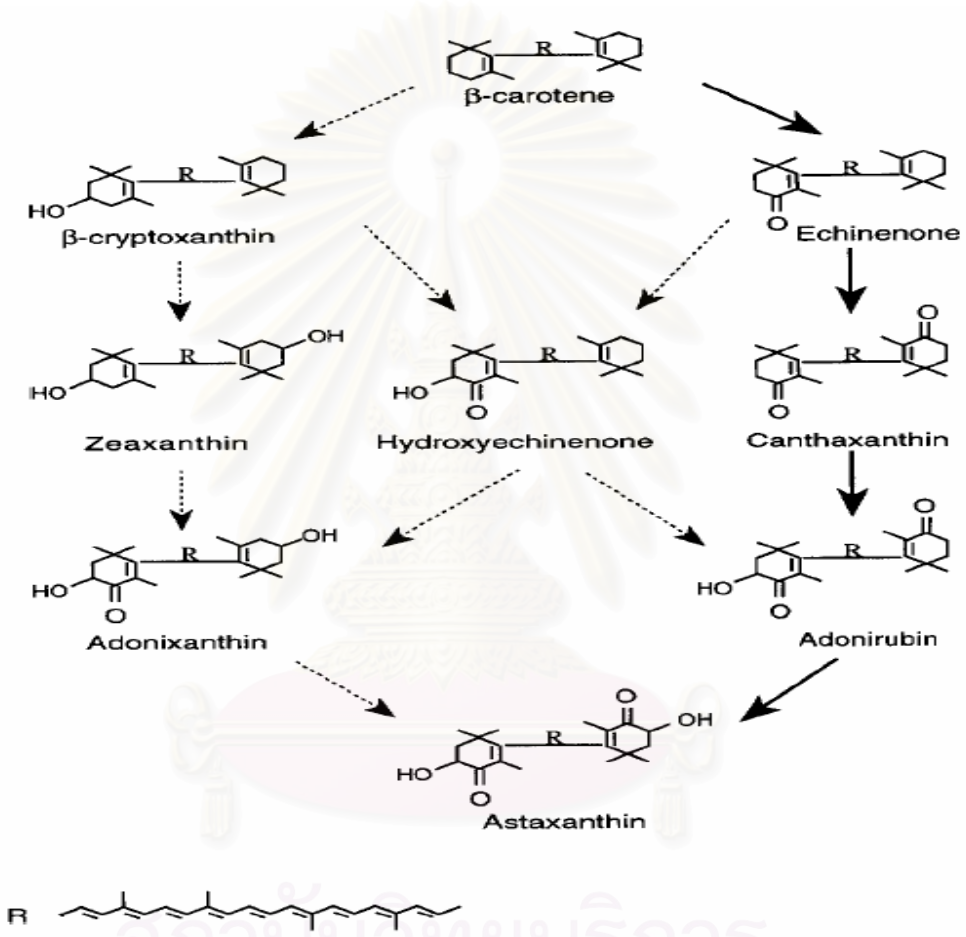
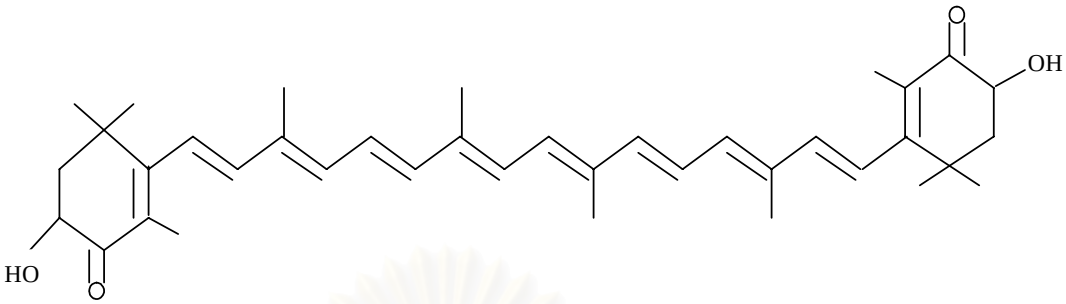
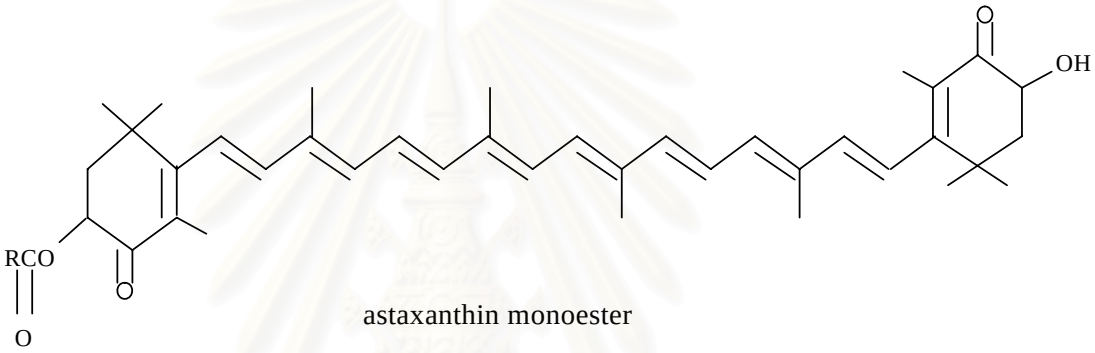


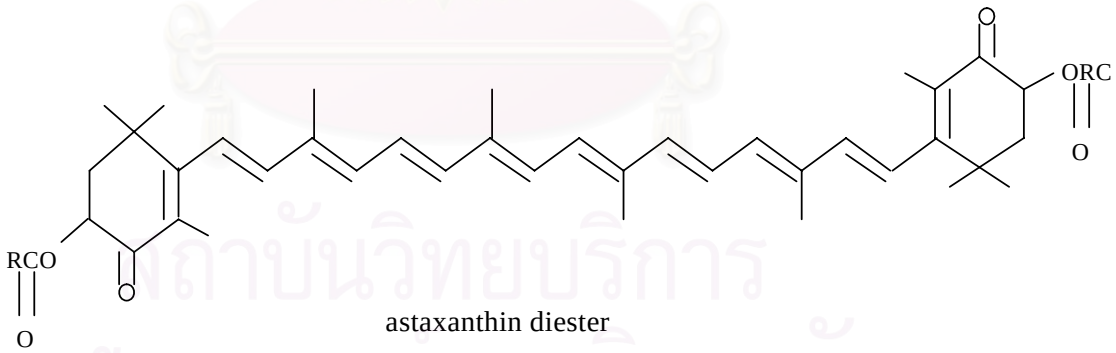
Figure 2.4 Pathway for astaxanthin biosynthesis in H. pluvialis (Logan, 1995)



astaxanthin



astaxanthin monoester



astaxanthin diester

Figure 2.5 Configuration of astaxanthin

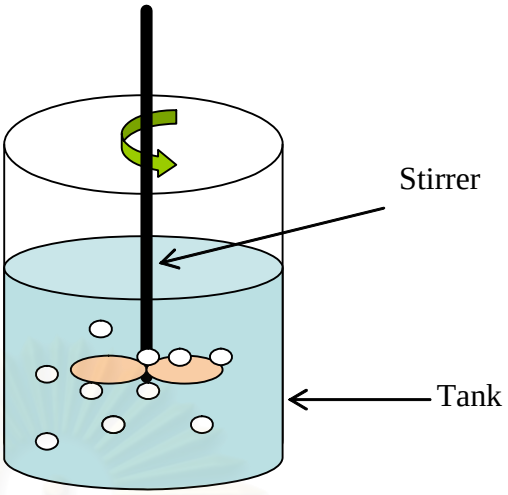


Figure 2.6 Configuration of stirred tank reactor

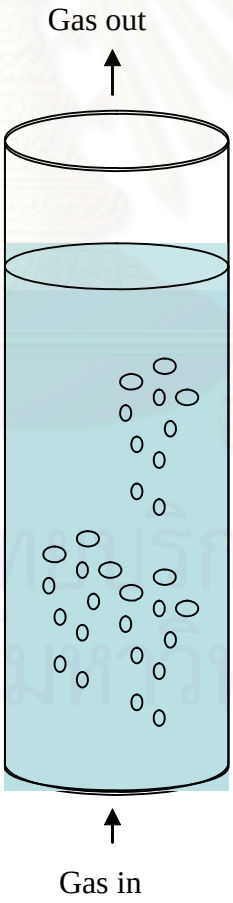


Figure 2.7 Configuration of bubble column

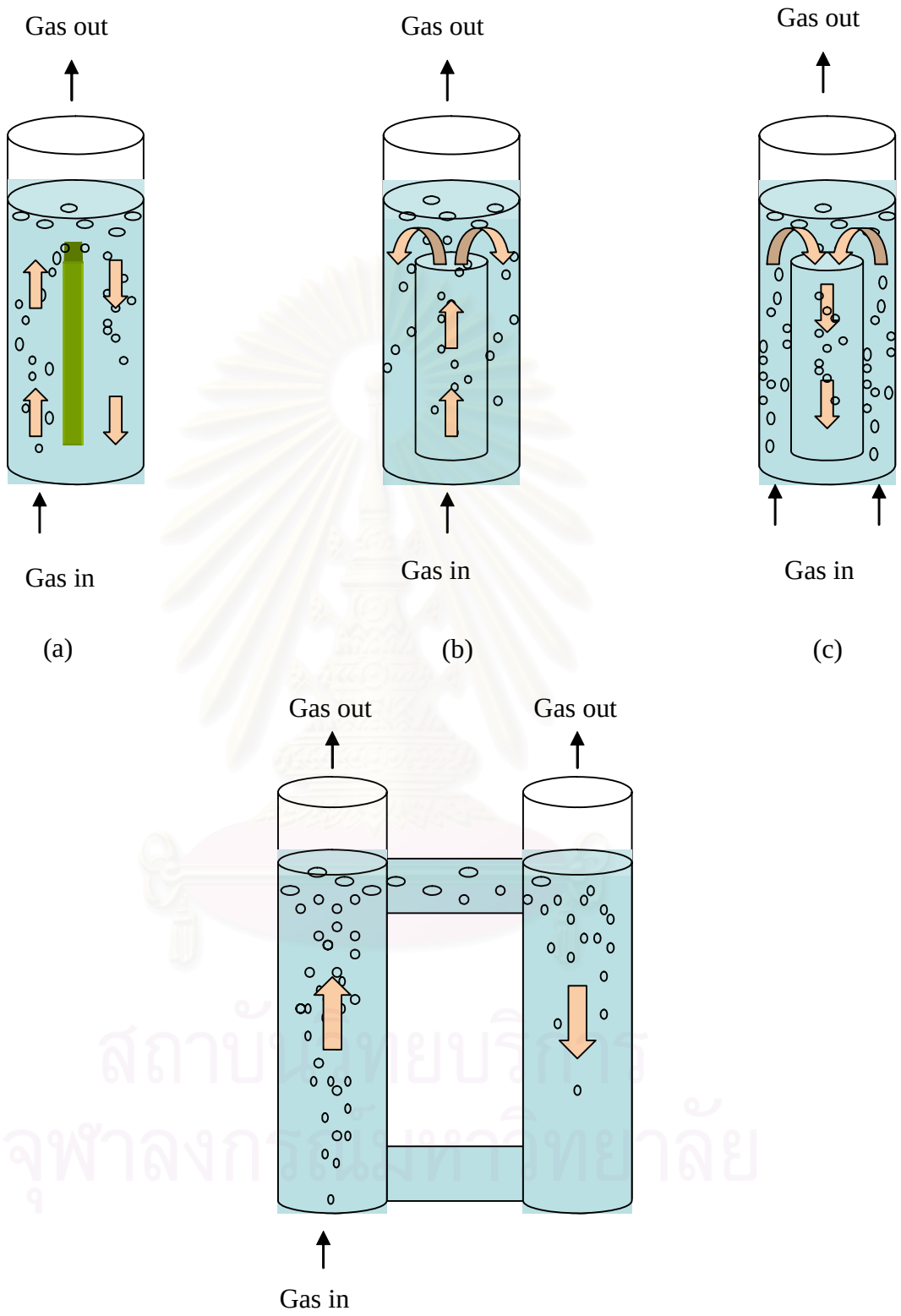


Figure 2.8 Configuration of airlift reactor: (a) split cylinder loop airlift reactor; (b)-(c) concentric tube airlift reactor; (d) external loop airlift reactor

CHAPTER III

Materials and Methods

3.1 Experimental Method

The experiment in this work can be divided into three parts:

(i) determination of suitable growth medium (in flask).
(ii) determination of suitable operating condition (in airlift bioreactor).
(iii) preliminary examination of astaxanthin production.

For the first part, the effect of the type of medium and vitamin were studied whereas the effect of aeration, the ratio between the downcomer and riser cross sectional area (A_d/A_r), gas velocity and light intensity were studied in the bioreactor. Finally, the condition very high concentration was chosen as the most appropriate condition for growing *H. pluvialis*. For astaxanthin production, astaxanthin is induced by changing the cell environment to stress promoting condition.

3.2 Determination of suitable condition

3.2.1 Algal Strain

A green alga, *H. pluvialis* strain NIES-144 was obtained from the National Institute of Environmental Studies, Tsukuba, Japan.

3.2.2 Medium

1) prepare M1, M6, Basal, BG-11, F1, Hong Kong and Basal:BG-11(1:1) mediums with chemical compositions as summarized in Table 3.1 (7 types of growth medium)
2) sterilize the mediums in autoclave at 121°C for 20 min

3.2.3 Preparation starter innoculum

1) inoculate 10% by volume of cell into 600 mL sterilized fresh F1 medium in 1,000 Erlenmeyer flask
2) incubate the flask at 27±1°C and supply continuous light intensity of 20 μ mol photon m^{-2} s^{-1} to the surface of the culture vessel
3) shake the flask twice a day manually
4) 7 day culture (green motile cells) was employ as starter culture of *H. pluvialis* for all experiment

3.2.4 Experimental procedure

3.2.4.1 Determination suitable growth condition in flask

1) prepare starter innoculum as described in Section 3.2.3
2) prepare the nutrient as described in Section 3.2.2
3) add 10% by volumn of innoculum into a 100 mL of M1 culture medium in 250 Erlenmeyer flasks
4) illuminate the flask with the fluorescent lamp (18 W) at continuous light intensity of 20 μ mol photon m^{-2} s^{-1} to the surface of the culture vessel. The temperature was controlled in the range of 27±1°C.
5) shake the flask twice a day manually
6) measure cell growth as described in Section 3.5
7) repeat Step 1-5 with different types of medium (M6, Basal, BG-11, F1, Hongkong and Basal:BG-11(1:1))

8) with the most suitable type of medium, repeat Step 1-5 with varying the vitamin B complex. Vitamin B complex used in this work was purchased from the government pharmaceutical organization which has the composition of vitamins B1, B6 and B12 as shown in Table 3.2. In the discussion hereafter the vitamin B complex will be refered to using the vitamin B12 content.

3.2.4.2 Batch experiment

1) inoculate cell into the sterilized fresh medium in culture vessel
2) culture cells until the stationary phase of growth is observed
3) harvest cell

3.2.4.3 Semicontinuous experiment

1) inoculate cell into the sterilized fresh medium in culture vessel
2) culture cells until the exponential phase of growth
3) a 50 percentage of volume of culture was harvested every three and four days and replaced with the fresh culture media. Each run was repeated three time

3.3 Determination of suitable operating condition

3.3.1 Experimental setup

The schematic diagrams of experimental setup in bioreactor are shown in Figures 3.1 and 3.2.

All bioreactors were made of a clear acrylic plastic. The airlift bioreactor was composed of the draft tube installed centrally inside of outer column. The configuration of airlift bioreactor and bubble column are shown in Figure 3.3 and 3.4, respectively and with their dimensions as detailed in Table 3.3.

The liquid culture in the bioreactor was agitated by rising air bubbles introduced into the base of the bioreactor through the sparger assembly. Ambient

air from an aquarium pump was metered through a flowmeter, sterilized with a 0.45 μm Gelman autoclavable filter, was passed into the culture at the base of the bioreactor. In the case with CO_2 addition, ambient air from an aquarium pump and CO_2 gas was metered through a flowmeter, and then bubbled through water. The CO_2 enriched air was introduced and was metered through a flowmeter, sterilized with a 0.45 μm Gelman autoclavable filter and then through the culture at the base of the bioreactor.

Light for the operating in bioreactor consisted of a vertically 18 W fluorescent lamp that provide adequate intensity. The illumination intensity incident to the bioreactor outer surface was measured in units of lux with a digital lux meter.

Photon flux density and irradiance may be readily interconverted as:

$$1\ \mu mol\ (m^{-2}\ s^{-1}) = 1\ \mu E\ m^{-2}\ s^{-1} = \frac{119.7}{\lambda}\ W\ m^{-2} = 50\ lux$$

where λ = the wavelength of the light in nanometer

3.3.2 Procedure

3.3.2.1 Preparation of the bioreactor

1) setup the bioreactor as described in Section 3.3.1
2) fill the water into the bioreactor
3) sparge ozone gas through the 0.45 μm Gelman autoclavable filter and a flow meter into the water at the base of the bioreactor for 1 h in order to clean the whole system
4) sparge the air through the 0.45 μm Gelman autoclavable filter and a flow meter into the water at the base of the bioreactor for 3-4 h to remove residual ozone in the water

3.3.2.2 Determination suitable growth condition in bioreactor

1) prepare starter innoculum as described in Section 3.2.3
2) prepare nutrient which yield highest growth obtain from Section 3.2.4.1, and prepare a bioreactor as described in Section 3.3.2.1
3) inoculate the bioreactor with 250 mL of cell with an initial density of 2×10^4 cells mL^{-1}
4) culture cells (duplicate) in the bioreactor where air was sparged through the bioreactor at superficial gas velocity of 0.4 cm s^{-1}
5) illuminate the bioreactor with the fluorescent lamp (18 W) at continuous light intensity of 20 μ mol photon m^{-2} s^{-1} to the surface of the bioreactor. The temperature is controlled in the range of 27±1°C.
6) measure cell growth as described in Section 3.6
7) repeat Steps 1-5 with the ratio between the downcomer and riser cross sectional area of 0.9 and 3.2
8) repeat Steps 1-5 with superficial gas velocity range indicated in Table 3.4
9) repeat Steps 1-5 with light intensity indicated in Table 3.4

Note that the samples was collected from the sampling point at the side of the reactor as indicated in Figure 3.1 and 3.2, respectively. The number of cells shown in the Results and Discussion Section included both vegetative cells and cysts unless stated otherwise.

3.4 Production of astaxanthin

1) repeat Section 3.3.2.2 with the most suitable conditions
2) when algae cells reached the stationary phase, increase the aeration (in terms of superficial gas velocity) from 0.4 to 2 cm s^{-1} (these levels were obtained from the results from Section 3.3.2.2: see Chapter 4 for the discussion)
3) test for astaxanthin accumulation by increasing the light intensity from 20 to 140 μmol photon m^{-2} s^{-1} (these levels were also obtained from the results from Section 3.3.2.2: see Chapter 4 for the discussion)

4) take 3-5 mL of sample for the determination of chlorophyll *a* and *b* and astaxanthin content
5) repeat Steps 1-3, but this time, keep the light intensity constant at 20 μmol photon m^{-2} s^{-1}, stop the aeration for a good settling of cells
6) replace 50% of the medium volume by distilled water to test for astaxanthin accumulation
7) take 3-5 mL of sample for the determination of chlorophyll *a* and *b* and astaxanthin content

Note that, in the sample collection in this experiment, aeration was firstly supplied into the reactor to generate adequate mixing before the sample collection from the side sampling port.

3.5 Determination of growth

Algal cell growth was determined by cell density, dry weight and specific growth rate.

3.5.1 Determination of cell density

Cell density was measured by microscope counting using an improved Neubauer haemacytometer.

1) take two 25 μL drop of culture and place them on clean microscope slide
2) place a clean coverslip carefully on the drop so that the drop is evenly dispersed under the coverslip
3) count cells under a microscope (objective 10 x). Count cells which touch the upper and left border but not those which touch the lower and right borders (see Figure 3.5)
4) do all counts at least in duplicate
5) calculate the number of cells as follow:

$$\text{Cells number (N; cell·mL}^{-1}) = \frac{n_1+n_2}{8} \times 10^3 \quad (3.1)$$

where n_1 and n_2 (cells) = number of cells counted in upper and lower grid

3.5.2 Determination of dry weight

Dry weight of algal cells was determined as follow:

1) dry a Whatman GF/C filter (47 mm in diameter) in the oven at 70°C for 24 h and weight
2) filter 30 mL of algae suspension using Bunchner setup connected to a vacuum pump through a Whatman GF/C filter
3) wash the algae twice with distilled water in order to free the algae from insoluable
4) dry the filter containing algae in oven under the above condition
5) cool the dried filter containing algae in a desicator for 20 min
6) weight the dried filter containing algae

$$\text{Dry weight (DW; g L}^{-1}) = \frac{(W_1-W_2)\times 1000}{V} \quad (3.2)$$

where W_1 = weight of filter plus algae (g)
W_2 = weight of filter (g)
V = volumn of sampling (mL)

3.5.3 Determination of specific growth rate

The specific growth rate was calculated by the following equation:

$$\text{Specific growth rate } (\mu; d^{-1}) = \frac{Ln(N_2)-Ln(N_1)}{t_2-t_1} \quad (3.3)$$

where N_1 and N_2 = cells density at t_1 and t_2 (cell·mL^{-}1)

t = time (day)

3.5.4 Determination of productivity

The productivity was calculated by the following equation:

$$\text{Productivity (cells mL}^{-1}\text{ day}^{-1}) = \frac{C_2-C_1}{t_2-t_1} \quad (3.4)$$

where C_1 and C_2 = cells density at t_1 and t_2 (cell·mL^{-1})

t = time (day)

3.6 Determination of chlorophyll and astaxanthin

1) centrifuge 3-5 mL of culture sample at 2,500 rpm for 20 min
2) discard the supernatant and wash the cell pellet with distilled water
3) add 2-3 mL of 90% acetone with the remaining cell pellet
4) break cells by manual homogenizer in order to extract the total pigment from the cells
5) repeat the extraction procedure until the cells debris was completely colorless
6) separate the mixture by centrifugation at 2,500 rpm for 15 min
7) collect the supernatant containing pigments
8) conduct all of the above process in darkness
9) fill both spectrometer cell with 90% acetone and find cell-to-cell blank
10) fill both spectrometer cell with the supernatant containing pigments determine chlorophyll *a* and *b* and astaxanthin content at 665 and 645 and 480 nm respectively
11) collect the measured absorbance by calculate the concentration of pigments from Strickland and Persons (1977) equation
12) calculate the concentration of pigment from equation

$$\text{mg pigment/m}^3\ (\mu\text{g/L}) = C/V \quad (3.5)$$

where V = volumn of filtered sample in litre

C = value obtained from the following equation

C(chlophyll *a*) = $11.6E_{6650}-1.31E_{6450}-0.14E_{6300}$

C(chlophyll *b*) = $20.7E_{6450}-4.34E_{6650}-4.42E_{6300}$

C(total carotenoid) = $4.0E_{4800}$

where E_i = extrinction values, at wavelengths indicated by the subscripts (A°)

Remark: It is assumed here that the total carotenoid extracted from the cell consisted mostly of astaxanthin and absorbance of 480 nm, which was originally designed for the measurement of total carotenoid (Francis et al., 1972 as refer to in Jeffrey et al., 1997) can be applied accordingly.

Table 3.1 Formulation of culture media

	M1(Chen et al., 1997) (per liter)	Basal (Ogbonna et al., 2001) (per liter)	F1 (Fábregas et al., 1998) (per liter)	BG-11 (Monsant et al., 2001) (per liter)	Hongkong (Zhang et al., 1999) (per liter)	M6 (Ogbonna et al., 2001) (per liter)
$CaCl_2 \cdot 2H_2O$	183.8 mg	25 g	9.78 mg	36 mg	73 mg	3.676 g
KNO_3	0.5 g		0.41 g		0.3 g	
$NaNO_3$				1.5 g		
Na_2HPO_4			0.03 g			
NaH_2PO_4	195 mg				35.5 mg	1.778 g
H_3PO_4	0.12 mg					12.37 mg
$K_2HPO_4 \cdot 7H_2O$		75 mg		40 mg		
KH_2PO_4		175 mg				
NaCl		2.513 g				
KOH		30.85 mg				
H_2SO_4		0.99 mg				
EDTA	18.6 g	49.34 mg		1 mg		
$C_6H_5FeO_7 \cdot 5H_2O$			2.21 mg			
$FeSO_47H_2O$		4.976 mg				417 mg
$MgSO_4 \cdot 7H_2O$	61.6 mg	74 mg	16.41 mg	75 mg	24.6 mg	1.231 g
$ZnSO_4$	0.72 mg				0.014 mg	71.89 mg
$CuSO_4 \cdot 5H_2O$	0.62 mg	1.572 g	0.008 mg		0.012 mg	62.42 mg
$Na_2MoO_4 \cdot 2H_2O$	0.07 mg		0.08 mg		0.001 mg	7.26 mg
$CoCl_2 \cdot 2H_2O$	0.05 mg				0.0005 mg	4.67 mg
$MnCl_2 \cdot 4H_2O$		1.445 mg				
MoO_3		0.705 mg	0.66 mg			
$CoNO_3 \cdot 6H_2O$		0.389 mg				
H_3BO_3		10.948 mg			0.003 mg	
Cr_2O_3			0.05 mg			
SeO_2			0.036 mg			
$CoCl_2 \cdot 6H_2O$			0.0078 mg			
$ZnSO_4 \cdot 7H_2O$		8.827 μg				
$EDTANa \cdot 2H_2O$					6.7 mg	0.372 g
$FeSO_4 \cdot H_2O$					8.3 mg	
$FeSO_4 \cdot H_2O$					0.016 mg	
Na_2CO_3				0.02 g		
$NH_4Fe(C_6H_5O_7)$			6 mg			
H_3BO_3				0.86 g		
$MnCl_2 \cdot 4H_2O$				1.81 g		
$ZnSO_4 \cdot 7H_2O$				0.222 g		
$Na_2MoO_4 \cdot 2H_2O$				0.39 g		
$CuSO_4 \cdot 5H_2O$				79 mg		
$Co(NO_3)_2 \cdot 6H_2O$				49 mg		

Table 3.2 Content of vitamin B complex (vitamins B1, B6 and B12)

vitamin B1 ($mg\ L^{-1}$)	vitamin B6 ($mg\ L^{-1}$)	vitamin B12 ($\mu g\ L^{-1}$)
5.3	0.4	4
10.7	0.8	8
16	1.2	12
21.3	1.6	16
26.7	2	20

Table 3.3 Geometric details of airlift bioreactor used in cell cultures of *H. pluvialis*

	Type of reactor	
	Airlift bioreactor	Bubble column
Total volumn (L)	3.6	3.6
Working volume (L)	3	3
Reactor diameter (cm)	10	10
Column height (cm)	60	60
Liquid height (cm)	46	46
Draft tube height (cm)	40	40
Diameter of draft tube (cm)	4.6,6.6	-
Bottom clearance (cm)	4	4

Table 3.4 Variation of growth factor in the determination of optimal condition growth

Culture condition	Type of reactor	Aeration	A_d/A_r	Superficial gas velocity ($cm\ s^{-1}$)	Light intensit ($\mu mol\ photon\ m^{-2}\ s^{-1}$)
aeration	glass bubble column	no aeration	-	-	
	airlift bioreactor	0%CO_2 (by volumn)	3.2	0.4 (batch)	20
	airlift bioreactor	1%CO_2 (by volumn)	3.2	0.4 (batch)	20
A_d/A_r	airlift bioreactor	1%CO_2 (by volumn)	0.9, 3.2	0.4-1 (batch)	20
superficial gas velocity	bubble column	1%CO_2 (by volumn)	-	0.4-1 (batch)	20
	airlift bioreactor	1%CO_2 (by volumn)	3.2	0.4-3 (batch)	
light	airlift bioreactor	1%CO_2 (by volumn)	3.2	0.4	20-60 (batch) 20 (semicontinuous)

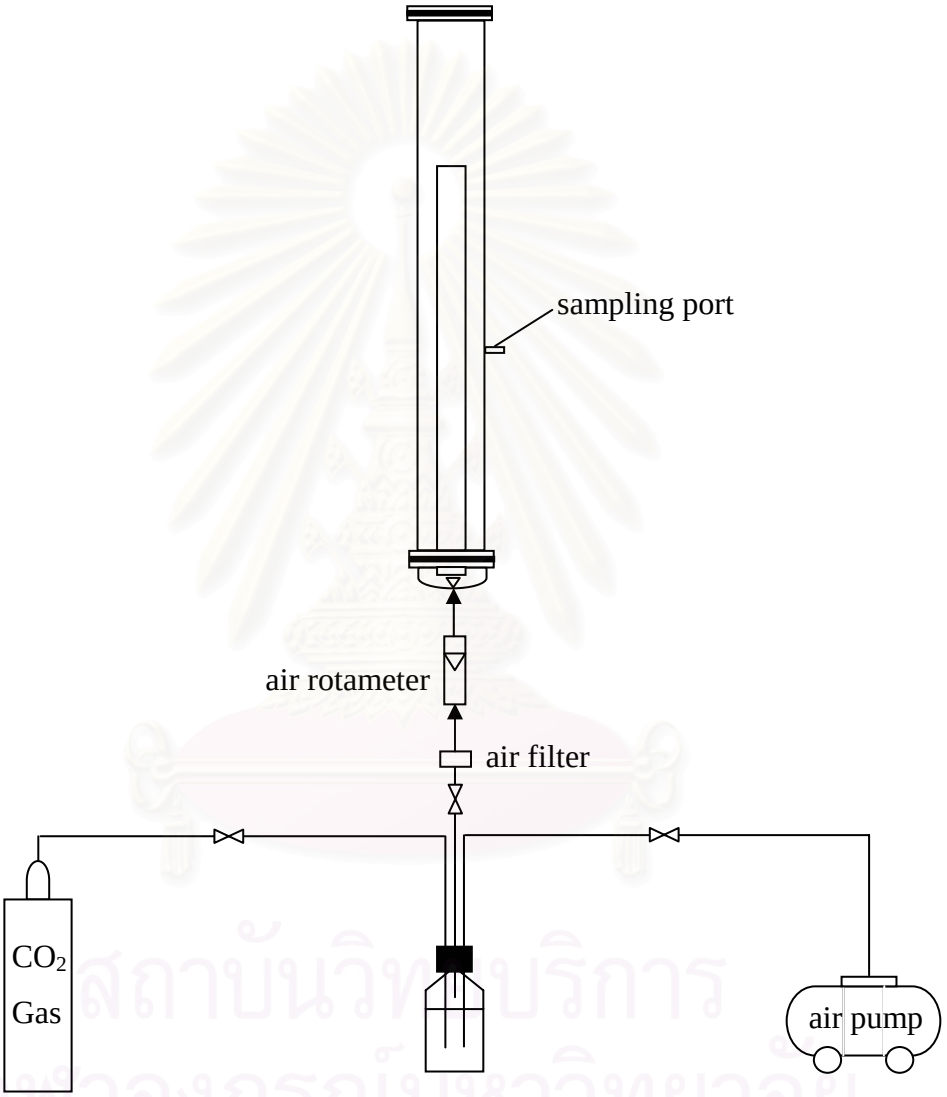
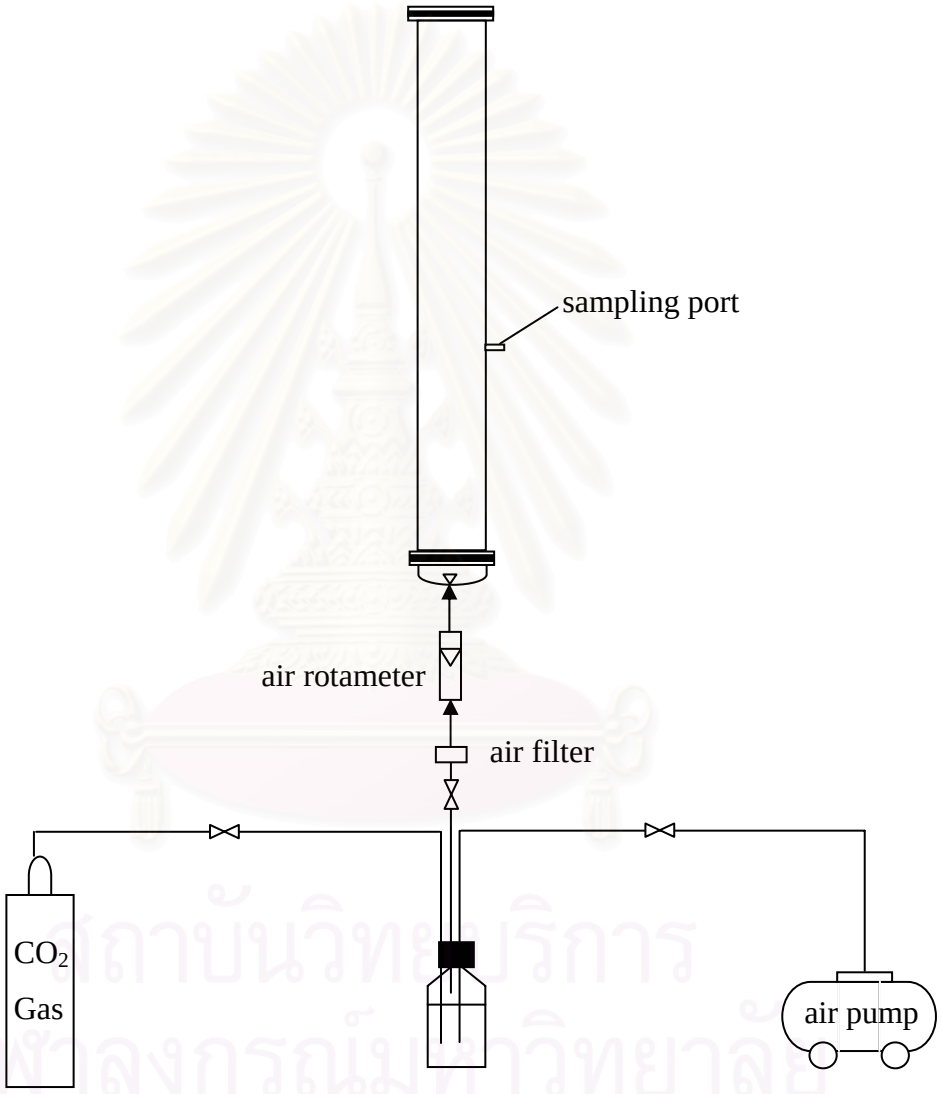


Figure 3.1 Experimental setup for airlift bioreactor



3.2 Experimental setup for bubble column

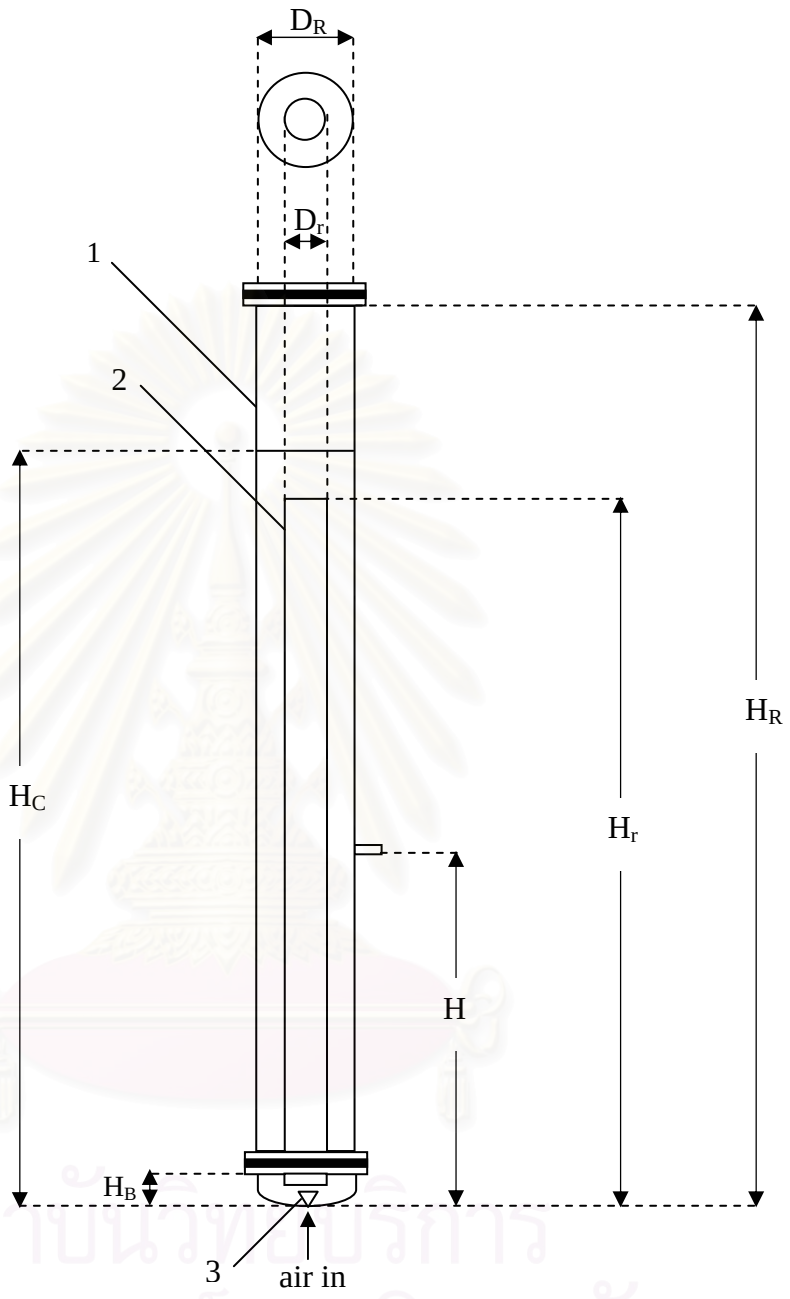


Figure 3.3 Schematic diagram of airlift bioreactor:

Symbol: 1, reactor column; 2, inner draft tube; 3, sparger; H_R, column height; H_r, draft tube height; H_B, clearlance height; H_C, culture broth height; H, sampling port height;

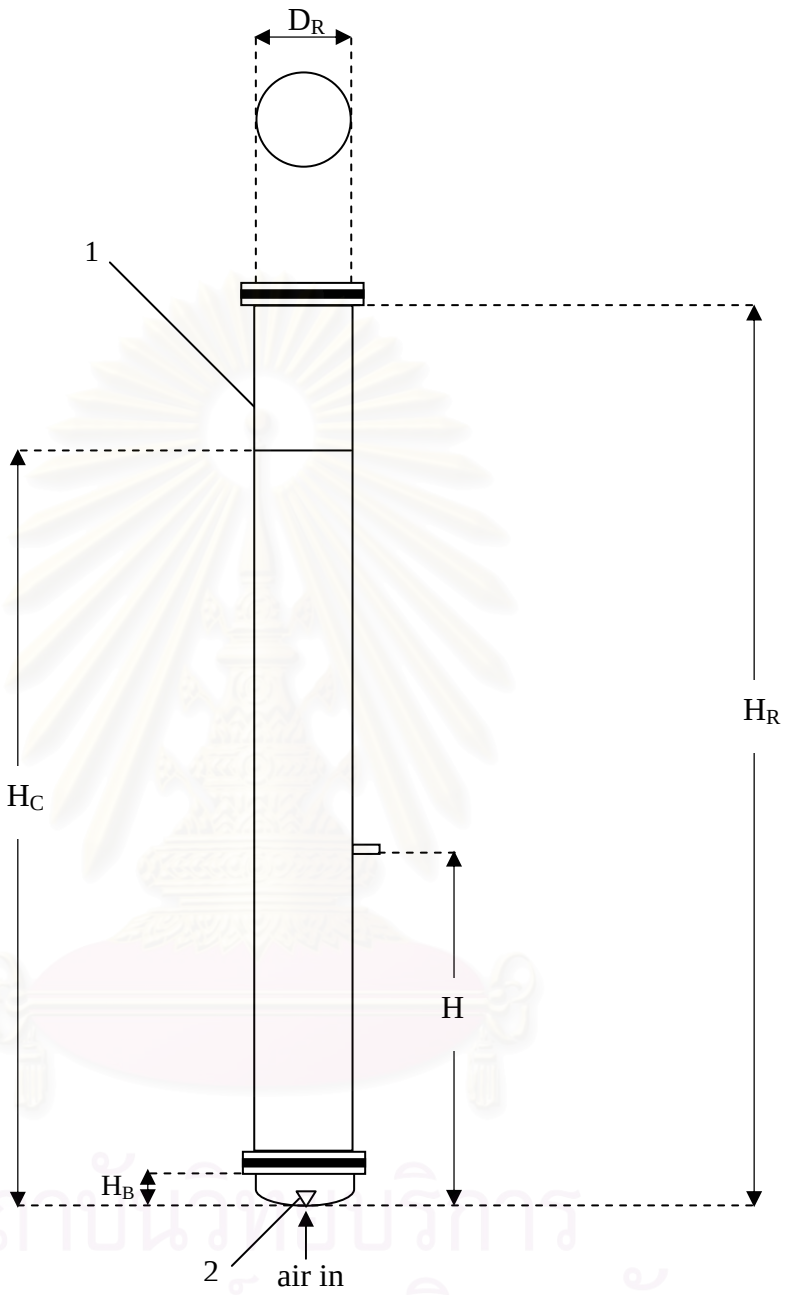
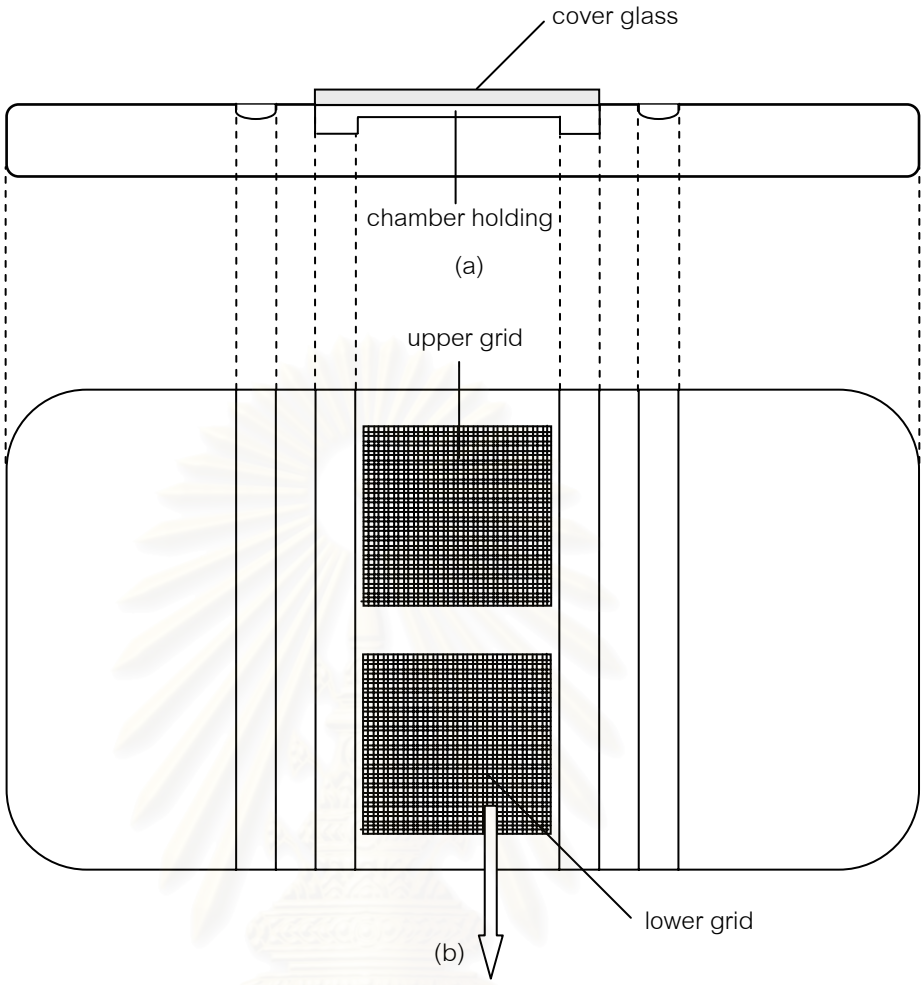
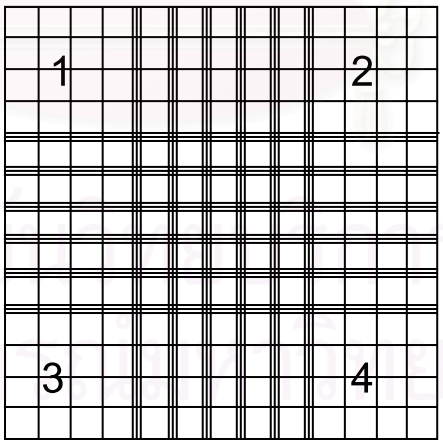


Figure 3.4 Schematic diagram of bubble column

Symbol: 1, reactor column; 2, sparger; H_R, column height; H_B, clearlance height; H_C, culture broth height; H, sampling port height; D_R, reactor diameter





(c)

Figure 3.5 (a) Side view of the cell counting chamber showing the cover glass and the space beneath it that holds a microalgae suspension. (b) Top view of the chamber. The chamber has two grids located in the center of the slide. (c) An enlarged view of the grid. The microalgae in the Squares 1, 2, 3 and 4 are used for cell count.

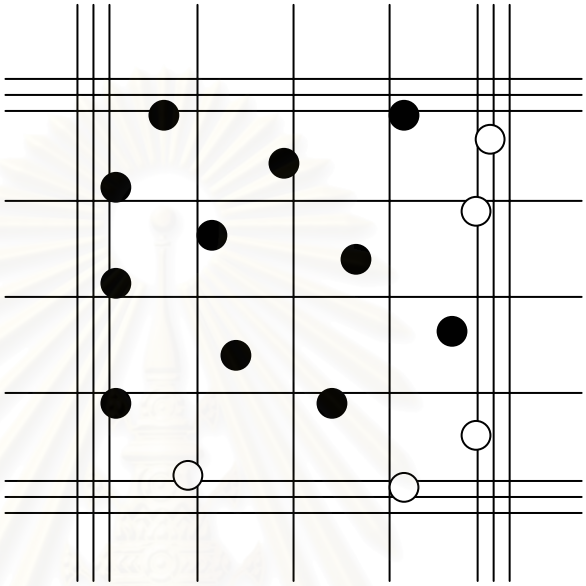


Figure 3.5 (d) Counting cell density

count the cells in the square and those which touch the top and left border (●). do not count the ones touching the right and lower border (○) (see down right corner).

CHAPTER IV

Results and Discussion

4.1 Determination of suitable growth medium

The cultivation of *H. pluvialis* for astaxanthin production involves two growth stages. The first stage is to obtain high cell density without the synthesis of the required product, astaxanthin. Cells grow in this stage is called vegetative cells. Astaxanthin only accumulates during the process of transformation of green vegetative cells to cyst (aplanospore stage). In the following discussion, each of the cultivation stage will be discussed in detail.

4.1.1 Effect of the type of medium

The medium is always important for an effective cultivation of microorganisms. Several attempts have been conducted to establish the suitable nutrient required for the growth of *H. pluvialis*. Often, each study reported one unique type of medium that was proven to be suitable for the growth of this alga. This simply means that the differences in environmental conditions could lead to a slight variety in the medium compositions. It was not the intention of this work to formulate a new recipe of the growth medium, however, it was aimed to investigate the suitability of the use of such various types of culture medium for the growth of *H. pluvialis*. Specifically, this section was set out to investigate whether there were differences in using the various reported medium types in growing *H. pluvialis*. Seven types of growth mediums were employed here as described in Chapter 3. The results from this experiment are displayed in Figure 4.1 which illustrates the effect of different culture mediums on cell density grown in the 250 mL Erlenmeyer flasks. Surprisingly, it was observed that the growth of *H. pluvialis* was greatly influenced by type of culture medium. The growth was

found to be worst in M1 and M6 mediums and was best in the F1 medium, in which the attainable maximum cell density was 5.44×10^4 cells mL^{-1}. The growth in the Basal:BG-11 medium was comparable to that in the BG-11 medium alone. The Hongkong medium also resulted in similar growth character with the Basal:BG-11 and BG-11 mediums. For most of the cultures grown in this experiment, cyst was observed when the growth entered the stationary phase. This meant that some cells already became inactive and no further cell division was expected. However, although the cells started to lose their flagella (as they converted to cysts), no accumulation of astaxanthin was apparent in M1, F1 and Hongkong mediums even after the 13 days cultivation. This implies that cells could still live without stress but not quite as active as the vegetative cells. With these findings, the F1 medium was selected as the medium for the subsequent experiments.

The results on the specific growth rate obtained from the various mediums were similar to that of the maximum cell concentration. This was because cells entered the stationary phase at approximately the same time (8 day) and therefore the medium that provided the highest cell density also gave the highest specific growth rate. In this experiment, the control medium gave the highest growth rate of 0.21 day^{-1}. This was approximately at the same level as the reported value by Tjahjono et al. (1994) who achieved the maximum specific growth rate of about 0.25 day^{-1} using Basal growth medium grown in mixotrophic condition with sodium acetate as a carbon source. However, this level was about one third of the maximum reported value for the growth of such alga was 0.58 day^{-1} which was the cultivation in the flask with Basal as growth medium growing in mixotrophic condition (Kobayashi et al. 1992).

4.1.2 Effect of vitamin B concentration

Vitamin B was often reported to have significant effects on the growth of microalgae. For example, thiamine (B1) was considered as a growth factor for microalgae, vitamin B12 was employed to stimulate growth but was not essential (Pringsheim, 1996). Fabregas and coworkers (1998) found that *H. pluvialis*

required thiamine together with vitamin B12 and biotin in order to achieve maximum growth. In contrast, Gong and Chen (1997) stated that vitamin B (including thiamine and vitamin B12) and also biotin had no significant effect on the growth rate of *H.Pluvialis* (with the growth medium being "M6" with the composition shown in Table 3.2). In this work, vitamins had not been included in the seven types of medium examined above. The objective of this section was therefore to demonstrate the effect of the addition of vitamin B complex (vitamin B1, vitamin B6 and vitamin B12) on the growth of *H. pluvialis*. Note that vitamin B complex was obtained from common pharmaceutical stores.

The growth curves from the cultivation of *H. pluvialis* in the F1 medium with varying concentrations of vitamin B12 were shown in Figure 4.2. It was obvious that the maximum cell density increased with the concentration of vitamin B12 within the concentration range employed in this work. The maximum cell density increased with the concentration of vitamin B12 up to 20 μg/L. The effect of vitamin was found to be strong at a low range of vitamin B12 (4 to 12 μg/L). Above 12 μg/L, the effect of the vitamin was only slight. Therefore the vitamin B12 at 12 μg/L was considered as an optimal level for the growth of *H.pluvialis* with the F1 medium. At this vitamin concentration, the maximum cell density increased by 75% as compared with cell growth in the medium without the addition of vitamin.

In addition, the result in Figure 4.2 illustrates that as much as 1.4% increase in the specific growth rate was obtained as the vitamin B12 level increased from 4 to 12 μg/L. Further increase in vitamin B12 from 12 to 20 μg/L resulted in a smaller rate of increase in the specific growth rate (only 0.6% increase). Hence, it was concluded here that the vitamin B12 at 12 μg/L was best for the cultivation of *H. pluvialis* in the F1 medium.

4.2 Cultivation of *H.pluvialis* in bioreactors

4.2.1 Comparison between performance of bubble column and airlift bioreactor

A variety of photobioreactors have been applied for algal culture. As *H. pluvialis* is highly sensitive to shear stress, reactors that induce high shear such as the commonly known stirred tanks are not recommended. Fortunately, *H. pluvialis* is a slow growth culture which does not require a high rate of mass transfer and mixing therefore, the use of high performance but energy intensive stirred tanks is not necessary. Pneumatic bioreactors immerge as an ideal alternative for such microorganism. In pneumatic systems such as bubble columns or airlift bioreactors, the mixing and mass transfer are induced just by the aeration which generates very low level of shear and also is much less energy intensive than the stirred tank. In particular, the flow pattern obtained in the airlift system could result in an effective light utilization along the length of the reactor as will be discussed later on in this section.

In this experiment, the cultivations of *H. pluvialis* in both airlift bioreactor and bubble column were investigated over the same range of superficial gas velocity. Figure 4.3 shows the comparison between the performances of these two systems which illustrated that the specific growth rate of *H. pluvialis* in the airlift bioreactor was always better than that in the bubble column at the same operating condition (same gas throughput). Note that the airlift used in this experiment had the ratio between downcomer and riser (A_d/A_r) of 3.2.

As stated earlier, the configuration in the airlift bioreactor provided a well defined flow pattern compared with the random flow pattern in the bubble column (Merchuk et al., 1998). Therefore most cells in the airlift system would circulate along the axial direction of the reactor and would be exposed to light which was supplied along the reactor length (see Figure 4.4 for a schematic fluid flow direction in the airlift system). In other words, the uniform flow pattern in airlift bioreactor led to a certain movement of cells from dark zone to light zone. In the bubble column, on the other hand, no clear flow pattern was induced and therefore

the movement of the cells inside the reactor was random, i.e. cells may have stayed at the regions with high or low light intensities for a long time without being recirculated. As cells were better exposed to light in the airlift bioreactor than in the bubble column, it was anticipated that more photosynthesis took place more significantly in the airlift system. Therefore a better cell growth was observed in the airlift system than in the bubble column. In addition, visual inspection always suggested that there were a number of cell agglomerations which resulted in the sedimentation of cells in the bubble column, the condition which was not found in the airlift bioreactor. Changes in morphology of algae were already observed in the bubble column even at the low level of aeration rate which indicated that the condition in the bubble column might not be suitable for the algal growth.

4.2.2 Effect of CO_2

Chemical analysis has shown that algal biomass consist of 40 to 50% carbon (Rubio et al., 1999). Hence, growth rate of photoautotrophic cultures essentially depends on a sufficient supply of carbon source for photosynthesis. It was previously reported that the principal carbon source of *H. pluvialis* was CO_2. Unlike other algae, *H. pluvialis* can also grow as heterogeneous culture which utilizes some other organic carbon sources in small amount and acetate is commonly used as a complementary organic carbon source (Chen et al., 1997; Dong and Zhao, 2004). The specific growth rate of vegetative cells under heterotrophic condition was, however, considerably lower than that obtained under photoautotrophic condition (0.22 day^{-1} compared to 0.32 day^{-1}) (Kobayashi et al., 1991). Although Kobayashi et al. (1997) reported the potential of obtaining high cell density in heterotrophic condition as the cells could remain in the vegetative form for a long time, no such achievement has so far been reported. This was possibly due to the fact that contamination became a significant problem for the heterotrophic culture.

In this work, *H. pluvialis* was grown in photoautotrophic condition with CO_2 as the main carbon source. To investigate the effect of CO_2 on the growth of the alga, experiments were performed in two airlift bioreactors, with and without the addition of 1% by volume CO_2 into the air stream supplied to the bottom of the system. Both airlift bioreactors were operated with superficial gas velocities of 0.4 and 2 cm/s and with A_d/A_r of 3.2.

Let us firstly discuss the experimental results obtained from the system running with the superficial gas velocity of 0.4 cm/s. The results in Figures 4.5 revealed that the maximum cell density and specific growth rate of 77 $\times 10^4$ cells mL^{-1} (2.79 g L^{-1} dry weight) and 0.45 day^{-1}, respectively, could well be obtained in the culture with the addition of 1% by volume CO_2. These were equivalent to a 68% increase in the max cell concentration, and 37% increase in the specific growth rate when compared with the growth in the system without the addition of CO_2. This clearly indicated that the growth rate of *H. pluvialis* was significantly influenced by the presence of CO_2.

The same figure illustrates that the influence of CO_2 on algal growth was not quite significant at higher gas throughput. At the superficial gas velocity of 2 cm s^{-1}, the addition of CO_2 could only marginally improve the growth of the alga in terms of maximum cell density (27% increases). The specific growth rate was reasonably enhanced (by 33%) but not in the same extent as that obtained at superficial velocity of 0.4 cm s^{-1}. This result was further discussed in the next section on the effect of aeration.

4.2.3 Effect of aeration

During the vegetative growth period, *H. pluvialis* is the algae with flagella which allow it to move around by itself. Hence, even though there is no mixing provided, the cells can move themselves to the location suitable for their growth. This is why it was important to initially check whether the mixing is beneficial for the growth of such micro-organism. Thus the experiment was performed in the airlift bioreactor with the superficial gas velocity of 0.4 cm s^{-1} and with the ratio

between cross sectional area of downcomer and riser of 3.2 compared with the experiment in the column without aeration, and the results are shown in Figure 4.6.

It was illustrated that *H. pluvialis* grew much better in the airlift bioreactor with the achievable maximum cell of 23.83 x 10^4 cells mL^{-1}, or 0.73 g L^{-1} dry weight, compared with 3.59 x 10^4 cells mL^{-1}, or 0.08 g L^{-1} dry weight, in the unaerated column. This indicated that the growth was significantly induced by the air supplement. Note that the growth rate in the system without aeration was extremely low and one could barely observe the increase in cell number even with more than 10 days of cultivation. The alga could perhaps take longer than 10 days to grow but that was out of the interest in this work.

Results demonstrated that the growth of *H. pluvialis* was stimulated by aeration. In airlift bioreactor, the aeration led to a better mixing, this implied that mixing of the microalgal culture was required for a better growth. This mixing could have several purposes such as preventing sedimentation, maintaining homogeneous conditions which helped in a better contact between the cells and the nutrients, etc.

This section also aimed at the examination of the effect of aeration rate on the cell growth, and therefore growth experiments in the airlift system (A_d/A_r = 3.2) operating with different levels of aeration rate in the range of 0.4-3 cm s^{-1} were compared. The results were shown in Figure 4.6. Interestingly, further increase in the aeration rate (above the superficial gas velocity of 0.4 cm s^{-1}) did not show benefits for the growth. In fact, growth seemed to be drastically slowed down by excessive aeration.

Increasing aeration rate induced mixing, liquid circulation and mass transfer between gas and liquid phases in the airlift systems (Krichnavaruk and Pavasant, 2002; Wongsuchoto et al., 2003 and Wongsuchoto and Pavasant, 2004). A higher mass transfer might also facilitate the removal of gases such as oxygen, preventing the accumulation of these gases which might adversely affect the growth rate (Tung et al., 1998). However, the cell culture of *H. pluvialis* in the

batch culture in the airlift system was negatively affected by an increase in superficial gas velocity. This was believed to be due to the shear stress caused by the high aeration rate which indicated that the cell of *H.pluvialis* was highly shear sensitive and even with the shear caused by aeration could badly deteriorate the growth.

This explanation was supported by several past reports. For instance, Gudin and Chaumont (1991) stated that the key problem in the cultivation of microalgae in photobioreactors was cell damage due to shear stress. Hata et al, (2000) illustrated that the culture of green vegetative cell in exponential phase of growth required a low liquid velocity due to its fragility. To further examine the effect of the aeration, the structure of *H.pluvialis* under various aeration rates was monitored. The results as summarized in Figure 4.7 illustrates that an increase in superficial gas velocity could significantly change the cell morphology from vegetative cells to non-motile green cells (see photos in Figure 4.8 and 4.9). In other words, the fraction of immature cyst became more dominant with an increase in the aeration rate. At the superficial velocity of 0.4 cm s^{-1}, a very small fraction of non-motile green cells was observed when compared to the number of vegetative cells. On the other hand, the number of vegetative cells could hardly be observed at the superficial velocity greater than 2.5 cm s^{-1}. The vegetative cells are more productive in view of cell multiplication. Therefore it would be difficult to obtain high cell density if the cell could not be maintained in vegetative form particularly in high shear stress condition. Note that cysts can also grow in size, but with a slow rate of cell division.

The best superficial gas velocity of growth of *H. pluvialis* was found at the lower limit of the experiment which was at 0.4 cm s^{-1}, with the maximum cell density and maximum specific growth rate were 77×10^4 cells mL^{-1} (2.79 g L^{-1} of cell dry weight) and 0.45 day^{-1} (Figure 4.6). Due to the equipment constraints, the air flow could not be accurately adjusted below this level, therefore it could not be concluded at this point that this 0.4 cm s^{-1} of superficial gas velocity was the optimal level. It is worth mentioned, however, that the specific growth rate obtained at this condition (0.45 day^{-1}) was significantly higher than most of the

reported data in literature. This is only second to Kobayashi et al., 1992 who achieved the specific growth rate of 0.58 day^{-1} in the small flask experiment growing in mixotrophic condition.

4.2.4 Effect of the ratio between the downcomer and riser cross sectional area (A_d/A_r)

In this section, the effect of design configuration in the airlift system was investigated. A basic design parameter in the airlift system is the ratio between cross sectional areas of downcomer and riser (A_d/A_r). For this experiment, this parameter could be simply altered by changing the draft tube size. The two sizes of commercially available clear column were employed, i.e. at 5 and 7 cm, which gave A_d/A_r of approx. 3.2 and 0.9, respectively.

Figure 4.10 depicts the specific growth rate of the alga in the airlift with the two A_d/A_r ratios. The results demonstrated that there were no significant impacts of this parameter on the growth of *H. pluvialis* (with the range of aeration from 0.4-1 cm s^{-1}). Often if the riser is large, bubbles will play an important role in controlling the growth as bubbles could disperse or block the light pathway. Our uncommunicated results (at the time of this report) illustrates that a high rate of aeration could lead to a low light intensity in the column as the blockage of light path (shading effect) from the swarm of bubbles was considerable. However, the aeration rate employed in this experiment was rather low, and the number of bubbles produced at any time was sparse and not adequate for the shading effect to occur. Therefore no effect of A_d/A_r could be observed from this set of experiment.

Note that in most experiments here, the A_d/A_r of 3.2 was selected as the default configuration as this would help in lowering the operating cost due to aeration.

4.2.5 Effect of light intensity

The experiment with light intensity was carried out in the batch cultivation mode using the airlift bioreactor with A_d/A_r of 0.9 and superficial gas velocity of 0.4 $cm \cdot s^{-1}$. Five different surface light intensities were tested and the results on growth profiles and the maximum cell density are shown in Figures 4.11.

The results demonstrated that the cell density and specific growth rate increased with an increase in the light intensity up to 20 μmol photon m^{-2} s^{-1}. Further increase in light intensity, on the other hand, could inhibit growth and resulted in lower cell density and specific growth rate. Hence, the optimum light intensity of growth of *H. pluvialis* was reported at 20 μmol photon m^{-2} s^{-1} where the maximum cell density and maximum specific growth rate were 77×10^4 cells mL^{-1} (2.79 g L^{-1} of cell dry weight) and 0.45 day^{-1}, respectively.

Figure 4.12 illustrates that at light intensity lower than 40 μmol photon m^{-2} s^{-1} (2,000 lux), almost all of the cells were in vegetative form. The light intensity over 2,500 lux or 50 μmol photon m^{-2} s^{-1} was likely to induce morphological change with the cells formation, i.e. cells changed from vegetative cells to mature cyst (Figure 4.13), with a concomitant accumulation of astaxanthin. This proved that astaxanthin accumulation in *H. pluvialis* could be induced at high light intensity. Similar finding was observed previously by Boussiba and Vonshak (1991) where the light intensity stimulating the accumulation of astaxanthin was shown to establish at over 90 μmol photon m^{-2} s^{-1} (4,500 lux). However, the result here demonstrated that astaxanthin could well be accumulated at the light intensity of as low as 40 μmol photon m^{-2} s^{-1} (2,000 lux). Moreover, cell growth was no longer observed when the light intensity increased to 60 μmol photon m^{-2} s^{-1} or 3,000 lux.

In terms of growth, the accumulation of astaxanthin was not a good sign as this was the condition where cell division began to cease. Therefore this condition must be avoided since the growth of vegetative cells was the main objective of the cultivation.

4.3 Semi-continuous culture of *H.pluvialis* in airlift bioreactor

Semi-continuous cultivation was conducted in order to examine the potential of having the cultural system that could produce cell continuously. This cultivation was carried out under the optimum condition obtained from the batch culture which were: A_d/A_r = 3.2, superficial velocity = 0.4 cm s^{-1}, light intensity = 20 μmol photon m^{-2} s^{-1} The results are shown in Figure 4.14. In the batch culture, the cell density was often found to increase until it reached a maximum of 77×10^4 cell mL^{-1} which occurred at about day 8 of cultivation. For the semi-continuous culture, the cultivation was started off as a batch culture with the initial cell density of 2×10^4 cell mL^{-1}. The cell was grown in the system for 6 days after which a 50% by volumn of culture broth was replaced with a fresh culture medium. The harvest cell density was approx. 40×10^4 cell mL^{-1}. It was proven that with this harvesting cycle, the cell could maintain its vegetative form, and in each cycle, cell density increased up to the level obtained in the previous cycle. The specific growth rate and productivity of semi-continuous culture were 0.31 day^{-1} and 5.52 cell mL^{-1} day^{-1}, respectively. This result was comparable to that reported by Hata et al. (2001) who successfully achieved the semi-continuous culture, but only in the small scale (in 500 cc Erlenmeyer flask) with a productivity of 6.8 cells mL^{-1} day^{-1}.

4.4 Preliminary examination of astaxanthin production

There are several factors inducing the synthesis of astaxanthin. Some nutrients have been reported to affect the synthesis of astaxanthin such nutrient deprivation, high light intensity and salt stress. This section intended to provide preliminary results on the stimulation of astaxanthin by changing two environmental factors, i.e. light intensity and nutrient composition.

4.4.1 Growth stages of *H.Pluvialis* during astaxanthin induction experiments

To produce astaxanthin, a two stage cultivation approach was used. The first stage was to cultivate the cells under the optimal growth conditions (as determined from the previous sections) in order to obtain high cell density culture. The second stage was designed to induce astaxanthin formation. This section describes the experimental results obtained from the growth of *H. pluvialis* under controlled conditions, i.e. light intensity = 20 μmol photon m^{-2} s^{-1}, A_d/A_r = 3.2 and superficial gas velocity = 0.4 cm s^{-1}.

In the first stage (day 0 - day 8) and most cells were in the vegetative form. The cell density increased as a result of high rate of chlorophyll synthesis (see Figure 4.18(a)). The culture was continued up to day 9, at this time, there was still no morphology change from green vegetative cell to cyst. The cultivation was then ready to be treated for the production astaxanthin.

In the induction stage, the aeration velocity was increased from 0.4 to 2 cm s^{-1}. Most of cells remained in green motile cells for another 4 days and then reached a stationary phase where they gradually turned into green non-motile cells which still continued to increase in size. During this period, the contents of chlorophyll increased continuously and cells started to accumulate a low level of astaxanthin. After 15 days of second stage, red cells of algae became apparent. This caused a reduction a level of chlorophyll but high astaxanthin synthesis (Figure 4.15). Figure 4.18(b) shows the airlift system for *H. pluvialis* with astaxanthin accumulation.

4.4.2 Effect of light intensity on astaxanthin production

In the first experiment, the effect of light intensity on the astaxanthin production was tested. The superficial gas velocity was increased to 2 cm s^{-1} and the light intensity was switched 20 μmol photon m^{-2} s^{-1}, which was employed for the cell cultivation in the first stage, to 150 μmol photon m^{-2} s^{-1}. At this condition, *H. pluvialis* began to convert to cyst and accumulated astaxanthin.

The results on astaxanthin production were compared to that obtained from the control culture where cells were left in the growth medium without changes in light intensity. The cell density, chlorophyll and astaxanthin profiles of the culture under high light intensity condition were shown in Figure 4.16. Under high light intensity culture condition, 83% increase in the astaxanthin content was obtained when compared with that from the control condition after 20 days of astaxanthin induction (Day 28). These results agreed with that of Harker et al. (1995) i.e. light was one of the most important factors for astaxanthin formation in *H. pluvialis* as cells responsed to stress with high light intensity by generating astaxanthin.

4.4.3 Effect of medium in astaxanthin production

In the second astaxanthin induction experiment, the aeration of the culture was stopped for about 3 h in order to obtain a proper settling of cells at the base of the reactor. The medium was then 50% replaced by the sterile distilled water and the superficial gas velocity was re-supplied to the column at 2 cm s^{-1}. The culture grew on the residual nutrient in the medium and entered the stationary growth phase very rapidly. After 10 days of stress condition, accumulation of astaxanthin appeared and chlorophyll level began to fall. The cell density, chlorophyll and astaxanthin profile of culture under this condition was shown in Figure 4.17. Under this treatment, 53% increase in the astaxanthin content could be achieved when compared with the control condition at day 22 after inducing astaxanthin (or day 30 from the start of experiment).

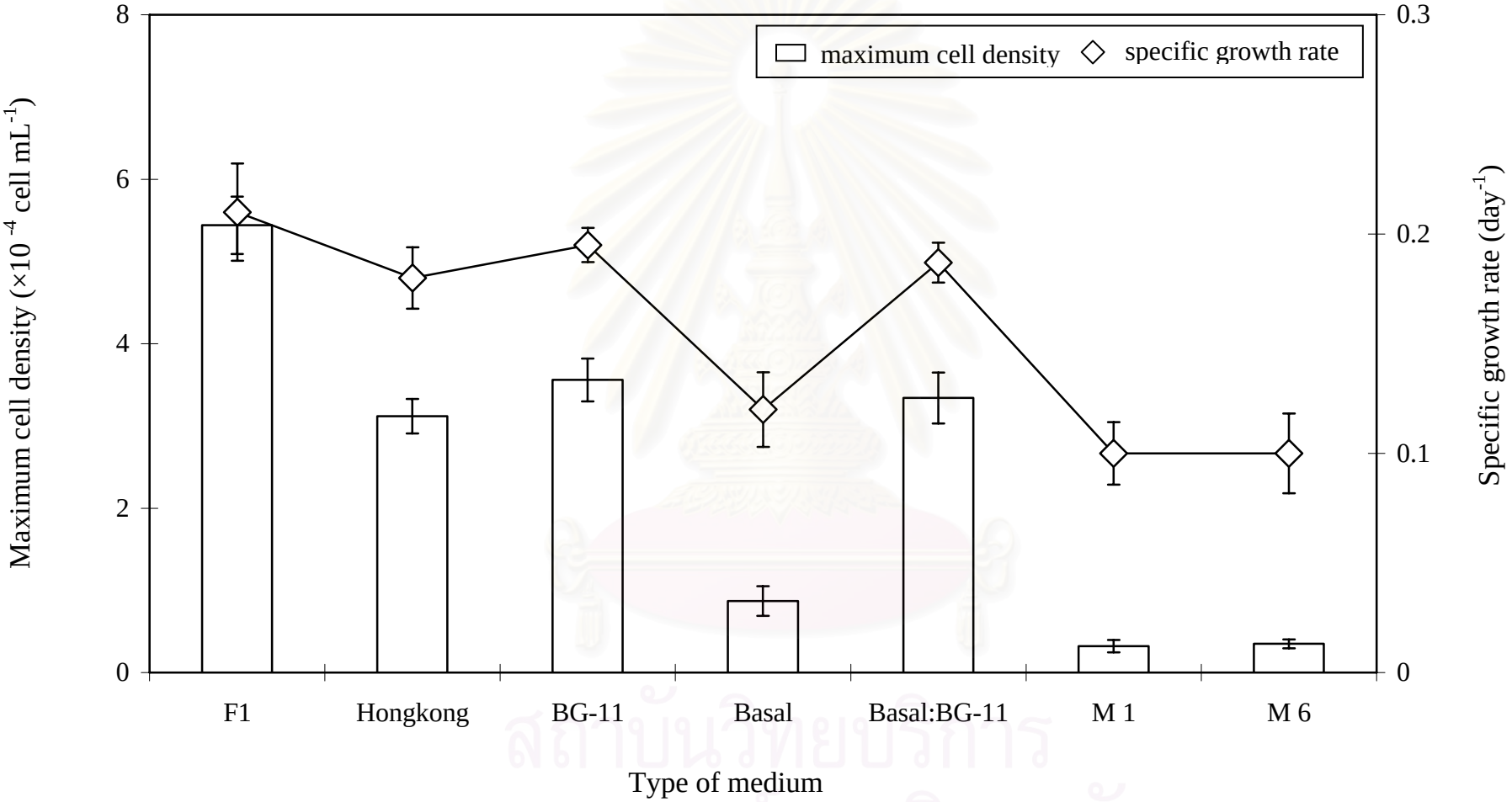


Figure 4.1 Maximum cell density and specific growth rate *H. pluvialis* with various medium

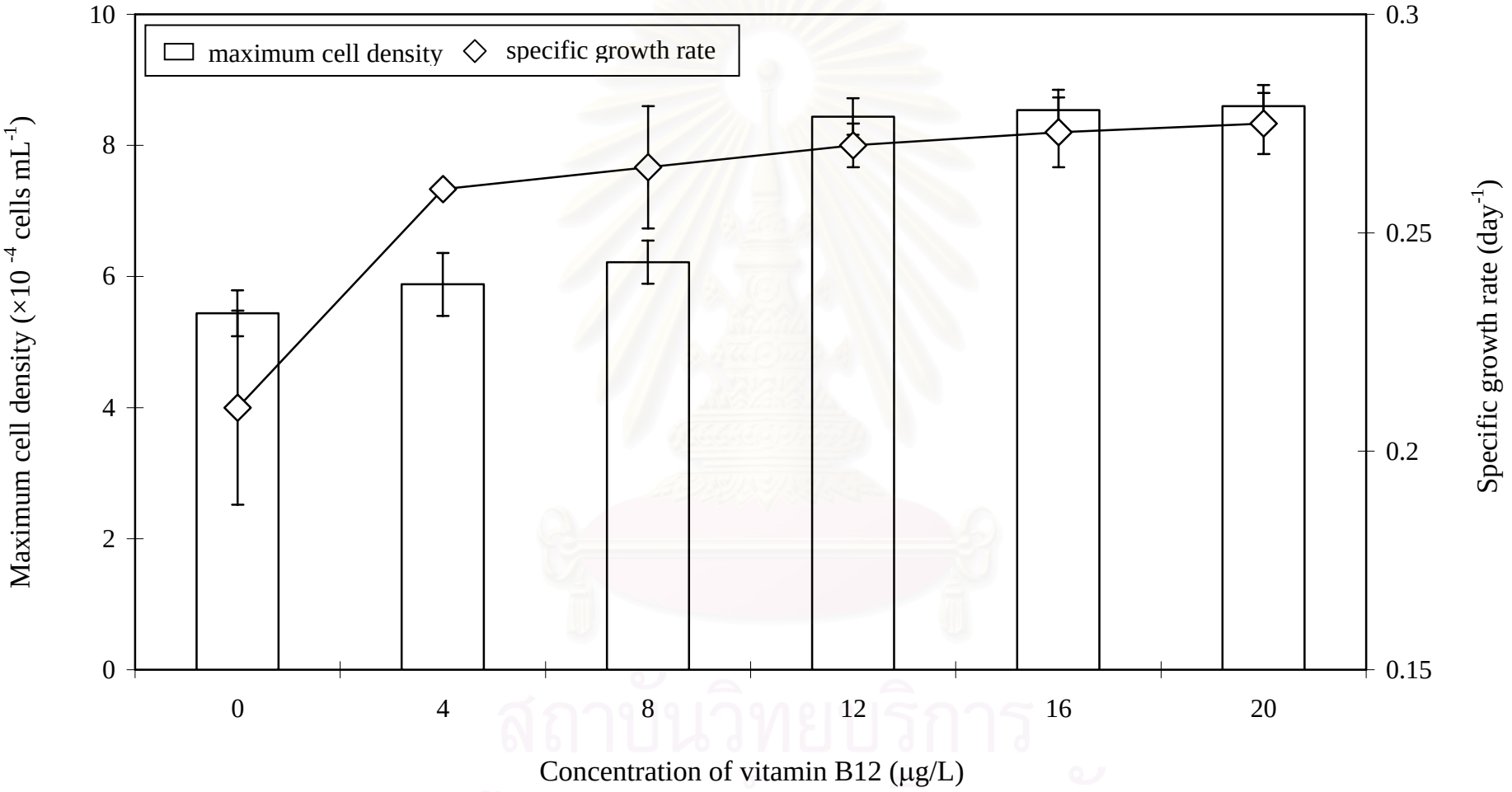
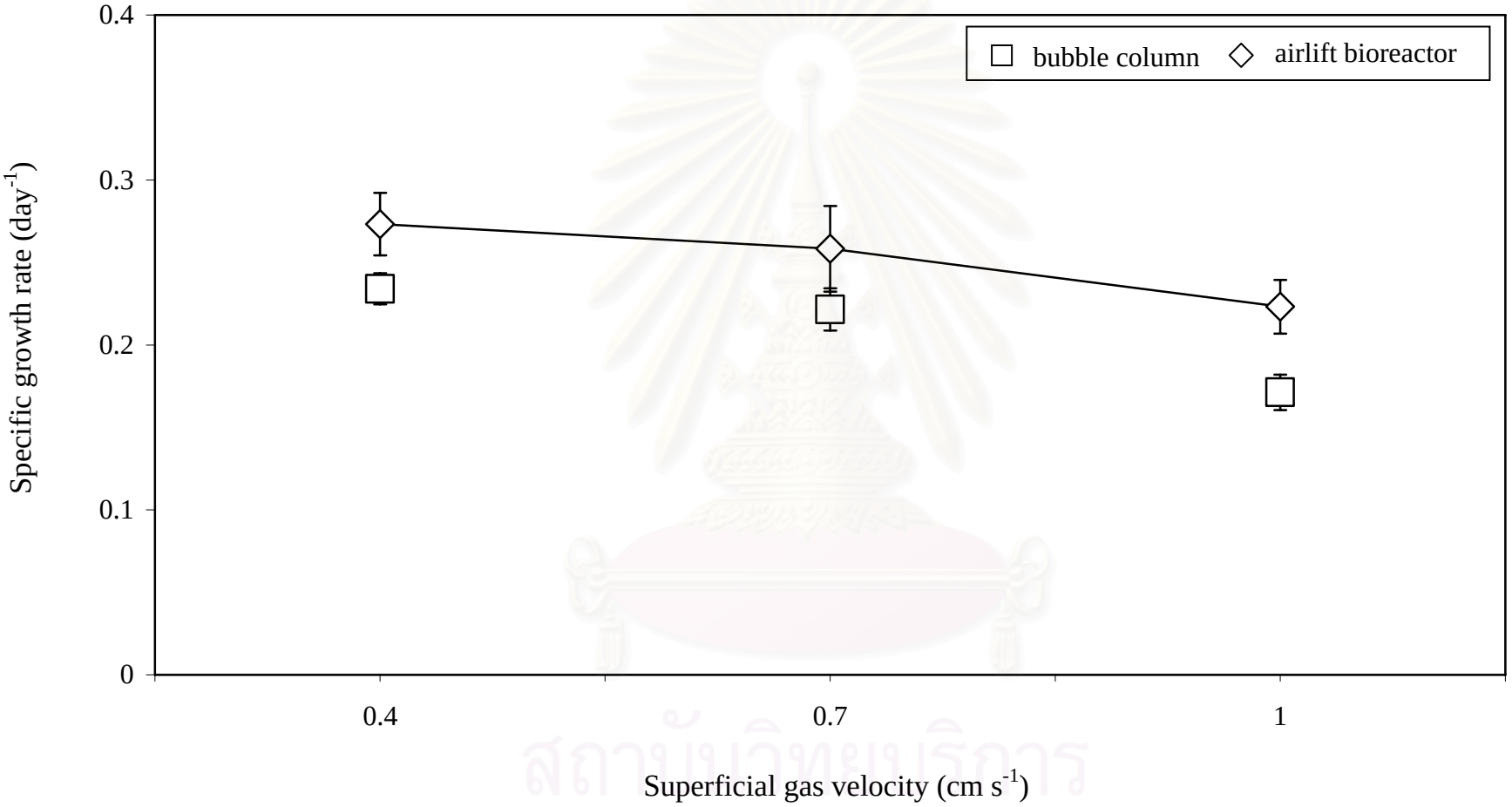


Figure 4.2 Effect of vitamin B12 on maximum cell density and specific growth rate of *H. pluvialis*



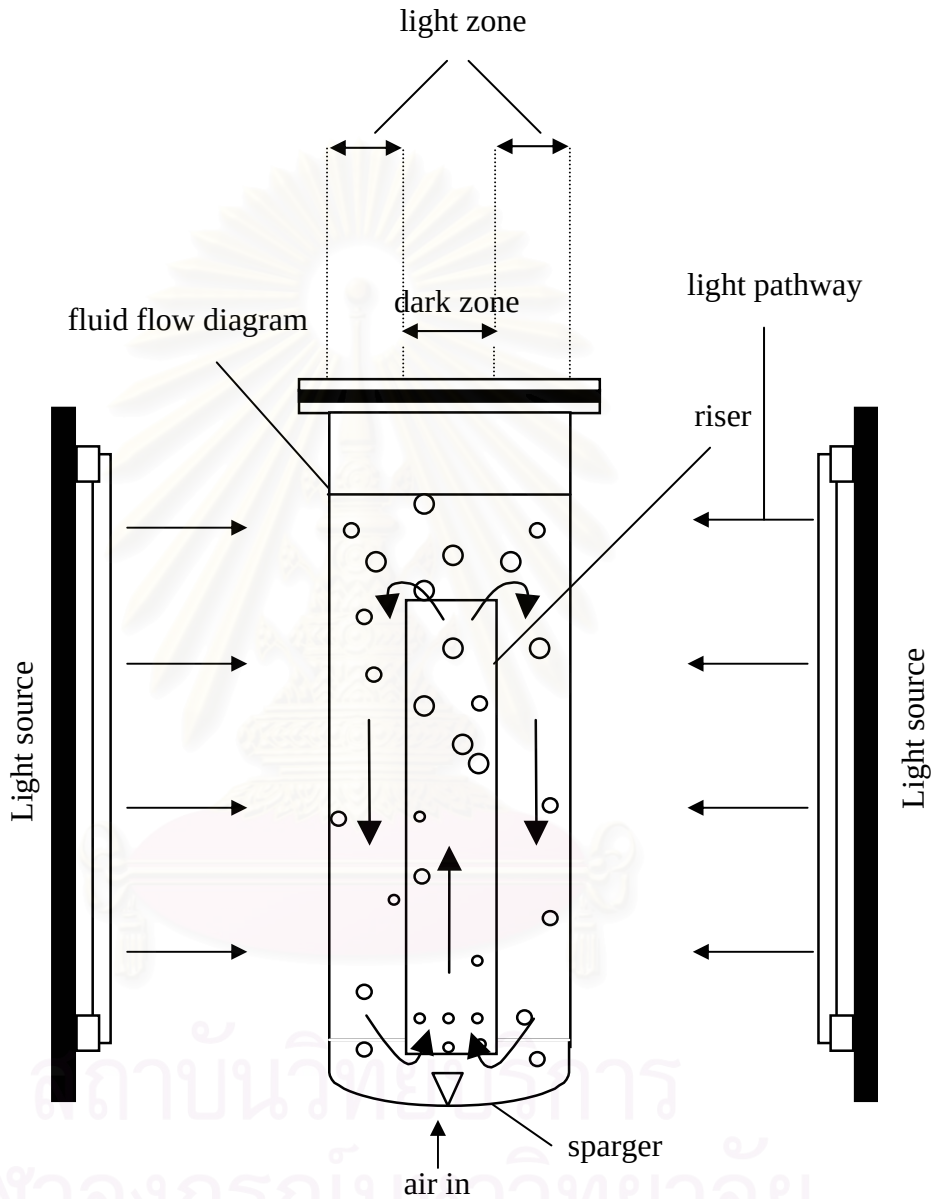


Figure 4.4 Schematic gas and liquid flow direction in the airlift system

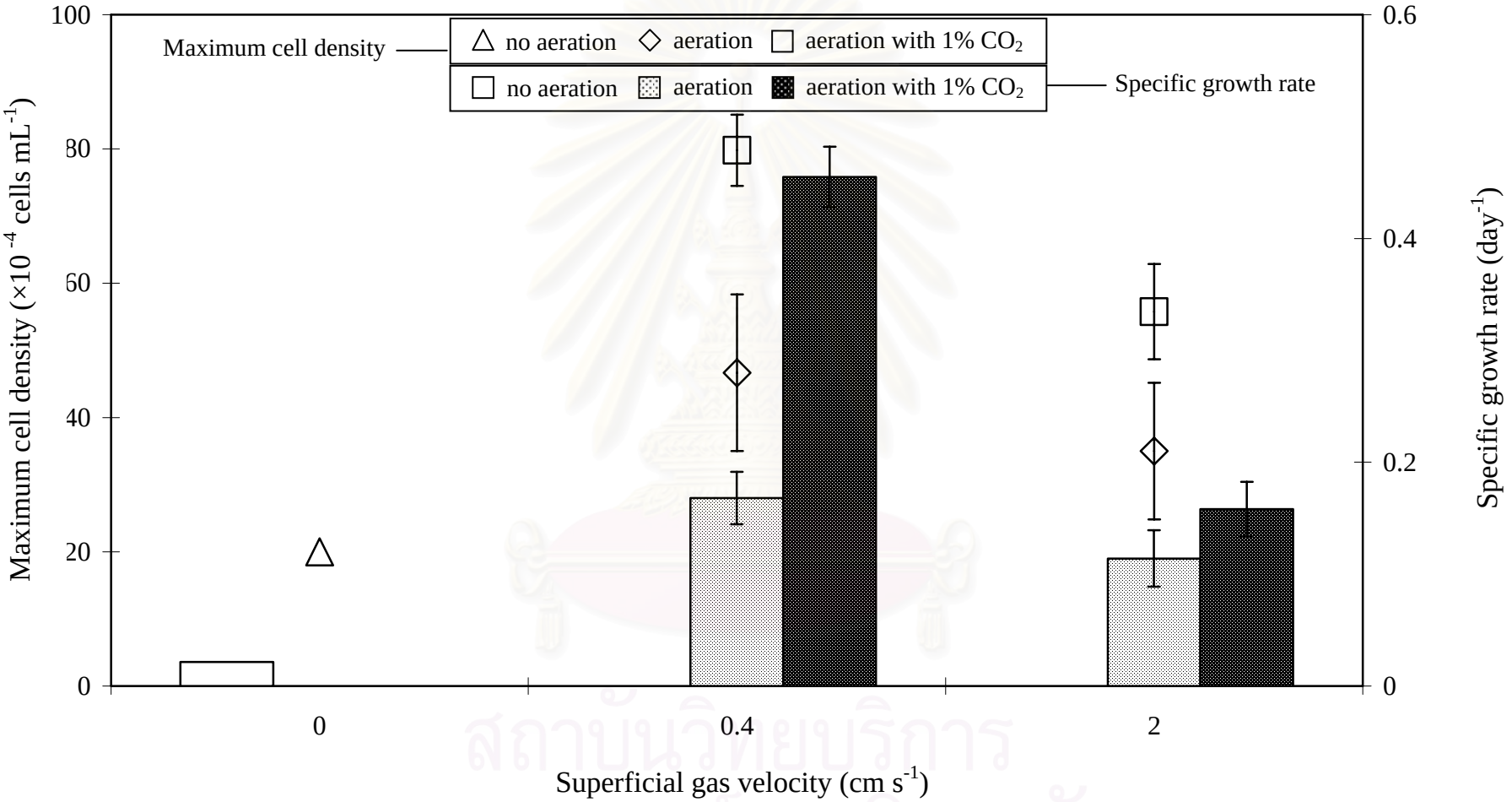


Figure 4.5 Effect of aeration and CO_2 on maximum cell density and specific growth rate of *H. pluvialis* at different superficial gas velocity

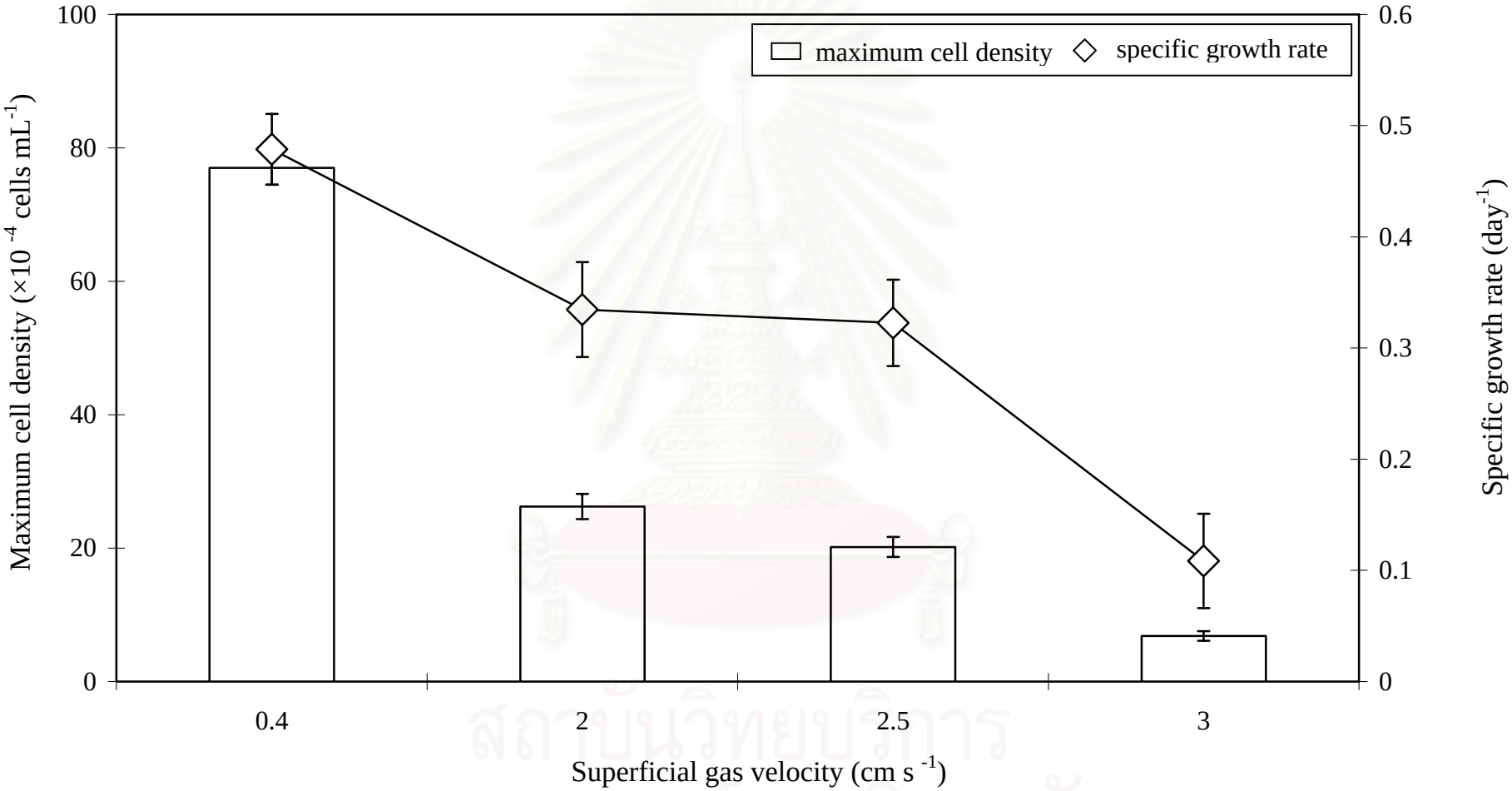


Figure 4.6 Effect of superficial gas velocity on maximum cell density and specific growth rate of *H. pluvialis*

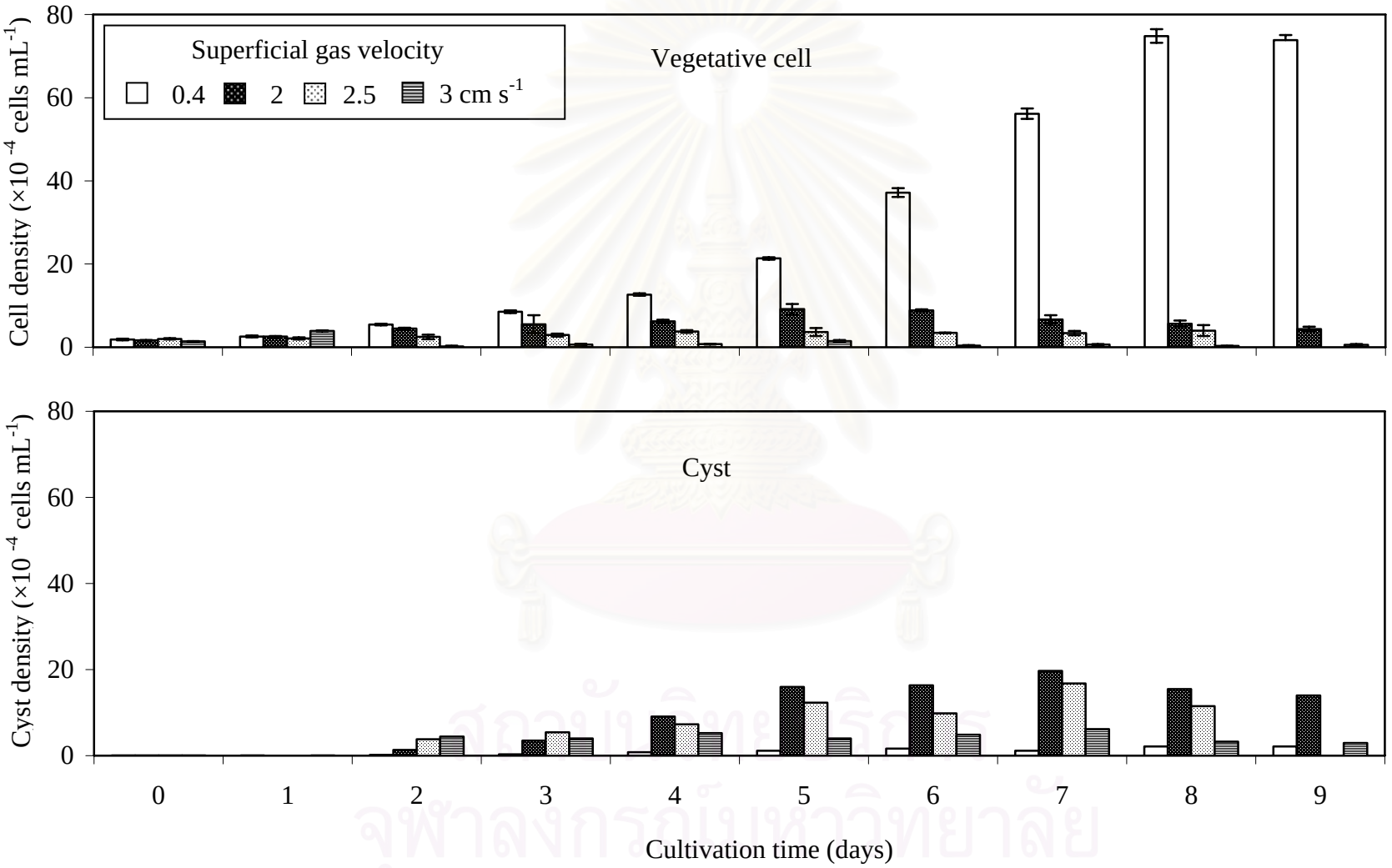


Figure 4.7 Density of vegetative cells and cysts of *H. pluvialis* at different superficial gas velocity

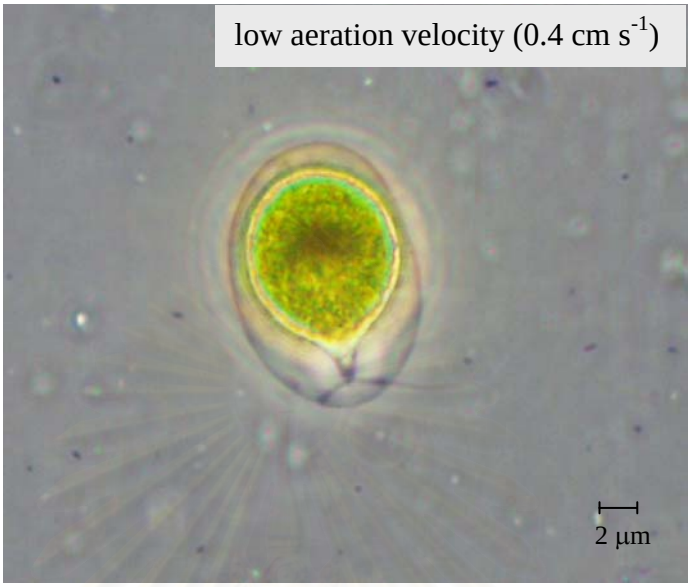


Figure 4.8 Vegetative form of *H. pluvialis*

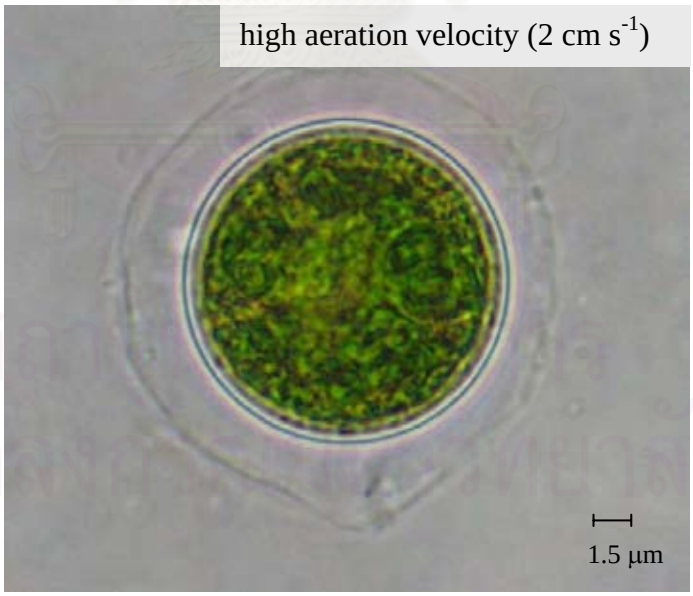


Figure 4.9 Non-motile cyst of *H. pluvialis*

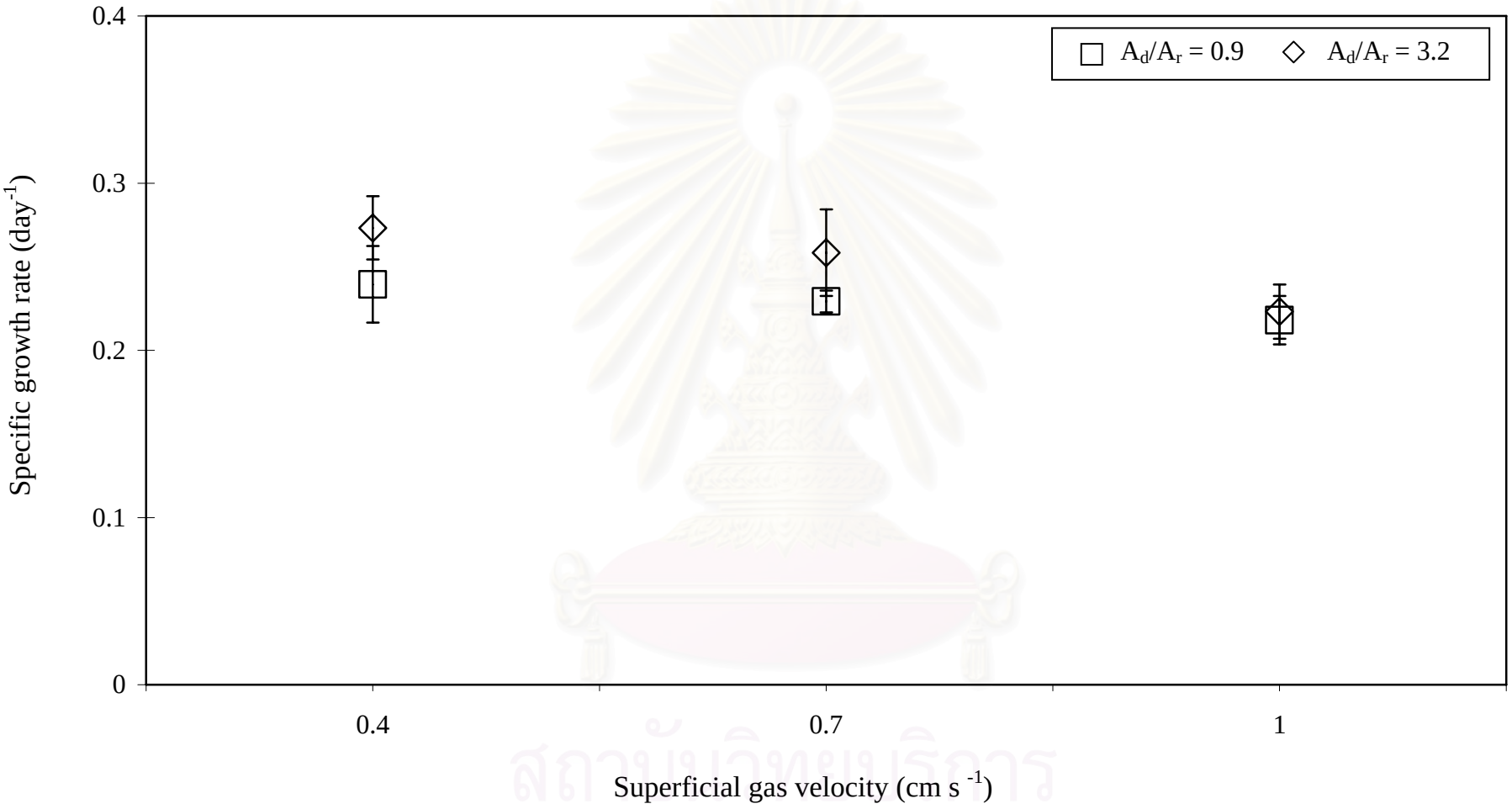


Figure 4.10 Effect of A_d/A_r on specific growth rate of *H. pluvialis* at different superficial gas velocity

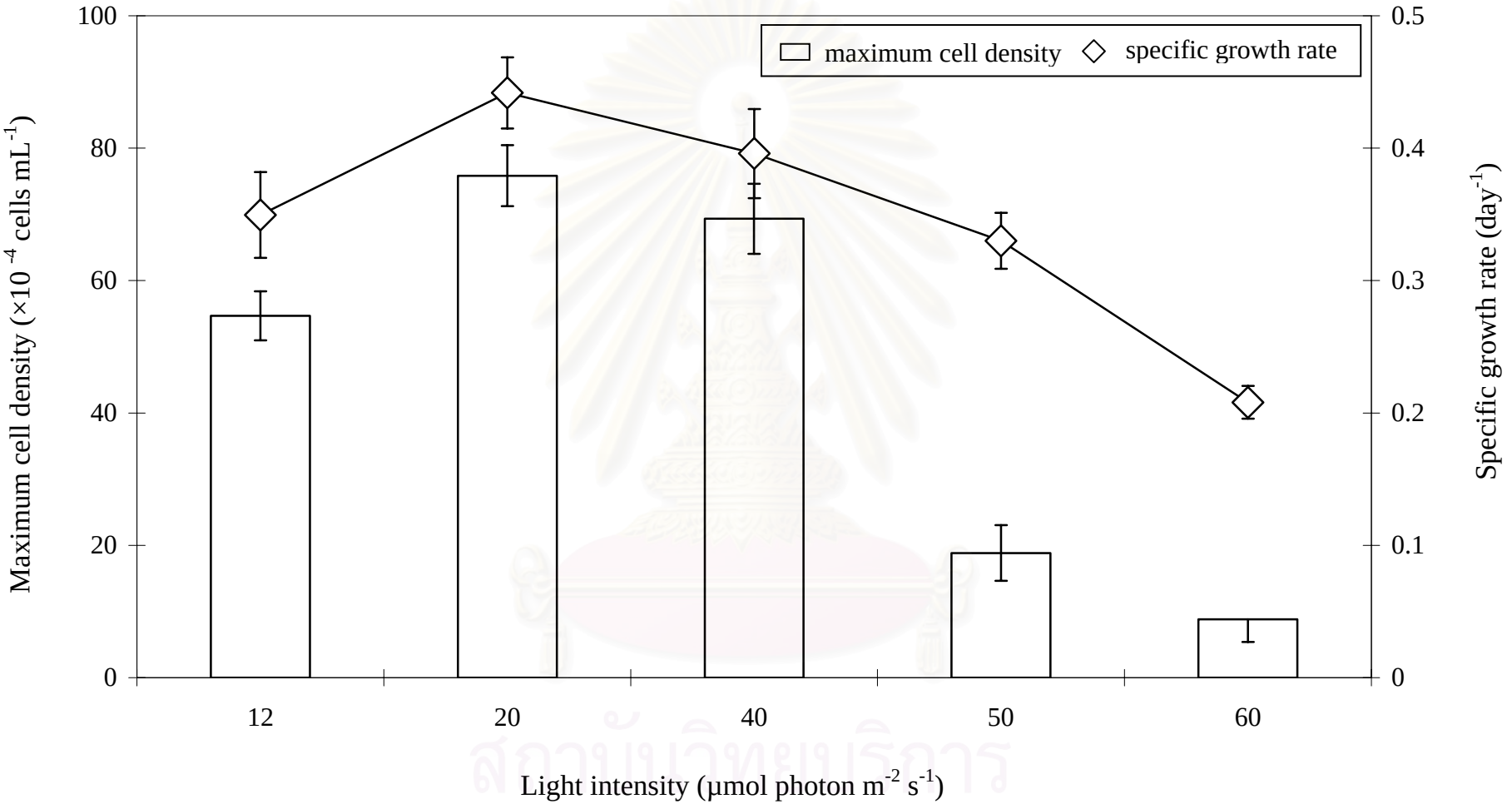


Figure 4.11 Effect of light intensity on maximum cell density and specific growth rate of *H. pluvialis*

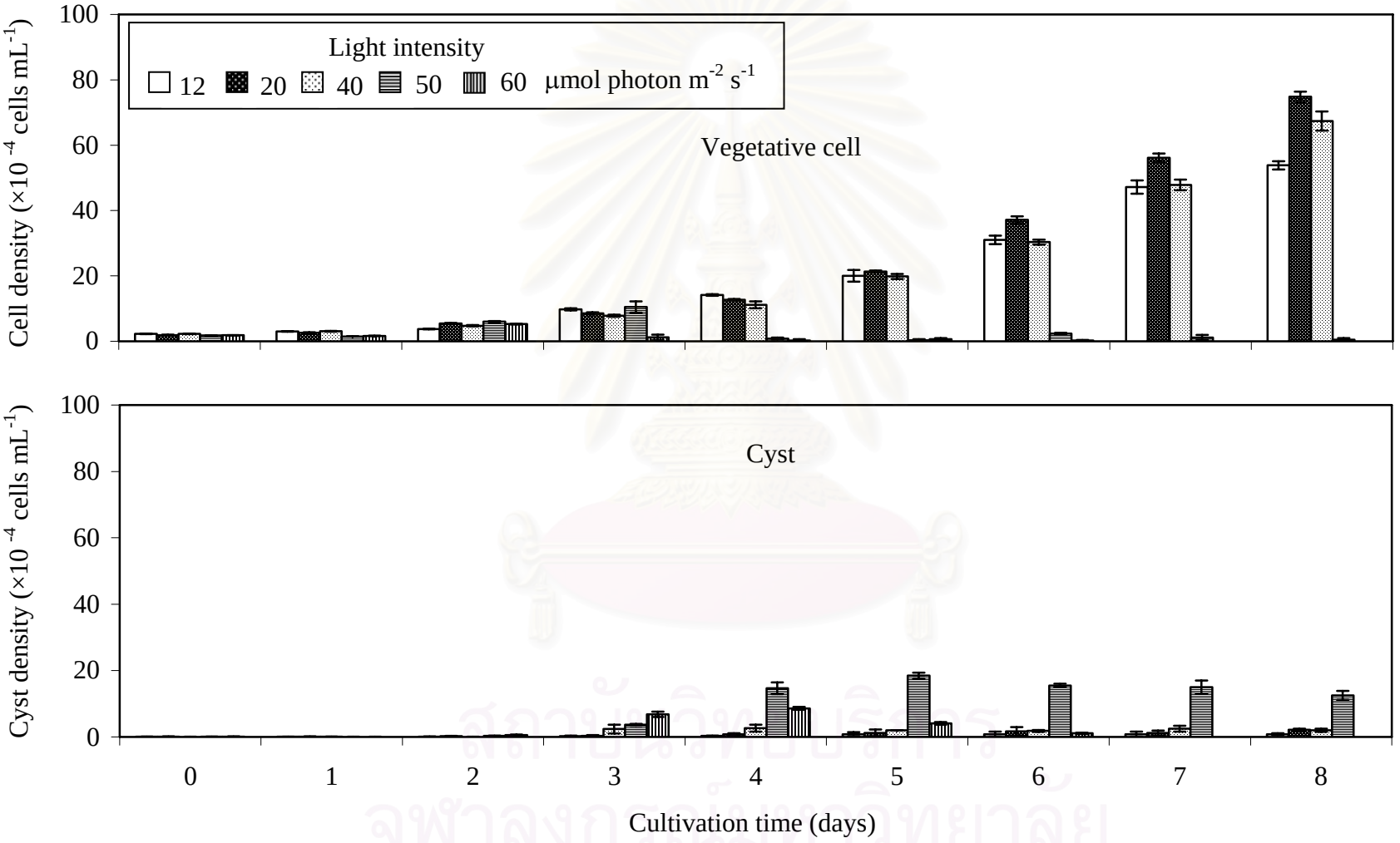


Figure 4.12 Density of vegetative cells and cysts of *H. pluvialis* at different light intensity

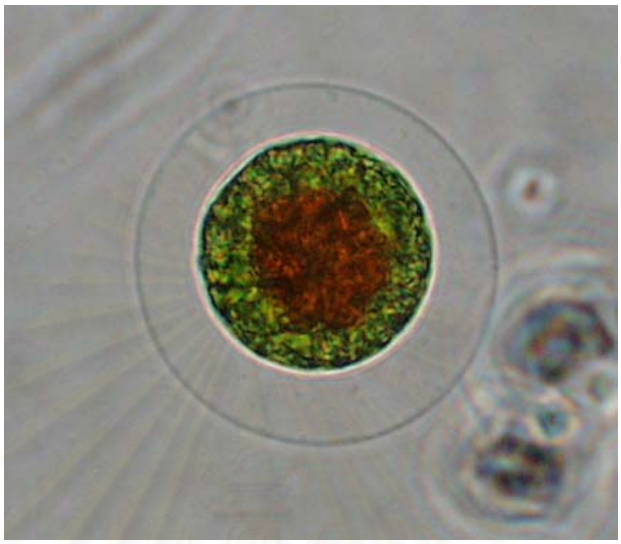


Figure 4.13 Cyst form (accumulation of astaxanthin observed as the redden region in the middle point of the cell) of *H. pluvialis* at high light intensity

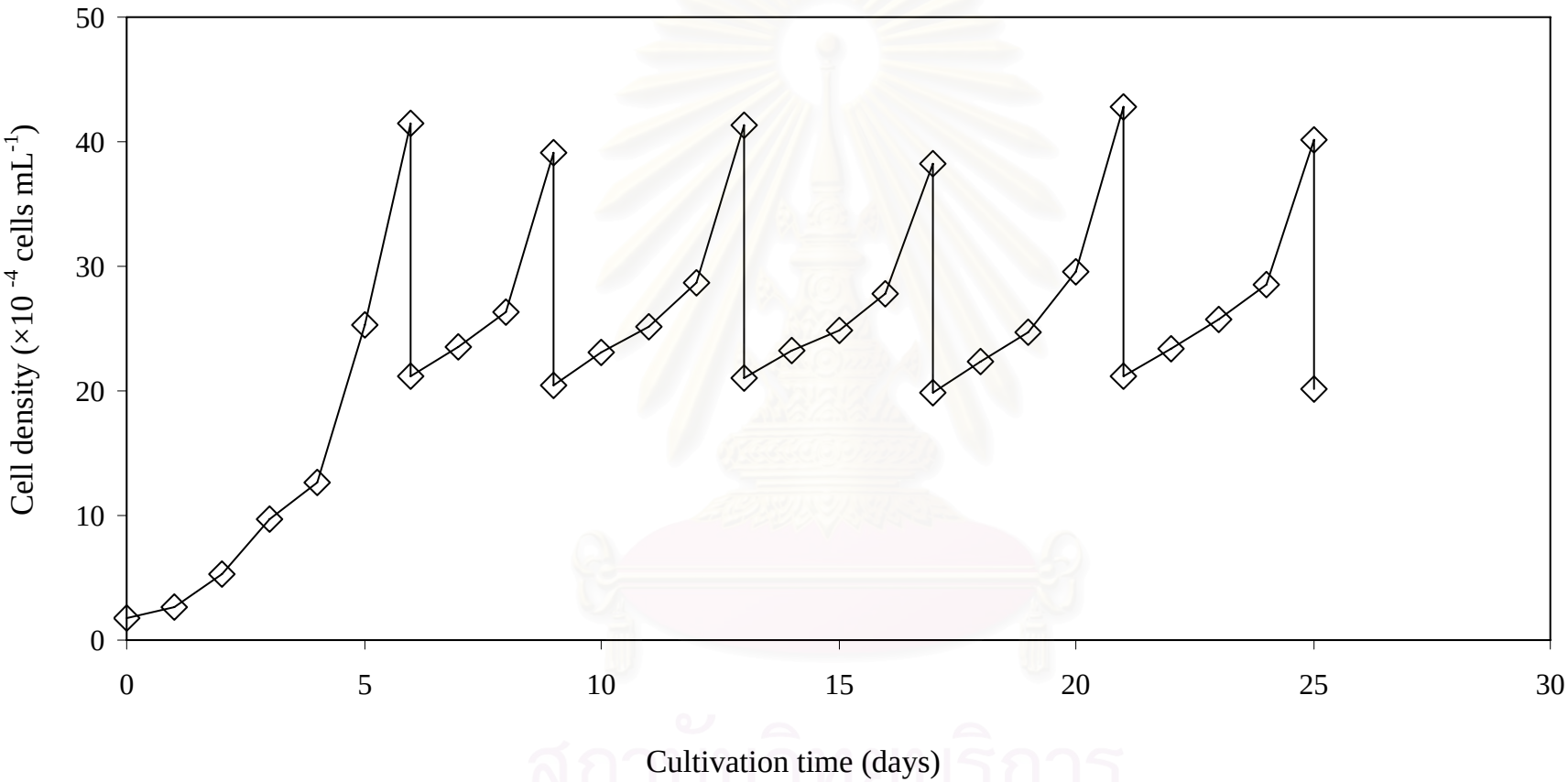


Figure 4.14 Cultivation of *H. pluvialis* under semi-continuous culture

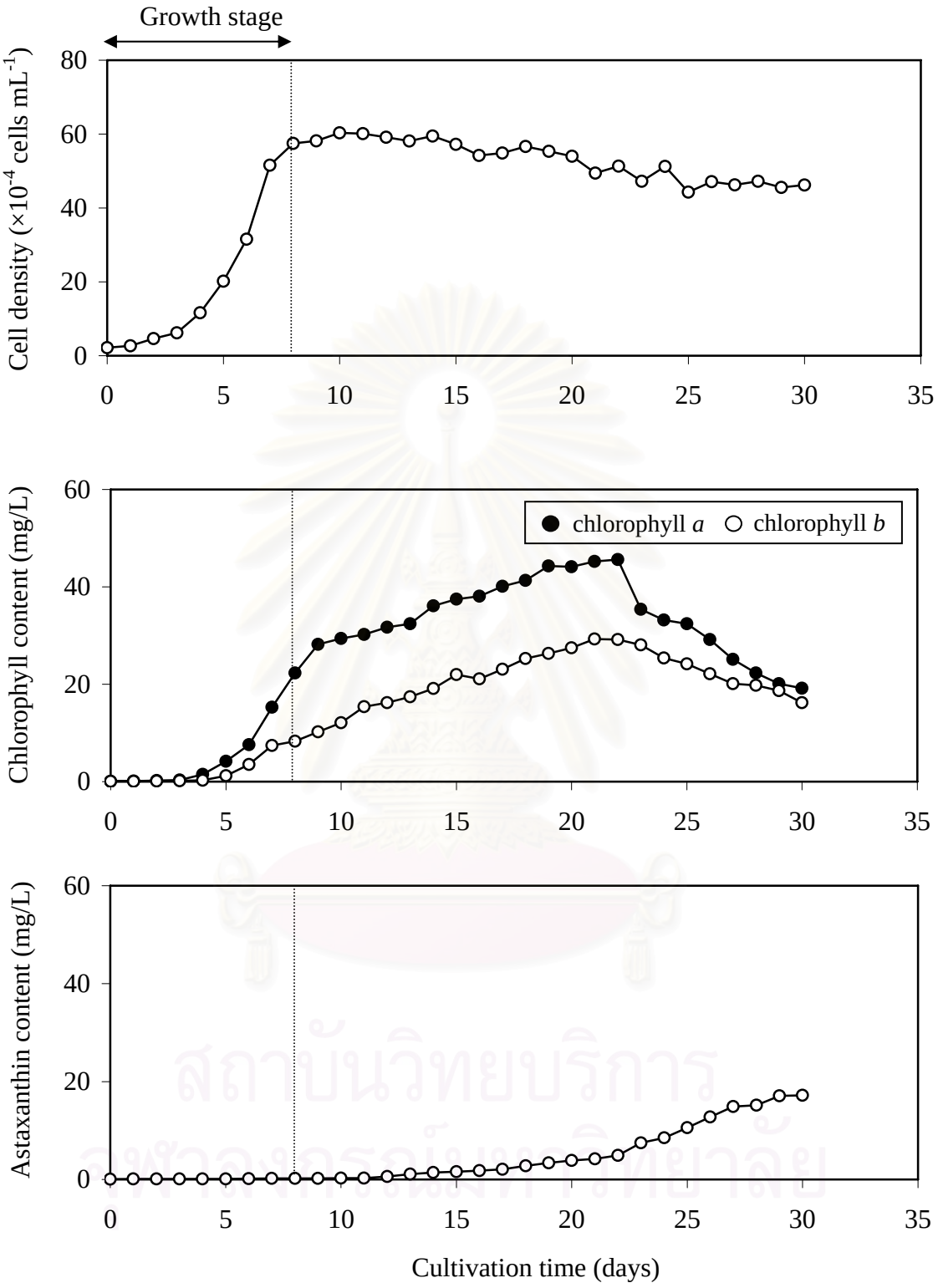


Figure 4.15 Growth and astaxanthin accumulation of *H. pluvialis* under control condition

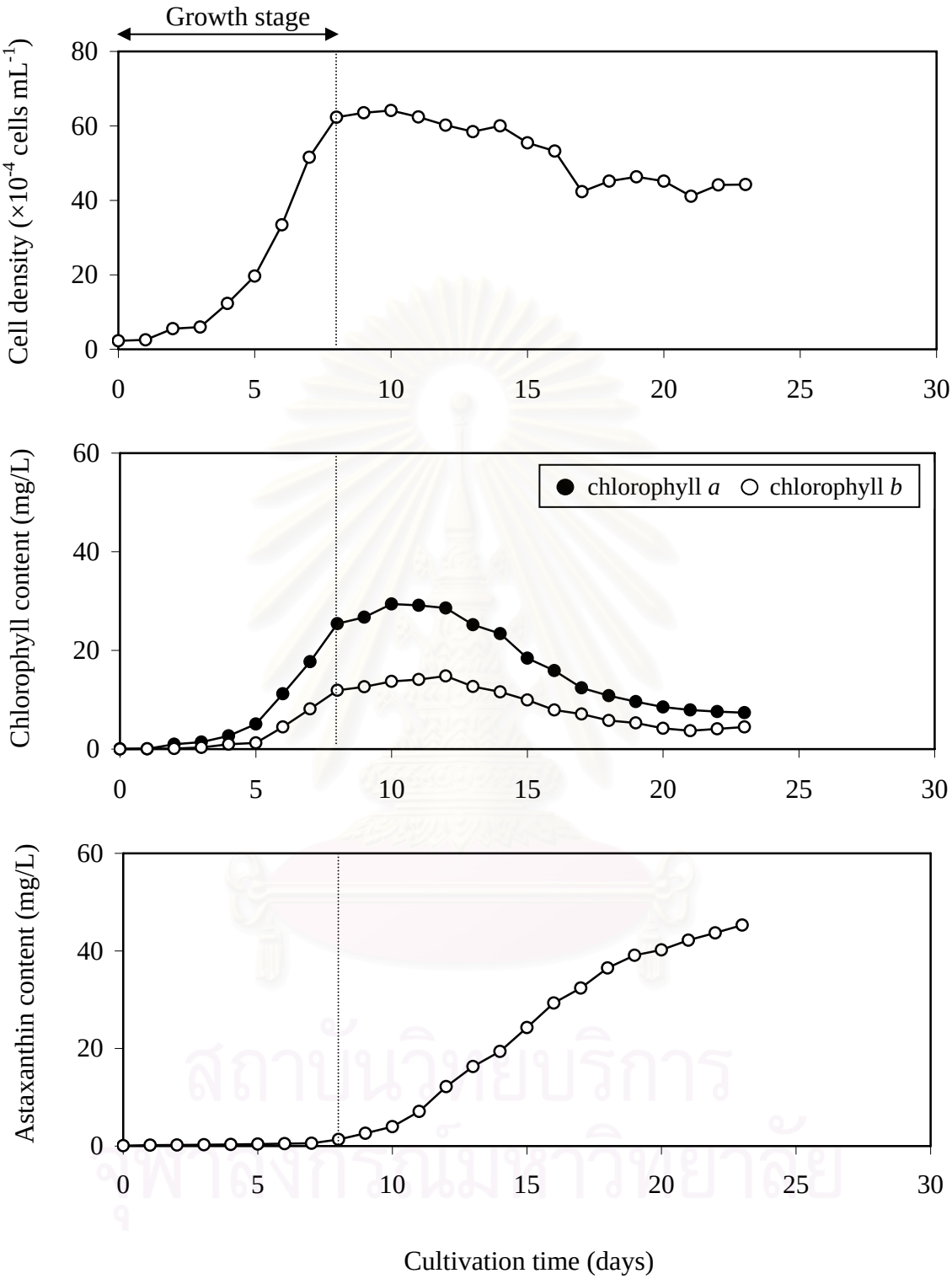


Figure 4.16 Growth and astaxanthin accumulation of *H. pluvialis* under high light intensity

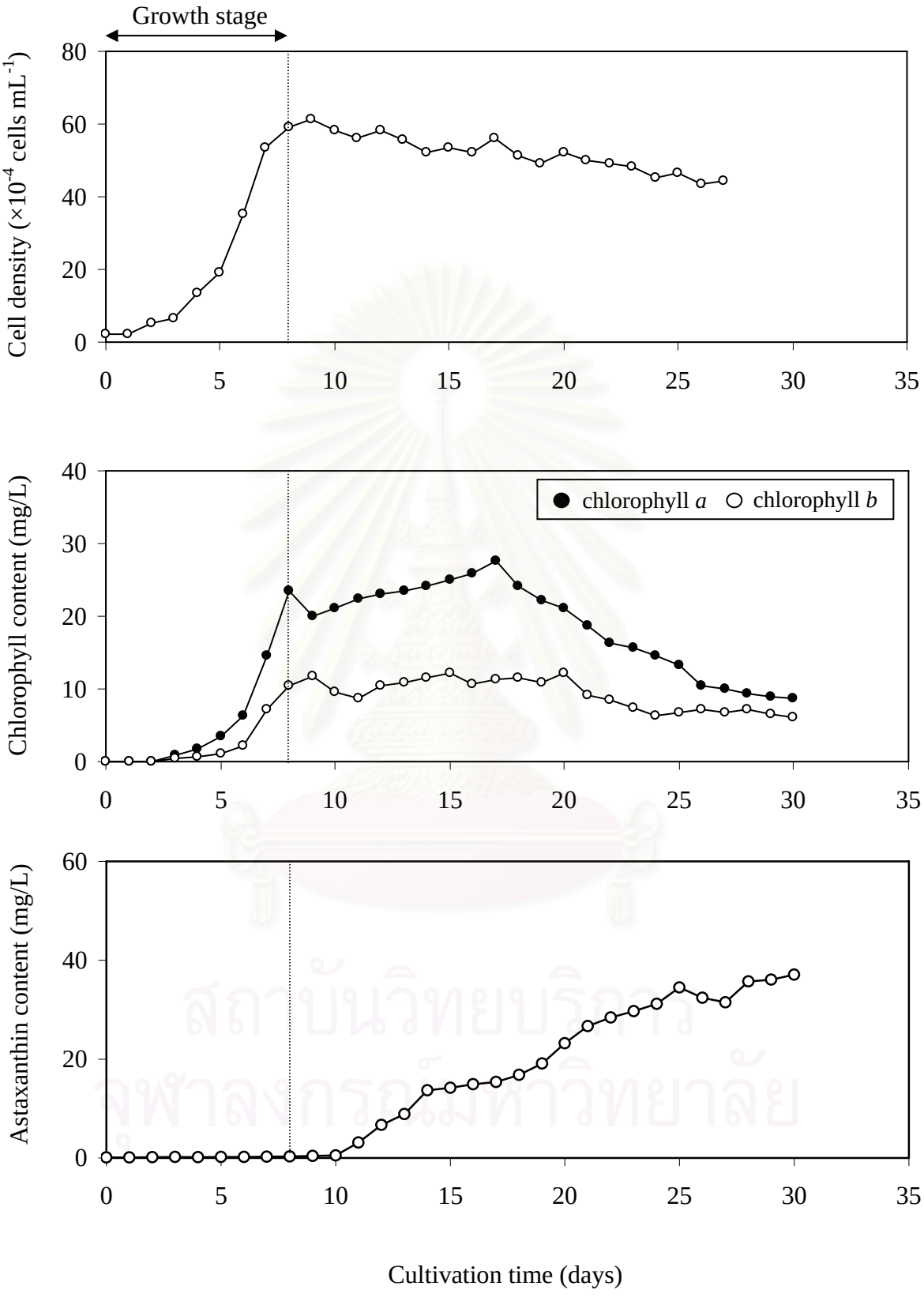


Figure 4.17 Growth and astaxanthin accumulation of *H. pluvialis* under dilute medium

Figure 4.18(a) Cultivation of *H. pluvialis* in airlift bioreactor: growth stage

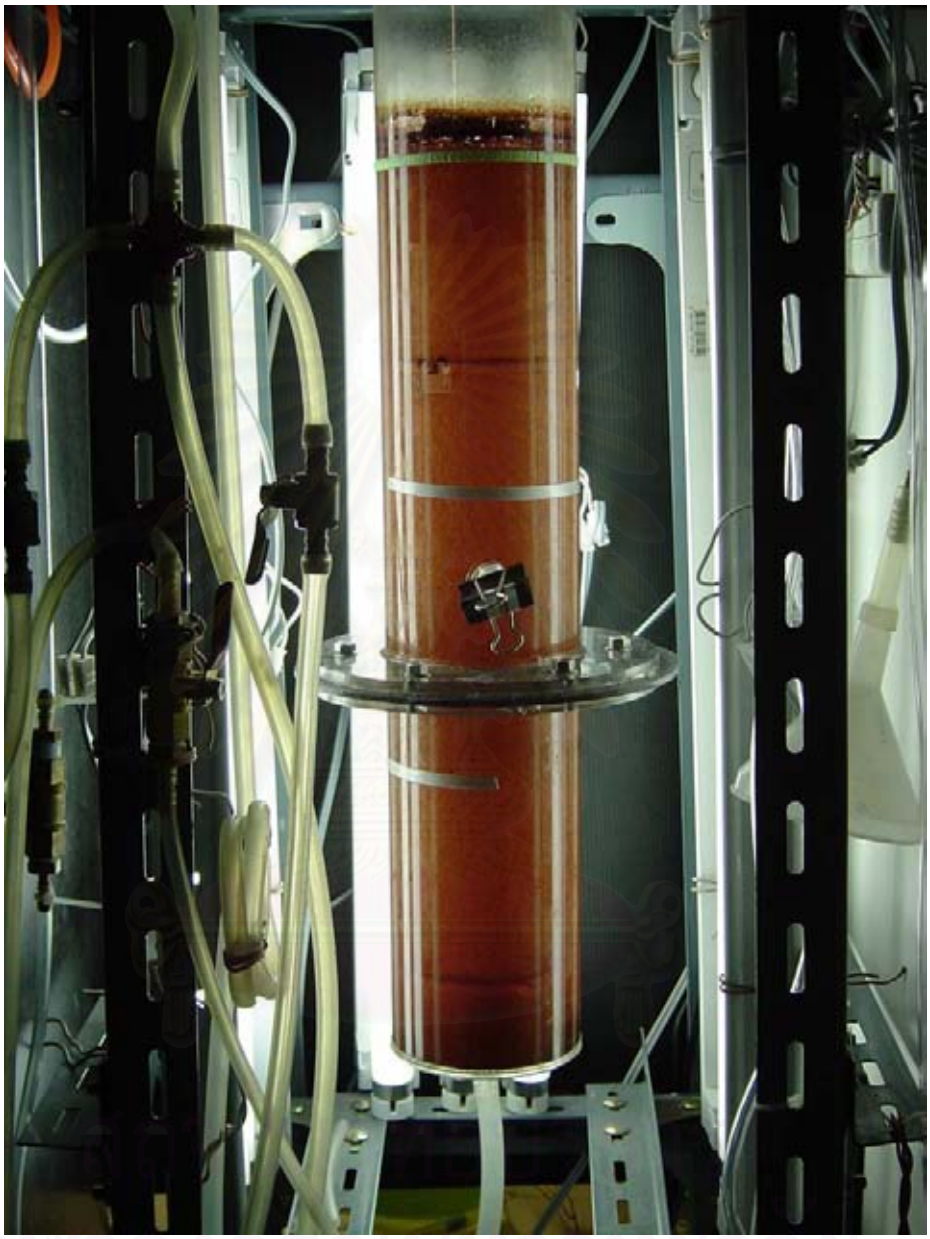


Figure 4.18(b) Cultivation of *H. pluvialis* in airlift bioreactor: induce astaxanthin stage

CHAPTER V

Conclusions and Recommendations

5.1 Conclusions

Major findings from this work can be summarized as follows:

1. High cell density of *H. pluvialis* NIES-144 was accomplished by culturing cells in the F1 medium (which was suggested by Fabregas et al., 1998) and the addition of vitamin B complex to this medium could appreciably enhance the cell density. The cell density of up to 8.44×10^4 cells mL^{-1} was obtained.

2. The cultivation of airlift bioreactor was demonstrated to outperform the bubble column at the same operating aeration velocity.

3. No appreciable effect of the ratio between downcomer and riser cross sectional area was noticed.

4. The addition of CO_2 could considerably enhance the growth rate of *H .pluvialis*.

5. The addition of air into the airlift bioreactor was crucial for a proper growth of the alga, but the aeration rate should be small, and in this work, the most appropriate aeration velocity (superficial velocity) was found at the lower limit of the pump which was 0.4 cm s^{-1}.

6. The most suitable light intensity for the growth of *H. pluvialis* NIES-144 in the airlift bioreactor was found to be at 20 μmol photon m^{-2} s^{-1}.

The culture with the light intensity of 20 μmol photon m^{-2} s^{-1}, at aeration velocity of 0.4 cm s^{-1} provided the maximum cell density at 77×10^4 cells mL^{-1} (2.79 g L^{-1} of cell dry weight) and with the maximum specific growth rate of 0.45 day^{-1}.

7. The semi-continuous culture was successfully implemented where the harvest could be performed every 4 days with the specific growth rate of 0.31 day^{-1}.

8. The accumulation of astaxanthin could be induced by increasing the light intensity and/or diluting the growth medium with water. The Astaxanthin content was concluded in Table 5.1.

Table 5.1 Astaxanthin content at various stressed conditions

Condition	Astaxanthin content			
	mg L^{-1}	mg in 10^6 cells	g dry weight L^{-1}	% astaxanthin content
Controlled experiment	17.2	37	0.14	0.8
Diluted medium	37.5	81	0.17	0.97
Increasing ight	45.3	102	0.22	1.28

5.2 Contributions

This work demonstrated that it was possible to design an airlift system for a semi-continuous production of *H.pluvialis* which is one of the most effective microorganisms that could produce the required, high antioxidant property, astaxanthin. The airlift system was proven to deliver a very high productivity of such alga, only second to the experiment by Hata et al (2001) who only carried out a smaller scale experiment using 500mL Erlenmeyer flasks. It should not be incorrect to say that this experiment is the first that proves the applicability of airlift system in growing such difficult-to-grow culture in relatively large bench scale equipment. The experiment could be operated for a long period without serious problems which indicates that there is a potential in upscaling this system to industrial level.

5.3 Recommendations

Due to a serious time constraint of the experimental work, several parameters could not be tested for their optimality in the cultivation of *H.pluvialis*. Therefore the following future works regarding the cultivation of the alga are recommended:

1. The optimal level of CO_2 in the air supply to the system should be examined.
2. The schedule for harvest in the semi-continuous culture should be determined to ensure the highest biomass productivity.
3. The upscale of the reactor should be investigated.

Note that there are also works required to complete the cycle of astaxanthin production such as the stimulation and extraction of astaxanthin. These works are available intensively in the literature and are not the main purpose of this work.

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APPENDICES

Appendix A

Experimental data

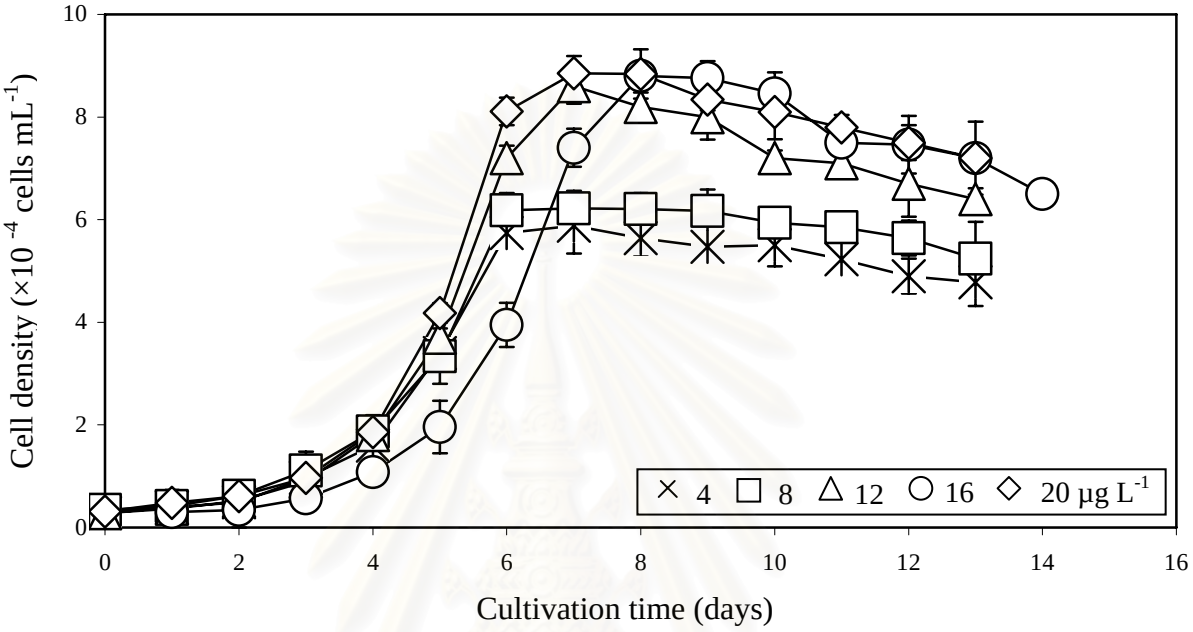

Figure A-1 Cultivation of *H. pluvialis* at different superficial gas velocity

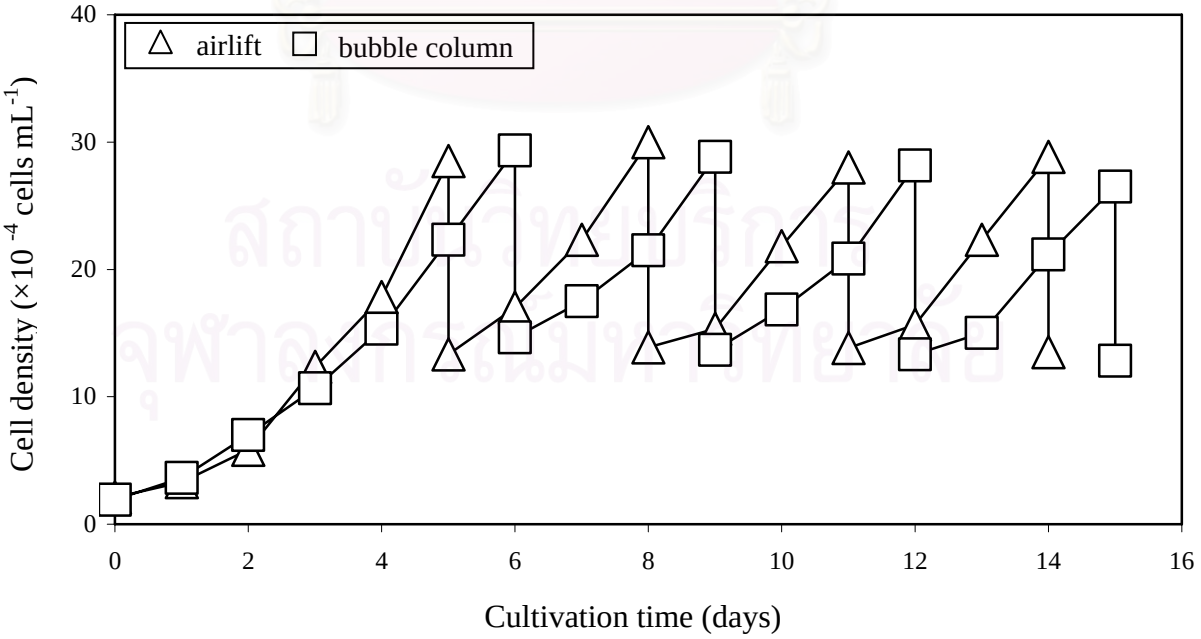

Figure A-2 Cultivation of *H. pluvialis* in airlift bioreactor and bubble column at 0.4 cm s^{-1} superficial gas velocity

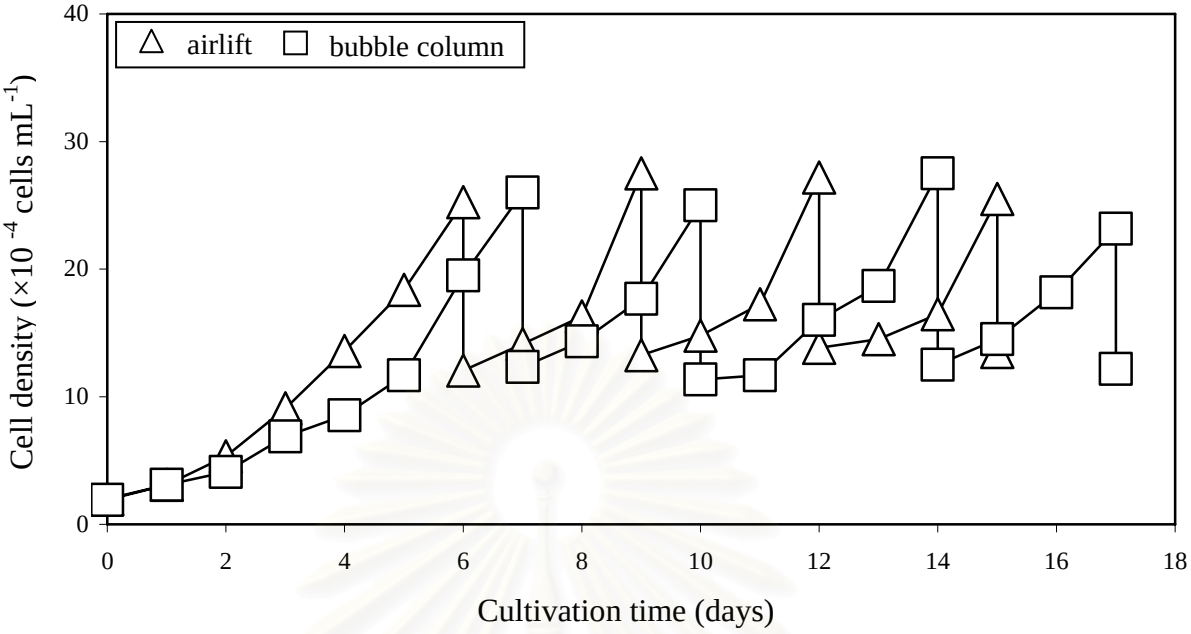

Figure A-3 Cultivation of *H. pluvialis* in airlift bioreactor and bubble column at 0.7 cm s^{-1} superficial gas velocity

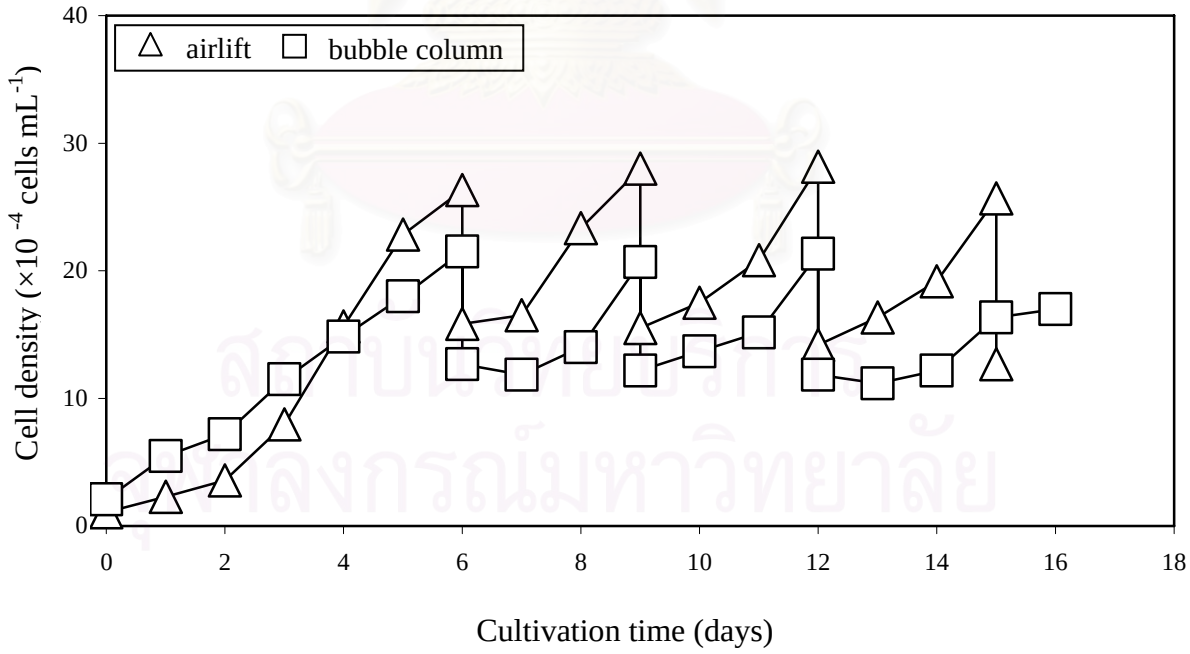

Figure A-4 Cultivation of *H. pluvialis* in airlift bioreactor and bubble column at 1 cm s^{-1} superficial gas velocity

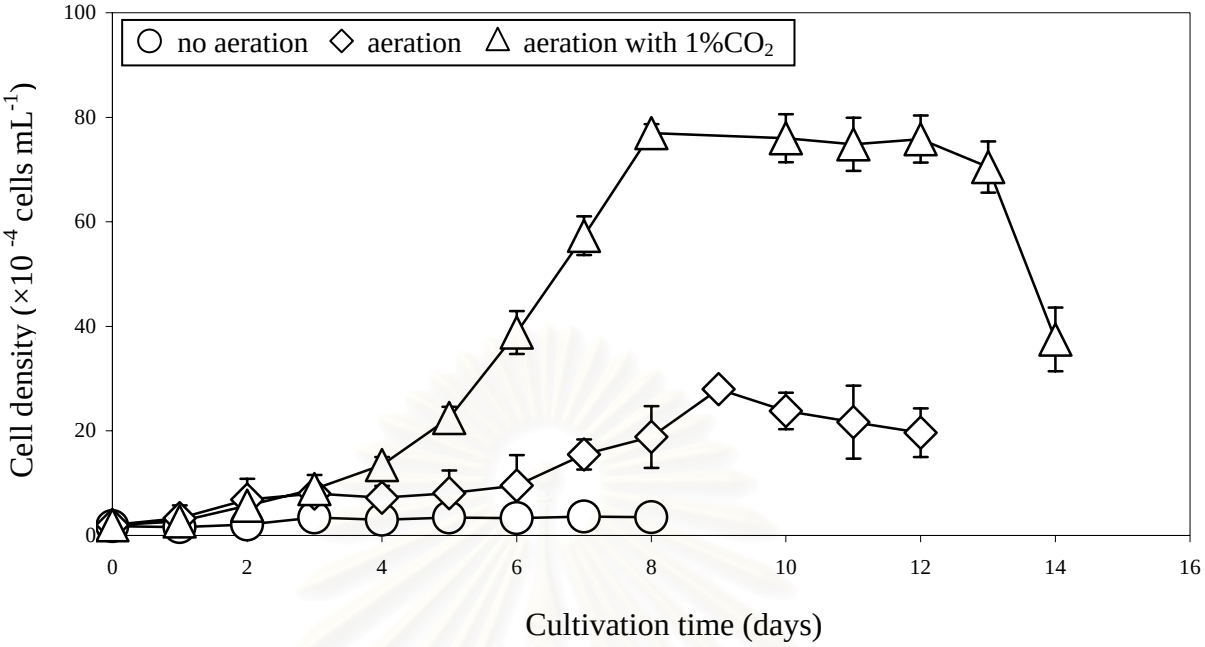

Figure A-5 Cultivation of *H. pluvialis* with different aeration in airlift bioreactor

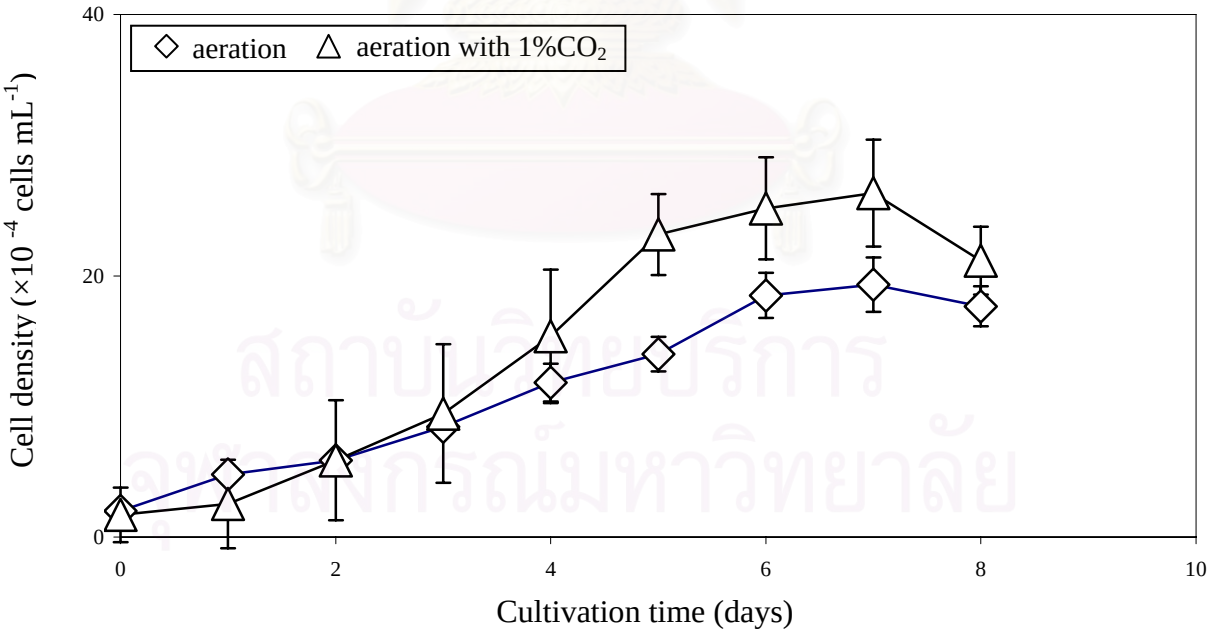

Figure A-6 Cultivation of *H. pluvialis* with different aeration in airlift bioreactor at 20.6 cm s^{-1} of superficial gas velocity

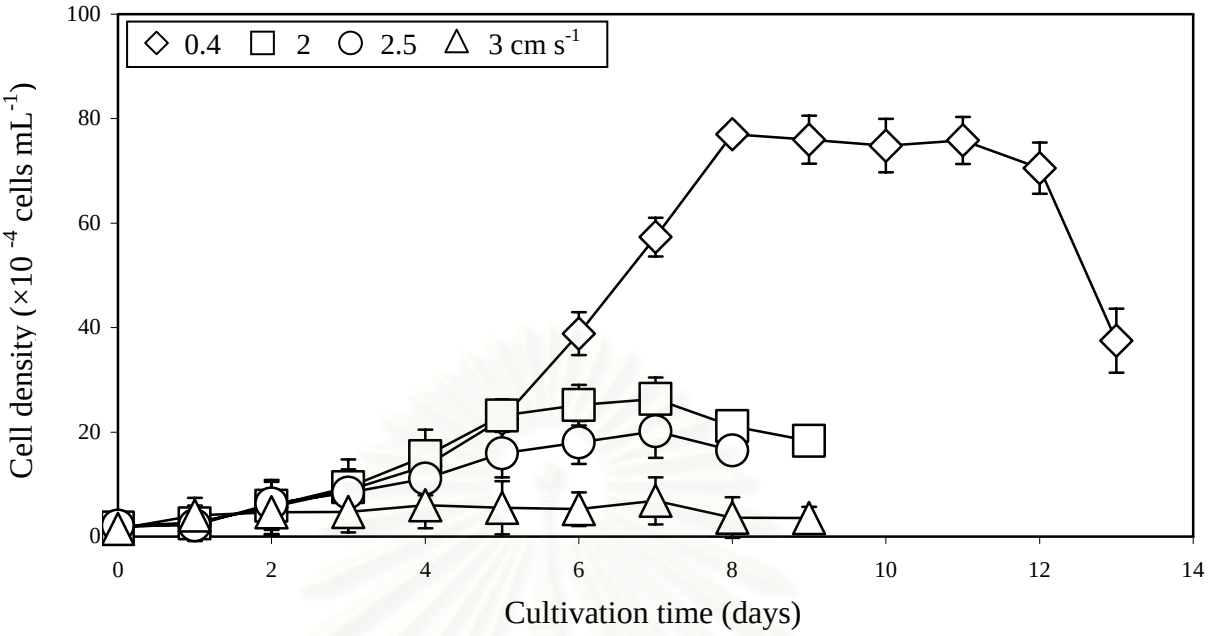

Figure A-7 Cultivation of *H. pluvialis* at different superficial gas velocity in airlift bioreactor

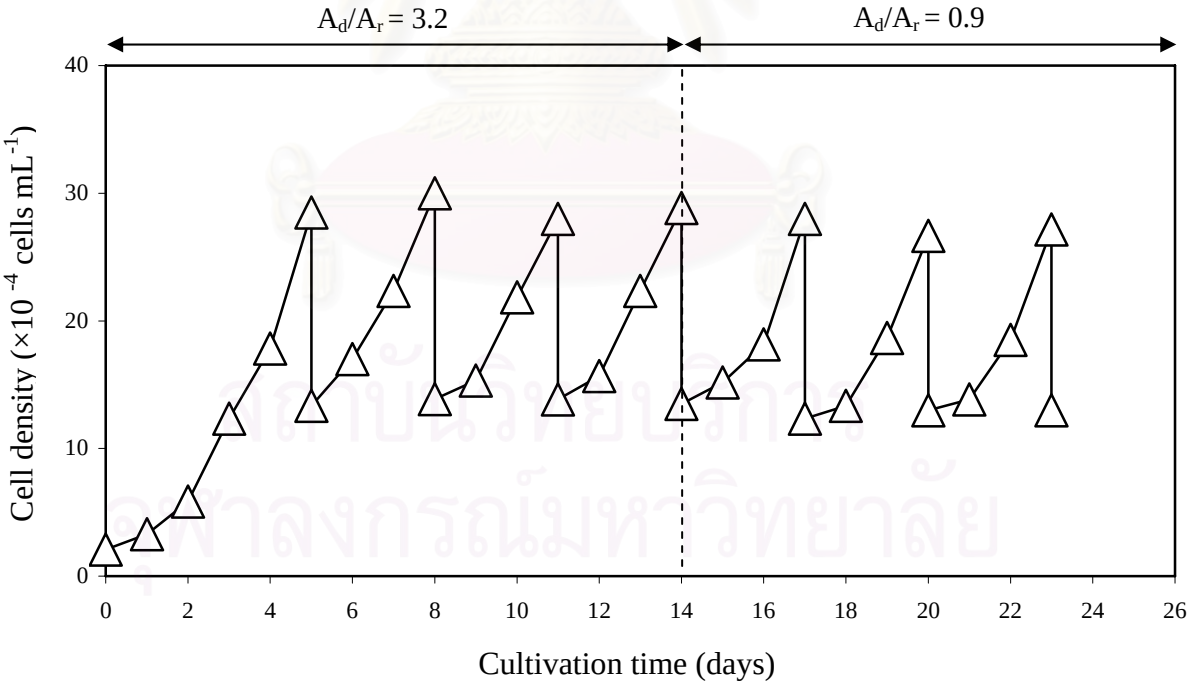

Figure A-8 Cultivation of *H. pluvialis* at 0.4 cm s^{-1} superficial gas velocity with different A_d/A_r

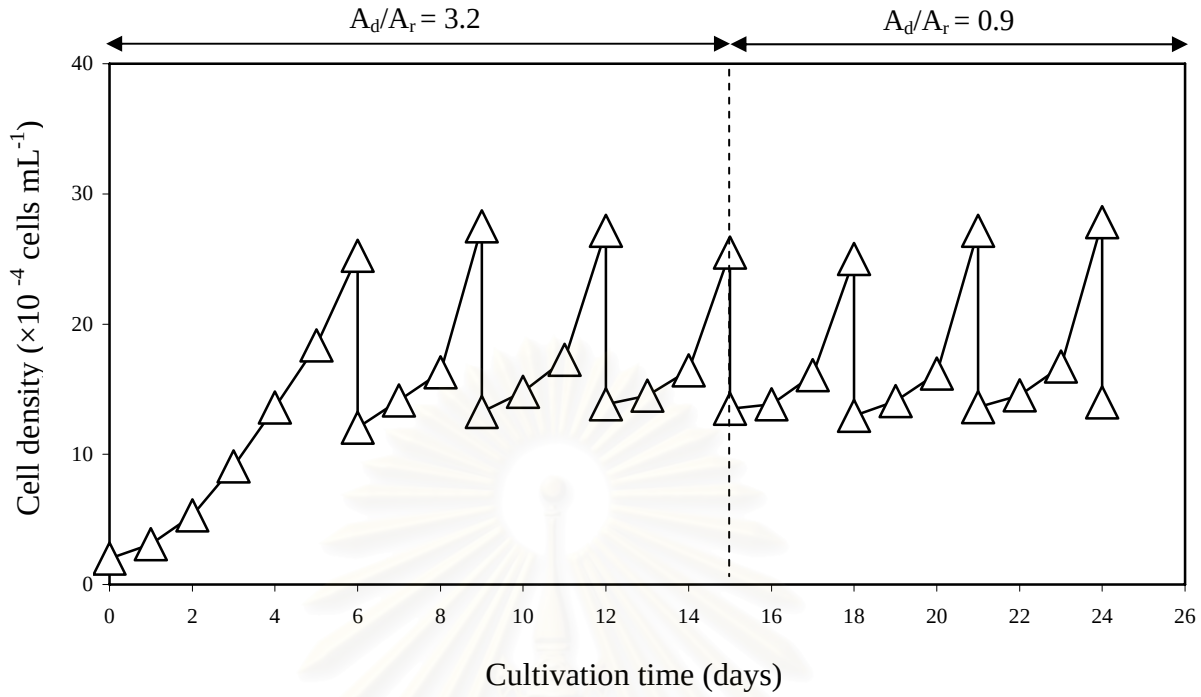

Figure A-9 Cultivation of *H. pluvialis* at 0.7 cm s^{-1} superficial gas velocity with different A_d/A_r

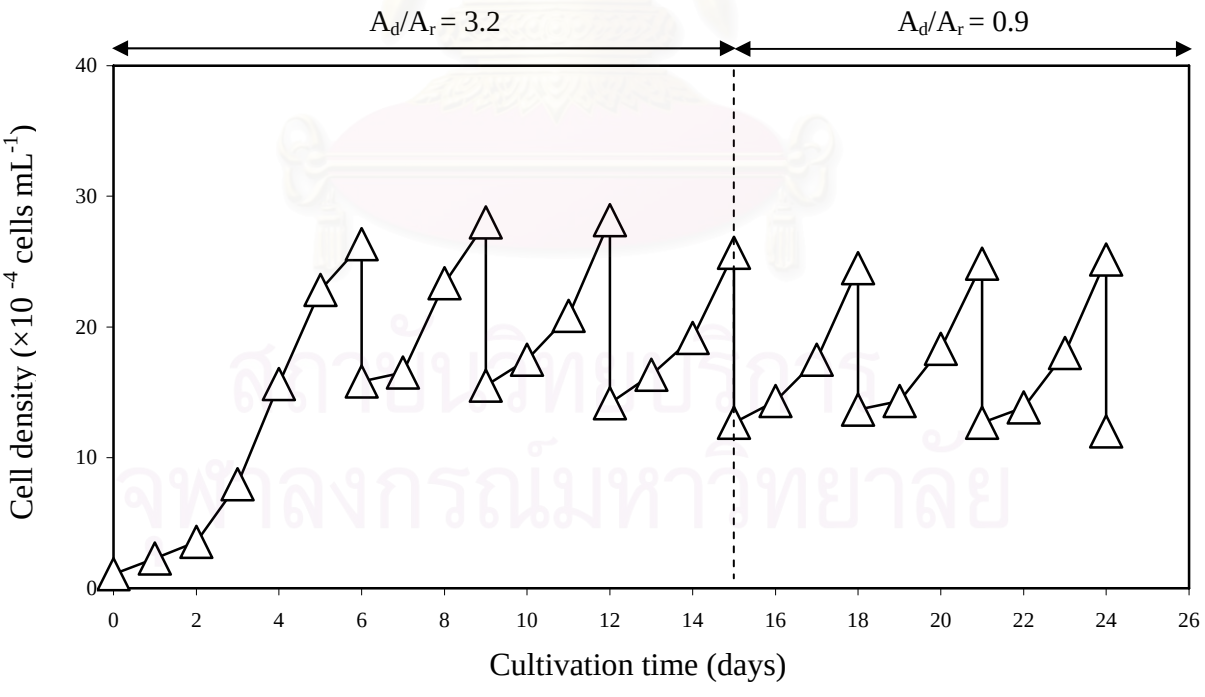

Figure A-10 Cultivation of *H. pluvialis* at 1 cm s^{-1} superficial gas velocity with different A_d/A_r

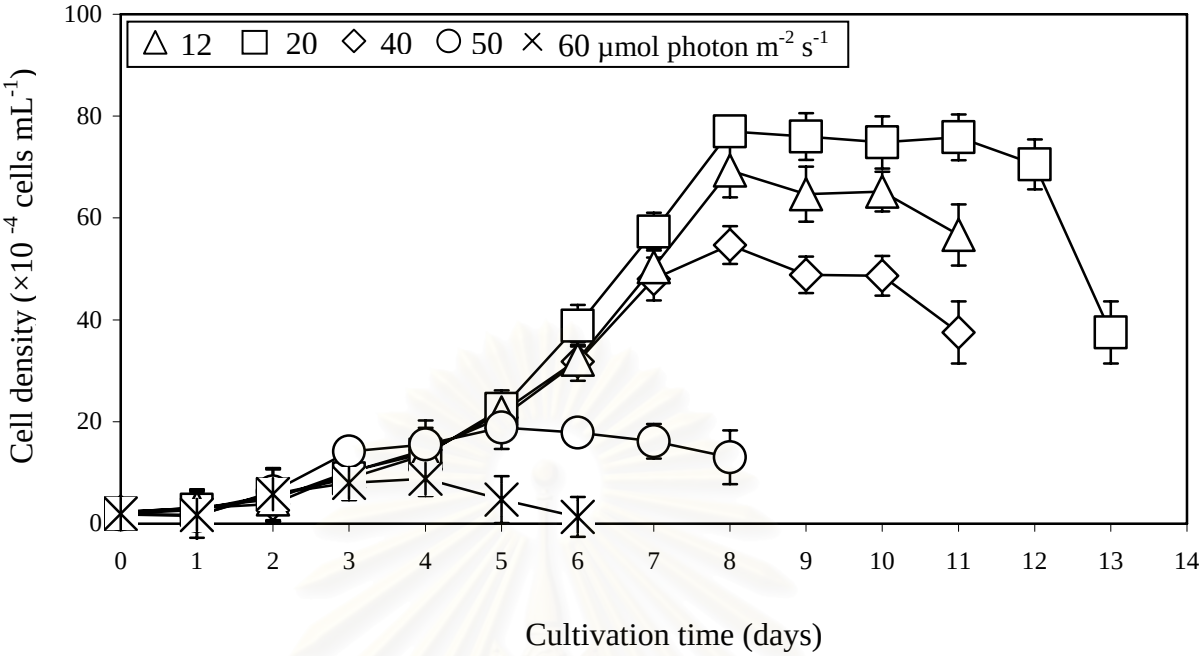

Figure A-11 Cultivation of *H. pluvialis* at different light intensity in airlift bioreactor

Appendix B

The Regional Symposium on Chemical Engineering 2003
Metro Manila, Philippines, December 1-3, 2003

Effect of Culture Media on Growth of *Haematococcus pluvialis*

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The unicellular microalga *Haematococcus pluvialis* has recently aroused considerable interest due to its capability to accumulate the ketocarotenoid astaxanthin, a derivative of β-carotene that is used as a food additive for pigmentation in aquaculture. The compound has also been shown to possess various positive actions, such as free-radical scavenging action, immunomodulation, and cancer prevention. *H. pluvialis* grows well in relatively low temperatures between 20-25 °C and pH between 6.8 and 8. Unlike some other algal species that grown in highly saline or highly alkaline waters, *H. pluvialis* grow in neutral freshwater, and are therefore more susceptible to contamination. Contamination problem is worsened when the alga is grown under heterotrophic conditions where carbon source is directly supplied in the culture medium. We therefore are interested in growing *H. pluvialis* under photoautotrophic condition.

In this paper, an initial study was presented, in which we determined the effect of various photoautotrophic culture media on growth of *H. pluvialis*, under continuous light, whose intensity was 2 klux. The temperature was controlled at 24 °C. The growth pattern in seven different culture media was monitored for a period of 14 days. And the effect of B vitamins was also determined for each medium. Among the photoautotrophhic media without B vitamins tried, Control medium or KNO_3 0.41 g l^{-1}, Na_2HPO_4 0.03 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 16.41 mg l^{-1},

$CaCl_2 \cdot 2H_2O$ 9.78 mg l^{-1}, $FeSO_4 \cdot 7H_2O$ 2.21 mg l^{-1}, $CoCl_2 \cdot 6H_2O$ 0.0078 mg l^{-1}, $CuSO_4 \cdot 5H_2O$ 0.0080 mg l^{-1}, Cr_2O_3 0.05 mg l^{-1}, $MnCl_2 4H_2O$ 0.66 mg l^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.08 mg l^{-1}, and SeO_2 0.036 mg l^{-1} was found to be the best for growth. Addition of B vitamins to the control medium resulted in increased maximum cell density by 75 %.

***Key words**: Haematococcus pluviails, astaxanthin, culture media, photoautotrophic cultivation*

INTRODUCTION

Astaxanthin or 3, 3'-dihydroxy-β,β-carotene-4,4'-dione, a red ketocarotenoid, naturally produced in large amount in unicellular microalga, *Haematococcus pluvialils*, has attracted tremendous interest as a food colorant and pigment in feed of aquaculture animals. Its anti-oxidative activities have been shown to be superior to its carotenoid analogues. *Haematococcus pluvialis* can be grown in heterotrophic, photoautotrophic, or mixotrophic condition. The two major stages of growth include the green vegetative stage and the enlarged red alplanospore stage. During the process of transformation of the green motile vegetative cells to the alplanospore stage, astaxanthin is accumulated. This occurs under the influence of increased light intensity and nutrient stress. Other factors inducing the synthesis of astaxanthin have also been extensively studied (Fábregas et al, 2000; Tripathi et al, 1999; Gong and Chen, 1998; Hata et al, 2001; Fabregas et al, 1998; Kobayashi et al, 1991). However, one of the main problems to be solved for the mass production of astaxanthin from *H. pluvialis* is the establishment of conditions for sustained production of vegetative cells, which greatly depends on the type of culture medium and culture conditions. *H. pluvialis* grows well in relatively low temperatures between 20-25 °C and pH between 6.8 and 8. Being freshwater algae requiring rather neutral growth condition makes *H. pluvialis* more susceptible to contamination than seawater algae or other alkali resistant algae as the conditions used for its growth is also favorable for growth of other microorganisms such as bacteria and protozoa. The contamination problem is worsened when the alga is grown under heterotrophic conditions where carbon source is directly supplied in the culture medium. We therefore are interested in growing *H. pluvialis* under photoautotrophic condition.

In this paper, an initial study was presented, in which we determined the effect of various photoautotrophic culture media on growth of *H. pluvialis*, under continuous light, whose intensity was 2 klux. The temperature was controlled at 24 °C. The growth pattern in seven different culture media was monitored for a period of up to 16 days. And the effect of B vitamins was also determined for each medium.

MATERIALS AND METHODS

Haematococcus pluvialis (NIES 144) was obtained from the National Institute of Environmental Studies culture collection. All culture experiments were performed in 250 ml flasks containing 90 ml of medium at 24 °C with continuous illumination provided by fluorescent

lights whose intensity was 2 klux. 10 ml of an inoculum (approximately 0.25×10^4 cells ml^{-1}) was used for each flask. During the experiment, the flasks were shaken manually twice a day. Seven culture media tested growth of *H. pluvialis* are 1) M1 (Chen et al, 1997) 2) Basal (Hata et al, 2001) 3) Control (Fábregas et al, 1998) 4) BG-11 (Montsant et al, 2001) 5) Hong Kong (Zhang et al, 1999), 6) M6 (Gong and Chen, 1998) in this study were previously used by various authors for *H. pluviailis* cultivation and were modified here such that carbon sources and vitamins were eliminated. The compositions of each culture medium were summarized in Table 1. Cell densities were measured daily by microscopecounting using a haematocytometer for up to 16 days. The effect of B vitamins was determined for each medium in which B1 (16.8 gl^{-1}), B6 (1.26 gl^{-1}), and B12 (12.6 gl^{-1}) were added.

RESULTS AND DISCUSSIONS

Effect of different culture media on cell density

The effect of different culture media without B vitamins on cell density is shown in Figure 1. It can be seen from this figure that the growth of *Haematococcus pluvialis* is greatly influenced by the type of culture media. The growth was found to be worst in M1 and M6 media and was best in Control medium, in which the maximum cell density was 5.44×10^4 cell ml^{-1}. This was obtained on day 12. Growth in Basal medium was inferior to that in Basal:BG-11 medium, which was comparable to BG-11 medium alone. The Hong Kong medium also resulted in similar growth to Basal:BG-11 and BG-11 media. The cell density during the first 8 days of the culture grown in Control medium was indeed similar to that grown in BG-11, Basal:BG-11, and Hong Kong media. However the cell density continued to increase in the case of Control medium, whereas in the other culture media, the growth started to enter the stationary phase, and declined shortly after that. For all cultures in this experiment, cell encystment was observed when the growth entered the stationary phase. Growth results of *H. pluvialis* determined by different authors differ significantly. It is rather difficult to compare these results obtained by different authors due to variations in experimental designs and bioreactors configurations, as well as different media like autotrophic, mixotrophic and heterotrophic media and culture conditions used. The cell densities obtained in the present study were not as high as those obtained in some other studies because other culture conditions were not optimized.

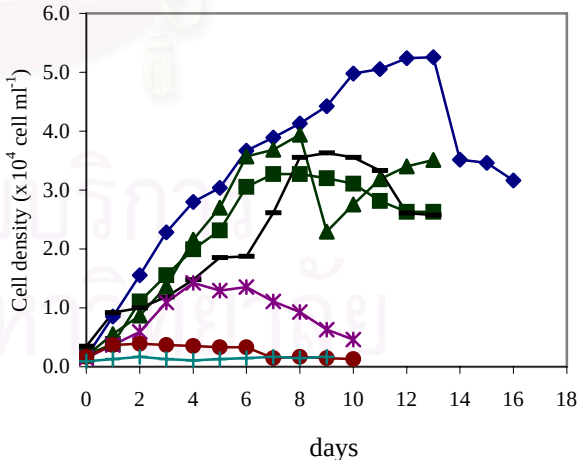

Figure 1. Cell density of *H.pluvialis* grown in different culture media. ◆ Contorl medium, ■ Hong Kong, ▲ BG-11, Basal, ━ Basal:BG-11, Basal, ● M1 and ┼ M6

Effect of B vitamins on growth of H. pluvialis

The growth in different culture media with added B vitamins is shown in Figure 2. The best medium for growth was still Control medium, which resulted in growth that was significantly higher than any other media tested. Addition of B vitamins showed significant improvement in cell density in the Control medium as can be seen in Figure 3. The maximum cell density was 8.6 $\times 10^4$ (75% increase), which was obtained on day 7. The rate of increase in cell density was improved to 1.2×10^4 cell ml^{-1} day^{-1}, from 0.48×10^4 cell ml^{-1} day^{-1} for culture in control medium without B vitamins. However, it did not significantly increase the cell density or the growth rate of *H. pluvialis* grown in the other growth media. In some cases, cell density was found to be lower when B-vitamins are added. The effect of B vitamins has been studied on growth of microalgae. Among vitamins, thiamine was established as a growth factor for this microalga, while B12 stimulated growth but was not essential (Pringsheim, 1966). Fábregas and coworkers found that *H. pluviails* requires thiamine (B1) together with the B12 and biotin in order to obtain maximal growth. (Fabregas et al., 1998), while other authors have found that biotin, thiamine and B12 have no significant effect on growth rate and final dry weight (Gong and Chen 1997). The present study shows that B vitamins addition affects growth of *H. pluvialis* in some media but does not affect growth in other media. This supports different results found by various authors.

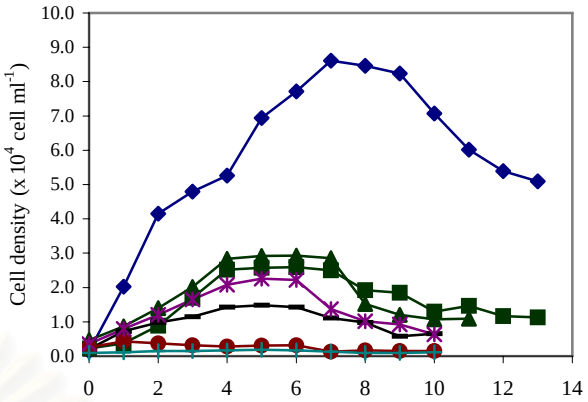

Figure 3. Growth of H. pluviailis in various meidia with ◆ B vitamins. ■ Control, ▲ BG-11, Basal: BG-11, Basal, ● M1, and ─┼─ M6

CONCLUSION

Of all the seven growth media tested, control media is best for the growth of H. pluvialis. The positive effect of B vitamins on the algal growth is significant in the control medium. However, this is not the case for other media tested.

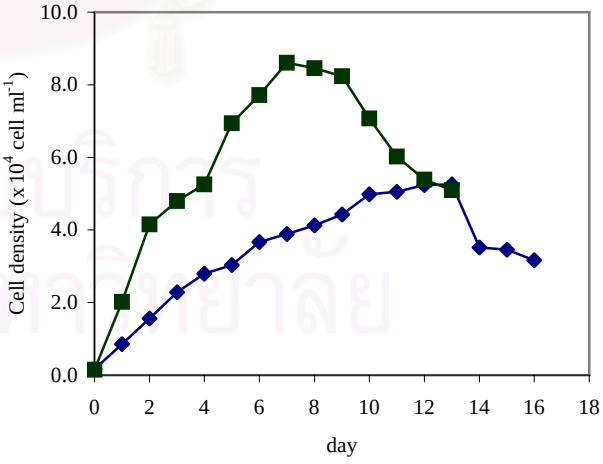

Figure 3. Effect of B vitamins on growth of H. Pluviailis in Cotrol media. ◆ Without , ■ With B vitamins.

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Table 1 Formulation of Culture media

	M1(Chen et al., 1997) (per liter)	Basal (Ogbonna et al., 2001) (per liter)	Control (Fábregas et al., 2001) (per liter)	BG-11 (Monsant et al., 2001) (per liter)	Hong Kong (Zhang et al., 1999) (per liter)	M6 (Ogbonna et al., 2001) (per liter)
$CaCl_2 \cdot 2H_2O$	183.8 mg	25 g	9.78 mg	36 mg	73 mg	3.676 g
KNO_3	0.5 g		0.41 g		0.3 g	
$NaNO_3$				1.5 g		
Na_2HPO_4			0.03 g			
NaH_2PO_4	195 mg				35.5 mg	1.778 g
H_3PO_4	0.12 mg					12.37 mg
$K_2HPO_4 \cdot 7H_2O$		75 mg		40 mg		
KH_2PO_4		175 mg				
NaCl		2.513 g				
KOH		30.85 mg				
H_2SO_4		0.99 mg				
EDTA	18.6 g	49.34 mg		1 mg		
$C_6H_5FeO_7 \cdot 5H_2O$			2.21 mg			
$FeSO_4 7H_2O$		4.976 mg				417 mg
$MgSO_4 \cdot 7H_2O$	61.6 mg	74 mg	16.41 mg	75 mg	24.6 mg	1.231 g
$ZnSO_4$	0.72 mg				0.014 mg	71.89 mg
$CuSO_4 \cdot 5H_2O$	0.62 mg	1.572 g	0.008 mg		0.012 mg	62.42 mg
$Na_2MoO_4 \cdot 2H_2O$	0.07 mg		0.08 mg		0.001 mg	7.26 mg
$CoCl_2 \cdot 2H_2O$	0.05 mg				0.0005 mg	4.67 mg
$MnCl_2 \cdot 4H_2O$		1.445 mg				
MoO_3		0.705 mg	0.66 mg			
$CoNO_3 \cdot 6H_2O$		0.389 mg				
H_3BO_3		10.948 mg			0.003 mg	
Cr_2O_3			0.05 mg			
SeO_2			0.036 mg			
$CoCl_2 \cdot 6H_2O$			0.0078 mg			
$ZnSO_4 \cdot 7H_2O$		8.827 g				
$EDTANa \cdot 2H_2O$					6.7 mg	0.372 g
$FeSO_4 \cdot H_2O$					8.3 mg	
$FeSO_4 \cdot H_2O$					0.016 mg	
$MnSO_4 \cdot 5H_2O$						
Na_2CO_3				0.02 g		
$NH_4Fe(C_6H_5O_7)$			6 mg			
H_3BO_3				0.86 g		
$MnCl_2 \cdot 4H_2O$				1.81 g		
$ZnSO_4 \cdot 7H_2O$				0.222 g		
$Na_2MoO_4 \cdot 2H_2O$				0.39 g		
$CuSO_4 \cdot 5H_2O$				79 mg		
$Co(NO_3)_2 \cdot 6H_2O$				49 mg		

BIOGRAPHY

Miss Kamonpan Kaewpintong was born on March 26th, 1981 in Bangkok. She finished her secondary school from Satriwitaya School in March, 1998. After that, she studied in the major of Electronic Engineering Faculty of Engineering at King Mongkut's Institute of Technology Ladkrabang. She continued her further study in Master's degree in Chemical Engineering at Chulalongkorn University. She participated in the Biochemical Engineering Laboratories and achieved her Master's degree in April, 2005.