OPTIMIZATION STUDY OF BIOMASS AND ASTAXANTHIN PRODUCTION BY *HAEMATOCOCCUS PLUVIALIS* UNDER MINKERY WASTEWATER CULTURES

by

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ABSTRACT

Haematococcus pluvialis (*H. pluvialis*) is a green freshwater microalgae species, well known for its ability for astaxanthin accumulation. In this study, minkery wastewater (MW) was used as nutrient resource for the cultivation of *H. pluvialis*, which was able to achieve an energy and cost-efficient production strategy. The findings indicated that *H. pluvialis* grew well in diluted MW. The maximum biomass and astaxanthin production was obtained in 1.5% MW cultures with successful removal of nitrogen, yielding at 906.33 ±34.0 mg L^{-1} and 39.72±1.69 mg L^{-1}, respectively. Acetate and NaCl were introduced as stress factors to improve the astaxanthin production. Response surface methodology found the optimal combination of stress factors for yield, consequently, resultant astaxanthin content can reach 67.95 ± 3.93 mg L^{-1} after 12-day induction period. These findings confirmed the potential use of MW in *Haematococcus* cultures for both biomass and astaxanthin production as compared to conventional culture medium.

LIST OF ABBREVIATIONS USED

A	Absorbance
AC	Microalgae concentration
ANOVA	Analysis of variance
ADP	Adenosine diphosphate
AO	Antioxidants
ATP	Adenosine triphosphate
BBM	Modified Bold's basal medium
Chl	Chlorophyll
Chl-a	Chlorophyll a
Chl-b	Chlorophyll b
C	Carbon, concentration
CCD	Central composite design
COD	Chemical oxygen demand
CO_2	Carbon dioxide
CPCC	Canadian Phycological Culture Centre
CRTR-B	β-carotene hydroxylase
CRTL-B	β-lycopene cyclases
CRTO	β-carotene-oxygenase
DMAPP	Dimethylallyl pyrophosphate
DMSO	Dimethyl sulfoxide
DW	Dry weight
EA	Energy absorption
GGPP	Geranylgeranyl pyrophosphate
Glu	Glutamate
H. pluvialis	*Haematococcus pluvialis*
ICD	Initial cell density

IPP	Isopentenyl pyrophosphate
ISC	Intersystem crossing
MW	Minkery wastewater
N	Nitrogen, dry weight in cultivation period
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NPQ	Non-photochemical quenching
OD	Optical density
P	Phosphorus, productivity, p-value
PAR	Photosynthetically active radiation
PBR	Photobioreactor
PDS	Phytoene desaturase
PSY	Phytoene synthase
PTOX	Plastid terminal oxidase
LED	Light emitting diode
LSP	Light saturation point
RISC	Reverse intersystem crossing
ROS	Reactive oxygen species
RSM	Response surface methodology
SCar	Second carotenoid
SC-CO_2	Supercritical carbon dioxide
SCF	Supercritical fluids
TN	Total nitrogen
TP	Total phosphorus
TSEQ	Triple-state energy quenching
UV-Vis	Ultraviolet-visible
V	Volume

VSS	Volatile suspended solids
W	Weight
X	Variable code
Y	Yield
ZDS	ζ-carotene desaturase

Greek letters

α	Alpha value
β	Regression coefficient
μ	Specific growth rate
λ	Wavelength

Subscripts

0	0^{th} day, constant coefficient
a	Chlorophyll a, weight after ashed at 550ºC, volume of extracts
b	Chlorophyll b, weight after dried at 105ºC, volume of culture samples
final	Final day of the cultivation period
i	Variable
initial	Initial day of the cultivation period
j	Variable
n	Number of replications, number of factors
t	t day, induction time
x	Nutrient parameters
470	Absorptivity at 470 nm
490	Absorptivity at 490 nm
652.4	Absorptivity at 652.4 nm

665.2	Absorptivity at 665.2 nm
680	Absorptivity at 680 nm

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CHAPTER I

INTRODUCTION

1.1 Microalgae

As the global energy demand increases with population growth, energy shortages have become one of the bottlenecks of current social and economic growth all over the world (Moreno-Garcia et al, 2017). With the introduction and commercialization of bioenergy, it is now a critical technology that can replace a proportion of traditional non-renewable energy sources, such as fossil fuels, to sustain the enormous energy market (Panis and Carreon, 2016). Bioenergy production derived from microalgae, is one of the most attractive substitute compared to other crop-derived bioenergy production (Mata et al., 2010). Microalgae are unicellular photosynthetic organisms that can be harnessed in several sources, including biofuels, food supplements, animal feed and nutrients, pharmaceuticals, colorants, cosmetics, and other chemical products (Moreno-Garcia et al., 2017). They have the following remarkable merits: (1) higher growth rate and a higher photosynthetic efficiency than terrestrial plants, (2) capacity to fix atmospheric carbon dioxide, thereby reducing atmospheric concentration of greenhouse gases, (3) ability to grow in diverse environments without competing for arable land and freshwater, (4) can absorb nutrients from most wastewaters and can be integrated into wastewater treatment systems under either autotrophic or mixotrophic conditions, (5) biomass residues after valuable lipid/polysaccharide/antioxidant extraction can be used as nitrogen sources as fertilizers for crops or animal feed (Cai et

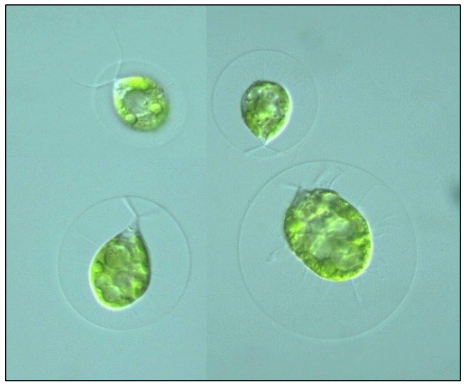
al., 2013; Munoz and Guieysse, 2006). There are more than 7,000 species of green microalgae existing in a variety of growth environments. Most microalgae can produce high contents of valuable compounds, such as protein, lipid, carbohydrates, fatty acids, vitamins, pigments and even accumulate secondary metabolites, such as astaxanthin, lutein and β-carotene under specific stress conditions (Shah et al., 2016; Panis and Carreon, 2016).

The possibility of producing biofuels using microalgae has been intensively studied in recent years (Cai et al., 2013; Abdel-Raouf et al., 2012; Kang et al., 2006). This is due to their higher productivity and economic feasibility and more environmental friendly production than those of first generation biofuels. In addition to their energy content, the amounts of bioactive molecules, such as carotenoids, fatty acid, polysaccharides, which can be extracted from microalgae biomass are important sources of nourishment, feed, colorants, and pharmaceuticals (Lemoine and Schoefs, 2010). Carotenoids are essential pigments that can naturally generate in all photosynthetic organisms including microalgae. These pigments have extraordinary functions on light absorbing, dissipation of excess light, and quenching excited state of chlorophyll (Collins et al., 2011). Recently, there has been a dramatic growth of interest in pigments on carotenogenic microalgae. Among these carotenoids, the most prevalent products are astaxanthin, β-carotene, lutein, canthaxanthin, and zeaxanthin (Solovchenko, 2013).

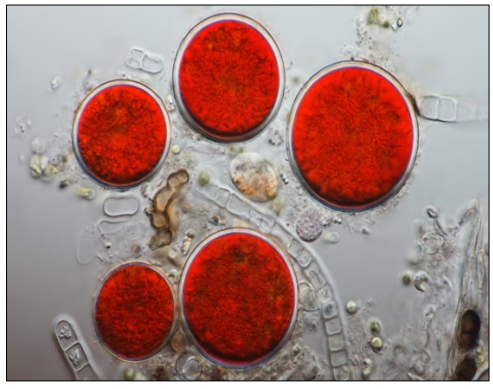
1.1.1 *Haematococcus pluvialis*

Haematococcus pluvialis (*H. pluvialis*) is a freshwater microalgae species that is mainly distributed in temperate regions around the world. It is well known for its ability for astaxanthin accumulation, which has extraordinary antioxidant and anti-inflammatory capabilities. This makes *H. pluvialis* production a potential opportunity for food and feed industries, pharmaceuticals and nutraceuticals as well (Higuera-Ciapara et al., 2006; Guerin et al., 2003). *H. pluvialis* has been described as having two phases of growth during its life cycle. Under optimal growth conditions, these algae grow as a green flagellated motile phase (Fig. 1a, accessed from http://protist.i.hosei.ac.jp/PDB/Images/Chlorophyta/Haematococcus/sp_2.html), while a non-motile non-flagellated red cyst phase (Fig. 1b, accessed from http://www.mikro-foto.de) is formed after exposure to stress conditions, such as nutrient depletion, enhanced salinity, high C/N ratio, increased light intensities or combined stress conditions (Collins et al., 2011; Kamath et al., 2008). Previously, researchers have reported that carotenoid fractions in *H. pluvialis* are different in the two phases. Carotenoids, such as lutein (75-80%) and β-carotene (10-20%), were mainly found in green vegetative cells; whereas around 80% at carotenoid as astaxanthin was found in the red cyst phase (Lorenz and Cysewski 2000; Renstrom et al., 1981). Pigment quantification analysis indicated that the concentrations of β-carotene and chlorophyll were stable as astaxanthin continued to accumulate as phases changed. High concentrations of carotenoid fractions were generated to protect

resting cells against photo-oxidative damage. Consequently, as the richest known resource of astaxanthin, *H. pluvialis* contains approximately 5% free astaxanthin, 10% astaxanthin diester, 70% astaxanthin monoester and the remaining 15% carotenoids were a mixture of β-carotene, lutein, canthaxanthin and other carotenoids of total carotenoids. Total astaxanthin content can be up to 4% weight of dry biomass, depending on growth conditions (Collins et al., 2011; Grunewald et al., 2001). Literature indicated that larger senescent vegetable cells, which are green immature cysts, have high capacity for astaxanthin production (Choi et al., 2011).



(a) Green flagellated motile phase



(b) Non-flagellated red cyst phase

Figure 1.1. *Haematococcus pluvialis* cells under microscope.

1.2 Potential bioproducts from *Haematococcus pluvialis*

Astaxanthin (3,3'-dihydroxy-b, b-carotene-4,4'-dione; $C_{40}H_{52}O_4$) is a high value keto-carotenoid, which is mainly found in aquatic animals. Astaxanthin has widespread applications, owing to its important biological functions, including protection against irradiation damage, promotion of oxidative stress resistibility, and enhanced

reproduction (Lemoine and Schoefs, 2010; Lorenz and Cysewski, 2000). Beutner et al. (2001) indicated that carotenoids, as biological antioxidants (AO), can prevent other molecules from being destroyed with degradation of carotenoids, by assimilating excited energy of singlet oxygen into carotenoids. Astaxanthin as an AO is reported to have stronger antioxidant capacity than that of β-carotene and Vitamin E (Shimidzu et al., 1996). Besides, *H. pluvialis* mostly accumulates lutein and β-carotene in the green motile phase prior to stress exposure, and they are also valuable carotenoids, which benefit human health (Lemoine and Schoefs, 2010). Since the *Haematococcus* algae astaxanthin has the traits mentioned above, they have been involved in various industrial applications. The global market for both synthetic and natural astaxanthin was estimated at USD 447 million in 2014 (Smith, 2015). Currently, synthetic astaxanthin is a major contributor to the commercial astaxanthin market (Lorenz and Cysewski, 2000). However, natural astaxanthin has been studied and found to be more favorable than the former, because of its extraordinary merits on application, function and security (Wan et al., 2014; Capelli et al., 2013).

1.3 Minkery wastewater as a nutrient resource

In order to achieve sustainable algae-based bioproduction, microalgae have been used in wastewater bioremediation processes, utilizing wastewater as a nutrient resource. This route has the advantages of reducing cultivation costs, natural resource inputs, and simultaneously obtaining high-value bioproducts (Farooq et al., 2013). Nitrogen (N) and phosphorus (P) are the main nutrients in various wastewaters, which

can easily be used for microalgae growth (Kang et al., 2007). In previous study, *H. pluvialis* was investigated for cultivation in diluted primary-treated sewage and primary-treated piggery wastewater (Kang et al., 2006). They grew well in these wastewaters and demonstrated successful uptake of nitrate and phosphorus after an 8-day cultivation period. In Nova Scotia, mink farming is one of the biggest agricultural industry, which offers many employment opportunities; and the industry was valued more than CAD$100 million dollars in 2010 (Statistics Canada, 2012). However, discharges of minkery wastewater pollute adjacent aquatic ecosystems due to its excessive content of nutrients, such as nitrate, ammonia, and phosphorus (Byrlinksy, 2011). Ji (2017) found that massive biomass production can be obtained when cultivated *Chlorella vulgaris* under diluted minkery wastewater. As mentioned above, microalgae have shown remarkable ability for nutrient uptake; and therefore, they support a sustainable approach for environmental remediation. In this way, mink wastewater can be used as a potential nutrient resource for *H. pluvialis* cultivation, and simultaneously, valuable bioproducts can be harvested for further useful applications.

1.4 Objectives

The overall goal of this study was to evaluate the technical feasibility of utilizing Nova Scotia's minkery wastewater resource for cultivating *H. pluvialis* with a specific purpose of generating high algal biomass and astaxanthin accumulation while biologically removing excessive nutrient contents from minkery wastewater. The specific objectives of this study were:

1) To determine the growth, nutrient removal capacity, biomass production and astaxanthin accumulation (under photoautotrophic induction) of green microalgae H. pluvialis under different minkery wastewater cultures;

2) To determine the effect of acetate and sodium chloride (NaCl) concentrations on astaxanthin accumulation by *H. pluvialis*; and

3) To determine the optimal levels of acetate and NaCl concentrations for maximum astaxanthin yield by *H. pluvialis*.

As will be discussed later in depth, for the purpose of inducing astaxanthin accumulation, this study tested a combination of nutrient starvation and high light intensity stress conditions as part of the photoautotrophic induction model, as well as acetate and NaCl (salinity) stress conditions as part of the mixotrophic induction model. Further, response surface methodology was used to determine the optimum levels of acetate and NaCl concentrations for the highest possible astaxanthin accumulation.

CHAPTER II

LITERATURE REVIEW

2.1 Biology of *H. pluvialis*

2.1.1 Classification, history, and distribution

Haematococcus pluvialis, also known as *Haematococcus lacustris* or *Sphaerella pluvialis*, is a green freshwater alga, which belongs to the class Chlorophyceae, order Volvocales, and family Haematococcaseae (Lorenz, 1999). *Haematococcus* was first observed by Girod-Chantrans in 1797 and described by Flotow in 1844. In 1899, Elloit Hazen published the life history of *Haematococcus*, presenting the alteration of cell morphology during its life cycle (Shah et al., 2016; Hazen, 1899). *H. pluvialis* is widely distributed in diverse environments around the world. It is primarily found in temperate regions and has been isolated in Europe, Africa, North America, and India (Suseela and Toppo, 2006). This species typically inhabits rock pools in temporary water bodies, such as ephemeral rain pools and artificial pools; it can also be also found on the rocks in brackish water, freshwater basins, and rocks in fish ponds (Shah et al., 2016). Proctor (1957) reported that, due to its distinguished and rapid ability of encyst formation, *H. pluvialis* can survive under conditions with extremely speedy fluctuations in light, temperature, and salinity levels that would be harmful to most microalgae.

2.1.2 Cellular morphology and life cycle

The details of cellular morphology of *H. pluvialis* were identified in 1934, which

is like most of other volvocalean unicellular green algae. During the life cycle, four types of cells were observed: microzooids, macrozooids, non-motile palmella, and hematocysts (aplanospores) (Elloit, 1934). Among four morphologies, microzooids, macrozooids, and non-motile palmella are normally referred as the 'green vegetative phase', while hematocysts are referred as the 'red non-mobile encysted phase'.

Flagellated green vegetative cells grow fast under favorite conditions with sufficient nutrient, 2-32 daughter cells are divided by mitosis in macrozooids stage (Wayama et al., 2013). When *H. pluvialis* are exposed to an unfavorable environment, macrozooids start to degrade the flagellates together with the cell size increasing. Subsequently, macrozooids gradually develop to non-motile palmella as an amorphous multilayered structure forms in the inner regions of the extracellular matrix or cell wall (Hagen et al., 2002). Continued adverse conditions (i.e., high light intensity, elevated temperatures, increased salt conditions, nutrient starvation) cause non-motile palmella (green vegetative phase) to accumulate massive astaxanthin content in the cell body, thereby transforming into the aplanospores (red cyst phase). Consequently, the structure of cells in this stage consists of a thick and rigid sheath and secondary cell wall of acetolysis-to be able to resist the extreme environmental conditions. Once *H. pluvialis* return to growing in optimal conditions, red aplanospores generate flagellated zoospores to initiate a new vegetative cycle (Shah et al., 2016; Lorenz, 1999). The life cycle of *H. pluvialis* under microscope is shown in Figure 2.1 (Shah et al., 2016).

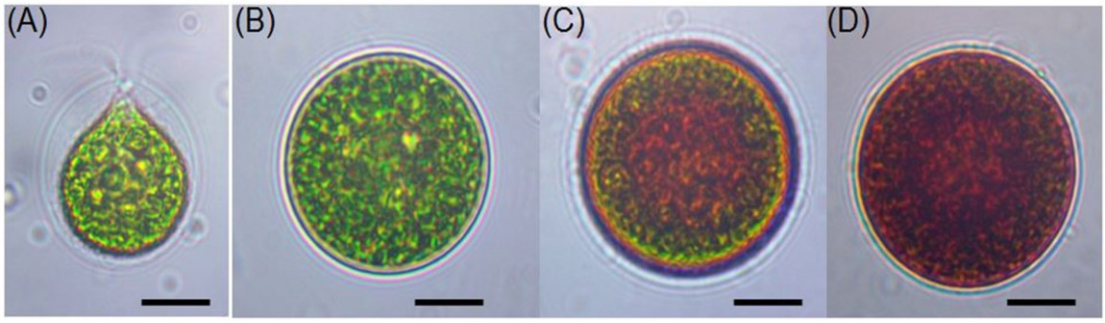


Figure 2.1. Light microscopic images of *H. pluvialis* cells in life cycle. (A) Green vegetative motile cells; (B) Green vegetative palmella cell; (C) Astaxanthin accumulation cell in transition to aplanospores; (D) Astaxanthin accumulated aplanospore cell. Scale bar: 10 μm.

2.1.3 Major biochemical compositions of *H. pluvialis*

Since the two-stage life cycle of *H. pluvialis* is atypical, the cellular composition of this microalgae is distinguished between vegetative cell stage and encysted stage. The typical composition of cells contains proteins, carbohydrates, lipids, and carotenoids (Shah et al., 2016).

2.1.3.1 Protein, carbohydrates, and lipids

In the vegetative stage, *H. pluvialis* are mainly composed of protein (29-45%), carbohydrates (15-17%), and lipids (20-50%) (Table 2.1). It was determined that there is a lower protein content in the red encysted stage of *H. Pluvialis*, approximately to 21-23%. Major amino acid composition in proteins contain aspartic acid, glutamic acid, alanine, and leucine (Lorenz, 1999). By contrast, massive increases in carbohydrate contents are synchronized with the conversion of the vegetative stage to red aplanospores, which may be up to 40% of the cell dry weight (DW) (Table 2.1). Starch are consumed in cells as the stress conditions prolonged (Shah et al., 2016). Total lipid

content, on the other hand, varies from 20 to 25% in the vegetative stage and increases to approximately 32 to 37% in the red encysted stage. Both of the two stages undergo the synthesis of neutral lipids, which occupy over half the present total lipid content. Glycolipid content almost doubles in red cells compared to the cells that grew in the vegetative stage, while the phospholipid content nearly stays the same with the green vegetative cells (Table 2.1). The predominant fatty acid components in *H. pluvialis* are palmitic, linoleic, and linolenic. According to comparative studies, the variations in the amount of these fatty acids are strongly associated with environmental factors depending on different strains of *H. pluvialis* (Lorenz, 1999). Boussiba and Vonshak (1991) reported that the increase in lipid contents is accompanied with the massive accumulation of astaxanthin. Since carotenoids are hydrophobic compounds, which can be dissolved in oil, it has been proven that fatty acids serve as a matrix for pigment solubilizing, thereby providing pigment accommodation (Boussiba 2000). Moreover, chlorophyll content declines dramatically as cells transit to the red encysted stage (Shah et al., 2016).

Table 2.1: Typical composition of *H. pluvialis* in vegetative and cyst stages.

Composition content (% of DW)	**Vegetative stage**	**Cyst stage**
Proteins	29-45	17-25
Carbohydrates	15-17	36-40
Lipid (% of total)	20-25	32-37
Neutral lipids	59	51.9-53.5
Phospholipids	23.7	20.6-21.1
Glycolipids	11.5	25.7-26.5
Carotenoids (% of total)	0.5	2-5

Composition content (% of DW)	Vegetative stage	Cyst stage
Astaxanthin (including esters)	n.d.	81.2
β-carotene	16.7	1.0
Lutein	56.3	0.5
Zeaxanthin	6.3	n.d.
Canthaxanthin	n.d.	5.1
Neoxanthin	8.3	n.d.
Violaxanthin	12.5	n.d.
Chlorophylls	1.5-2	0

Adapted from Grewe and Griehl (2012); n.d. means no data.

2.1.3.2 Carotenoids

Carotenoids are tetraterpenoid pigments that have a diversity of functions in organic photosynthesis, such as light-harvesting, quenching chlorophyll triplet states, scavenging reactive oxygen species (ROS), and dissipation of excess energy (Collins et al., 2011; Guedes et al., 2011; Grünewald et al., 2001). According to its chemical structure, carotenoids can be classified into different groups. Carotenes, which only consist of linear hydrocarbons without any substituent, can be cyclized at one or both ends of the molecule (i.e., α- and β-carotene, lycopene). Xanthophylls, which are the oxygenated derivatives of carotenes, which present the -OH groups (i.e., zeaxanthin), the =O groups (i.e., canthaxanthin) or both of the two groups (i.e., astaxanthin and lutein). Some carotenoids have more complex structures, such as the epoxy groups (i.e., violaxanthin) and the acetylated groups (i.e., dinoxanthin and fucoxanthin) (Raposo et al., 2015; Botella-Pavía and Rodríguez-Concepción, 2006). On the other hand, carotenoids can also be classified by different biological functions, known as primary and secondary carotenoids (SCar). Normally, primary carotenoids are located in the

thylakoid membrane of the chloroplast and serve for photosynthesis and other basic metabolism processes of microalgae. In contrast, secondary carotenoids are referred as the carotenoids that are neither localized in the chloroplast nor play a major role in photosynthesis (Krishna and Mohanty, 1998). Various microalgae species, such as *Chlorella zofingiensis*, *Dunaliella salina*, and *H. pluvialis*, have been found to accumulate massive secondary carotenoids in lipid vesicles after exposure to stressful environmental conditions through carotenogenesis (Collins et al., 2011). Reactive oxygen species, such as singlet oxygen ($^1O_2$), H_2O_2, superoxide ion radicals (O_2^-) and hydroxide radicals (OH^-) can be generated by these photochemical (photosynthesis) and non-photochemical stresses (Lemoine and Schoefs, 2010). As a result, oxidative stress triggers due to the generation of unbalanced ROS, which damages cellular components, including proteins, DNA, and membranes. Simultaneously, the production of secondary carotenoids can be stimulated, which assimilates molecular oxygen via a carotenogenesis pathway, thereby reducing formation of ROS (Li et al., 2008; Elstner, 1982). Furthermore, the secondary carotenogenesis is considered as a survival mechanism for microalgae under stressful environments, which can be used to store energy and carbon (Lemoine and Schoefs, 2010). Much evidence has shown that oxidative stress caused by reactive species is highly related to several diseases, cancers, and malfunctions. For instance, some reactive species restrain the gene expressions and stimulate the development of some diseases, such as neurodegenerative disease and cardiovascular disease (Raposo et al., 2015; Pashkow, 2011). Carotenoids, as an

important AO, can be added to diets for protection from these diseases owing to their multiple and stronger antioxidant activities (Raposo et al., 2015). Various high-value carotenoid products from microalgae species and current applications are summarized in Table 2.2.

Table 2.2. High value carotenoid products from microalgae and their current uses.

Products	**Algae sources**	**Applications**	**References**
Astaxanthin	*Haematococcus pluvialis, Chlorella zofingiensi, Phaffia rhodozyma*.	Pigment (aquaculture); Antioxidant; Anti-Inflammation;	Borowitzka et al. (2013) Ambati et al. (2014)
β-carotene	*Dunaliella salina, Spirulina platensis, Chlorella Caulerpa taxifolia*	Food supplement; Pro-vitamin A; Antioxidant	Collins et al. (2011); Gao et al. (2012)
Lutein	*Scenedesmus spp., Muriellopsis sp.*	Antioxidant	Schwartz et al. (2012)
Canthaxanthin	*Chlorella spp., Coelastrella striolata*	Pigment (aquaculture, poultry and food)	Nasrabadi and Razavi (2010) Raposo et al. (2015)
Zeaxanthin	*Chlorella ellipsoidea; Porphyridium cruentum*	Antioxidant; Food colorant	Koo et al. (2012)

The protective role of carotenoids is essential to photosynthetic organisms due to their ability to quench highly ROS. The pathway of production and quenching of singlet oxygen and triplet chlorophyll (^{3}Chl*) is described in Figure 2.2 (Varela et al., 2015). Chlorophyll can be excited into a state called singlet chlorophyll (^{1}Chl*) through direct energy absorption (EA) from light, and then continues to rise to ^{3}Chl* via intersystem crossing (ISC). Singlet oxygen ($^1O_2$*) can be generated during the

process of triple-state energy quenching (TSEQ) of ^{3}Chl* by molecular oxygen. Since singlet oxygen ($^1O_2$*) is a majority ROS, the generation of carotenoid can protect cells by single-oxygen quenching (SOQ) or by deactivating TSEQ processes (as shown in Figure 2.2). The resulting triple state carotenoids from both pathways can safely return to the ground state carotenoids due to the excess energy is released as heat (Telfer et al., 2008). Reverse ISC (RISC) may rarely occur to transfer ^{3}Chl* back to ^{1}Chl*. Moreover, the process of non-photochemical quenching (NPQ) can lower the level of ^{1}Chl*, thereby reducing the formation of both ^{3}Chl* and $^1O_2$* (Erickson et al., 2015).

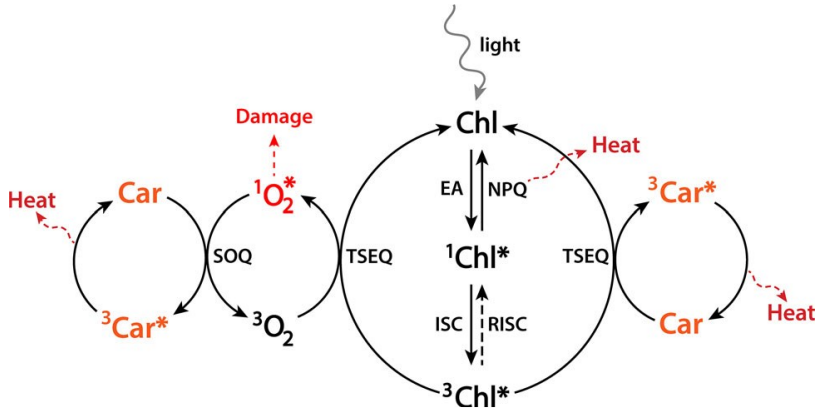


Figure 2.2. Production and quenching of singlet oxygen ($^1O_2$*), singlet (^{1}Chl*), triplet (^{3}Chl*) chlorophyll, and the protective role of carotenoids (Car).

During the vegetative stage of *H. pluvialis*, the primary compositions of total carotenoids are lutein (75-80%), β-carotene (10-20%), other primary carotenoids, and chlorophyll *a* and *b*. In the red encysted stage, the proportion of total carotenoids in cell dry weight would dramatically be improved and the primary carotenoids in the vegetative cells are replaced by secondary carotenoids, mainly occupied by astaxanthin (up to 80% of the total carotenoids content) (Dragos et al., 2010). The predominant

astaxanthin are deposited in lipid droplets as fatty acid esters of astaxanthin (Shah et al., 2016).

2.2 Introduction of astaxanthin

As mentioned in the previous section, astaxanthin (3,3'-dihydroxy-b, b-carotene-4,4'-dione) is the major SCar that can be mass accumulated during the cyst stage of *H. pluvialis*. It is also known as red ketocarotenoid, and is widely used in nutraceuticals, pharmaceuticals, aquaculture, poultry industry, and cosmetic industries (Zhang et al., 2016; Lorenz and Cysewski, 2010), due to its powerful AO capacity. Under unfavorable conditions, diverse functions of astaxanthin have been proposed, including screening the excess photosynthetically active radiation (PAR), neutralizing free radicals and inhibiting the generation of ROS, it serves as sink for the excess photoassimilates, and energy storage (Solovchenko, 2013; Boussiba, 2000). Cerón et al. (2007) reported that the AO capacity of astaxanthin is 38 times higher than that of β-carotene, and 500 times higher than that of Vitamin E. In addition to this, *in vitro* studies demonstrated that diester-astaxanthins are 1.6 folds more effective than monoesters and 2 folds more potent than free astaxanthin (Cerón et al.,2007). Interest in application of astaxanthin as sources of AO has been growing. Detailed information about astaxanthin including chemical characteristics and biosynthesis pathways will further be discussed in this section.

2.2.1 Structure, classification, and functions of astaxanthin

Astaxanthin belongs to xanthophylls, which contains conjugated double bonds with the presence of hydroxyl (-OH) group and keto (C=O) moieties on each terminal ring (Figure 2.3). This characteristic of conjugated double bond highlights the AO activity of astaxanthin by donating electrons and converting free radicals into stable products (Guerin et al., 2003). It has both lipophilic and hydrophilic properties, which make it possible to be esterified (Higuera-Ciapara et al., 2006; Hussein et al., 2006). Besides, there are three different stereoisomers of astaxanthin in nature: (3S, 3'S), (3R, 3'S), and (3R, 3'R), which can be distinguished according to their configuration of hydroxyl group on the terminal rings in C-3 and C-3'.

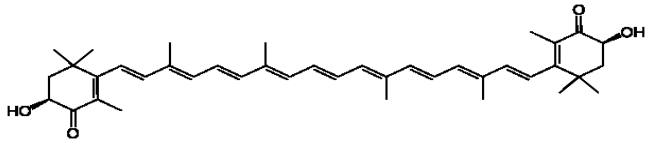


Figure 2.3. Structure of astaxanthin.

Since there are two carbons located at the 3 and 3' positions of the rings with two hydroxyl groups in astaxanthin, hydrogen atoms of hydroxyl group at terminal rings are considered as the radical trapping site, which are responsible for the efficient AO activity (Goto et al., 2001). As mentioned in earlier, most of the astaxanthin molecules are present as fatty acid esters, which enable them to protect the cells due to coexistence of hydrophilic and hydrophobic properties. Previous research implied that astaxanthin esters might be employed as stabilizers to maintain the high AO capacities in both hydrophilic and hydrophobic conditions (Lemoine and Schoefs, 2010;

Kobayashi and Sakamoto, 1999). Furthermore, the terminal ring of astaxanthin rests in hydrophilic layers, while the polyene chain is located in lipophilic area, which spans the biological membrane and enhances the defense response by linking cell membrane from inside to outside (Ambati et al., 2014). In addition, Li et al. (2008) reported that *H. pluvialis* has two antioxidative mechanisms: antioxidative enzymes in vegetative stage and the beginning of astaxanthin generation, and antioxidative astaxanthin in cyst formation stage. Therefore, astaxanthin as AO in *H. pluvialis* is utilized to provide a secondary-defense strategy for long-term stress conditions. These antioxidative defense mechanisms do not only protect cells from stress conditions but also facilitate cell transformation (Lemoine and Schoefs, 2010).

2.2.2 Applications of astaxanthin

2.2.2.1 Astaxanthin application in human health

Plenty of published studies both *in vitro* and *in vivo* animal models have confirmed that astaxanthin possesses a variety of mechanisms that assist and improve human and animal health, and it is extensively used in nutraceutical industry (Shah et al., 2016; Yuan et al., 2011). Oxidative stress plays critical roles in many age-related disorders (Beatty et al., 2000). For instance, cataract and macular degeneration are two major age-related eye diseases, which can cause loss of vision in older populations (Chiu and Taylor, 2007). Although astaxanthin has never been isolated from human eyes, they are useful in preventing cataract and macular degeneration by protecting eyes against light-induced oxidative injuries (Santocono et al., 2006; Guerin et al., 2003).

Compared with other AO, astaxanthin can cross the blood-retinal-brain barrier and exists in the retina, while other antioxidants cannot (Guerin et al., 2003). Moreover, astaxanthin has ability to absorb blue light, which can interact with the retina, thus inducing photo-oxidative processes (Lorenz and Cysewski, 2000). Natural astaxanthin has also been used in cosmetic production for skin protection, which helps tissues against ultraviolet (UV) light-mediated photo-oxidation. In this approach, it could not only protect the skin from sunburn and photo-oxidation, but also repair the previously damaged skin (Guerin et al., 2003). Immune response cells are sensitive to oxidative stress and cell membrane can be injured by free radical releasing from phagocytic action (Hughes, 1999). Some *in vitro* and *in vivo* research in animal model found that astaxanthin significantly influenced immunomodulation activities and enhanced murine immunocompetent cells (Okai and Higashi-Okai, 1996). Several studies have proposed that astaxanthin, as a potent AO and high capacity anti-inflammatory agent, is a potential therapeutic agent against atherosclerotic cardiovascular disease, which is caused by a variety of oxidative stresses and inflammatory diseases (Fassett and Coombes, 2011). Astaxanthin also showed an effect on remedying exercise-induced muscle damages by reducing ROS, because ROS contributes to aggravating role in the inflammation (Tracy, 1999; Dekkers et al., 1996). Similarly, with immune response cells, tissues in nervous system are also susceptible to the oxidative stress. Diet with high astaxanthin has confirmed to lower the associated risks in neurodegenerative diseases (Guerin et al., 2003; Facchinetti et al., 1998). Furthermore, activity of

astaxanthin against cancer has been studied, and Guerin et al. (2003) indicated that anti-cancer activities in astaxanthin might be involved with slowing development of cell growth, modulating immune responses regulation, and inducing formation of xenobiotic-metabolizing enzymes.

2.2.2.2 Astaxanthin in aquaculture and poultry industry

Currently, astaxanthin has great potential in the aquaculture industry, not only due to its ability to impact the colour characteristics of commercially valuable fish and other marine organisms but also to provide essential supplements for their growth and reproduction (Shah et al., 2016). Salmonid aquaculture feeds have been found as the largest potential consumer of natural astaxanthin produced by *H. pluvialis* (Lorenz and Cysewski, 2000). Evidence has shown that utilization of *Haematococcus* astaxanthin meal has resulted in enhanced flesh and skin coloration, egg quality, antioxidant systems, and an improvement in the survival rates of fry of marine organisms, such as Salmonids, rainbow trout, shrimp, and ornamental fishes (Shah et al., 2016; Sheikhzadeh et al., 2012; Parisenti et al., 2011; Ako and Tamaru, 1999). Beside aquaculture feeding, *H. pluvialis*-derived natural astaxanthin is widely used by poultry feed manufacturers as well. It has proven to improve pigmentation on egg yolk, enhance the production of breeding, and decrease mortality of hens and chicken (Yang et al., 2006).

2.2.3 Commercial market, safety, and challenges of astaxanthin

The current commercial astaxanthin world market is primarily dominated by synthetic astaxanthin. The major resources are contributed by NHU in China, BASF in France, and DSM in the Netherlands (Li et al., 2011). The total value of astaxanthin synthesis is estimated at more than $200 million, which corresponds to 130 metric tons of product annually. The average market price is above $2,000 per kilogram, and the cost is estimated at $1,000 per kilogram (Shah et al., 2016; Milledge, 2011). Recent investigations showed that microalgae-derived astaxanthin was only responsible for less than 1% of the total commercialized market, ascribed to its higher product prices than those of synthetic products and technological challenges of large-scale cultivation and harvesting of microalgae (Koller et al., 2014). Synthetic and natural astaxanthin can be distinguished from their chemical structures. Synthetic astaxanthin consists of the isomers (3S, 3'S), (3R, 3'S), and (3R, 3'R) with a proportion ratio of 1:2:1 and is non-esterified, while astaxanthin derived from microalgae consist mostly of (3S, 3'S) or (3R, 3'R) isomers and can be esterified by fatty acids (Li et al., 2011; Higuera-Ciapara et al., 2006). Currently, with increasing concerns about food safety of chemically synthesized astaxanthin, natural astaxanthin from biological sources has become the preferred choice for marketing (Han et al., 2013). In fact, synthetic astaxanthin, which is derived from petrochemicals raise the risks on food safety, pollution, and sustainability; thus, it is not allowed for human consumption and animal feed (Li et al., 2011). Régnier et al. (2015) reported that natural astaxanthin extracted

from *H. pluvialis* had about 90 times higher intracellular AO than that in synthetic astaxanthin. The chemical components and structures of natural astaxanthin improve its bioavailability and biological activities (especially antioxidant activity), which highlight the commercial value of the carotenoid. Spiller and Dewell (2003) conducted a randomized, double-blind, placebo-controlled clinical study on *Haematococcus* astaxanthin. The findings demonstrated that astaxanthin can be safely consumed by healthy adults at doses of 6 mg/day for 8 weeks. Compared with the regular anti-inflammatory drugs, *Haematococcus*-derived astaxanthin supplementation was observed to be similarly effective or even more effective than anti-inflammatory drugs in 92% of comparisons (Guerin et al., 2003). Due to the growing market demand on natural astaxanthin, the market value is estimated at 1.1 billion USD and projected to reach 670 metric tons by 2020 (Shah et al., 2016). In the case of *Haematococcus* astaxanthin, the market value is estimated in a range from USD 2,500/kg to USD 15,000/kg, depending on products' purity (Koller et al., 2014; Pérez-López et al., 2014). To date, natural astaxanthin has been used by humans mostly as nutritional supplements. The safety of astaxanthin is under trial at present, even though it has been indicated as safe by multiple studies (Ambati et al., 2014; Yang et al., 2013). Future studies need to focus on better understanding of the accumulation mechanisms of astaxanthin in human body. Moreover, natural astaxanthin is still expensive, so it is also important to develop innovative technologies for scaling up the commercial production of microalgae-derived astaxanthin to meet the global demand in the

foreseeable future and improve their competitiveness compared to their synthetic alternatives (Pérez-López et al., 2014).

2.2.4 Biosynthesis pathway of astaxanthin in *H. pluvialis*

Biosynthesis of astaxanthin in *H. pluvialis* is a complex process, which is highly influenced by stress conditions (Shah et al., 2016). During the induction period, the accumulation of astaxanthin corresponds with triacylglycerol and is deposited in the cytosolic lipid bodies. Primary steps that are involved in this carotenogenesis include the assembly of carbon skeleton, desaturation, cyclization and oxygenation (Solovchenko, 2013). The detail process of astaxanthin biosynthesis pathway is presented in Figure 2.4 (Cunningham and Gantt, 1998).

Isopentenyl pyrophosphate (IPP) is the precursor and the key intermediate of carotenoids in green microalgae. Normally, IPP can be synthesized via a mevalonate pathway or non-mevalonate pathway, takes place in the chloroplast (Eisenreich et al., 2001). Previous comparative analysis of biosynthesis pathways of astaxanthin in *H. pluvialis* indicated that IPP formation in this species is mostly synthesized by the non-mevalonate pathway, which is also known as DOXP pathway due to the first formation of 1-deoxy-D-xylulose-5-phosphate (Gwak et al., 2014; Lichtenthaler, 1999). Then, IPP is converted to its allyl isomer dimethylallyl pyrophosphate (DMAPP) via isomerization. Furthermore, the molecule of geranylgeranyl pyrophosphate (GGPP), a C_{20} compound, can be produced by attachment of three IPP molecules and one DMAPP molecule. The symmetric molecule of the phytoene (a C_{40} compound) is

produced, resulting from the assistance of phytoene synthase with two GGPP molecules in a reaction of catalysis. Phytoene serves as a precursor for astaxanthin and other carotenoids, and expression of the phytoene synthase gene up-regulates in *H. pluvialis* cells under uncomfortable conditions, converting vegetative cells into cyst cells (Gwak et al., 2014; Steinbrenner and Linden, 2001; Cunningham and Gantt, 1998). Sequential formation of lycopene, an unsaturated compound, is generated through four desaturation steps catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS) with plastid terminal oxidase (PTOX) (Li et al., 2010). Desaturation reactions elongate the conjugated carbon-carbon double bonds system, leading to colorless carotenoids, starting from ζ-carotene, being converted into colored carotenoid (lycopene) (Cunningham and Gantt, 1998). Cyclization occurs when the linear molecule lycopene is catalyzed by the enzyme β-lycopene cyclases (CRTL-B). It is known that cyclization is the branching point in the carotenogenesis process, resulting in the production of α-carotene and β-carotene, which are precursors for other carotenoids and play an essential role in the formation of cyclic secondary carotenoids in microalgae (Solovchenko, 2013).

In the final oxygenation steps in *H. pluvialis* cells β-carotene, as the precursor for astaxanthin synthesis, is consequently converted into astaxanthin and its esters by the involvement of enzymes β-carotene-oxygenase (CRTO), and β-carotene hydroxylase (CRTR-B), localized in the lipid vesicles (Solovchenko, 2013; Gao et al., 2012; Collins et al., 2011). The type of astaxanthin isomers depends on the spatial orientation on the

hydroxyl groups attached in the chiral carbon (Shah et al., 2016). Since major genes and enzymes participate in the carotenogenesis process, recent studies have focused on developing transgenic engineering to improve the conversion efficiency of β-carotene to astaxanthin in *H. pluvialis* (Lemoine and Schoefs, 2010).

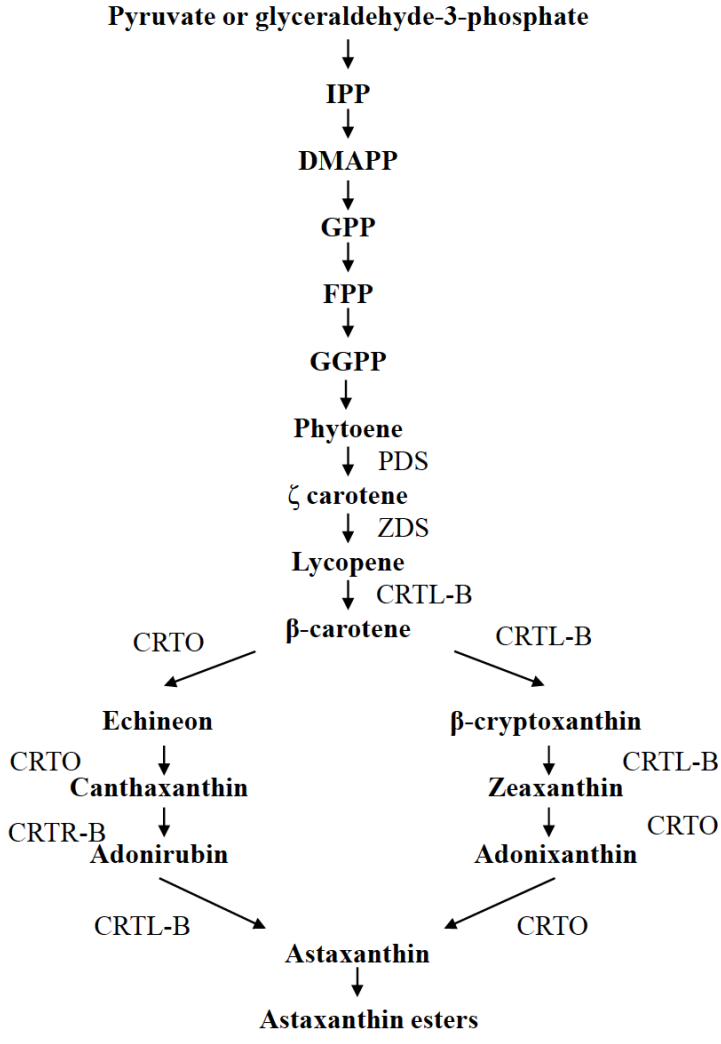


Figure 2.4. Pathway of astaxanthin biosynthesis in *H. pluvialis*. CRTL-B—β-lycopene cyclase; CRTO—β-carotene oxygenase; CRTR-B—hydroxylase of β-ring; GGPS geranylgeranyl pyrophosphate isomerase; PDS—phytoene desaturase; PSY—phytoene synthase; ZDS—ζcarotene desaturase.

2.3 Cultivation and processing of *H. pluvialis* for biomass and astaxanthin production

2.3.1 Culture conditions for vegetative stage

In microalgae production system, it is necessary to select appropriate culture parameters including light, temperature, pH, growth medium, etc. to improve the yields of bio-product. Additionally, Laws and Chalup (1990) proved that most of these parameters have different optima for bio-product processing as well as having significant effects on microalgae growth models.

2.3.1.1 Light

Light is the main energy source for autotrophic growth and photosynthesis activities in terrestrial plants as well as microalgae. In a photoautotrophic cultivation model, microalgae convert light energy into chemical through photosynthesis where photons are absorbed as nutrients (Mohsenpour et al., 2012). As an important growth parameter, both the light quality and intensity are considered to have essential impacts on microalgae cells with respect to productivity, co-product synthesis and distribution. Although a wide range of solar radiation can be captured at the ground level, microalgae can only absorb a fraction of solar radiation in the range of 400-700 nm for photosynthesis, depending on various types of pigments. This fraction of wavelength is called photosynthetically active radiation (PAR), which represents 43% of the total solar radiation. Consequently, the efficiency of photosynthesis in microalgae is quite diverse because of the different pigments' absorption spectra. (Pires et al., 2017; Saefurahman et al., 2013). Moreover, light intensity varies based on the geographic

location and environmental conditions. In most cases, microalgae do not require high light intensities during the cultivation period due to photoinhibition, which would take place when the amount of the light is beyond the absorption capacity of the chloroplast and the chloroplast reaches its light saturation point (LSP) (Han et al., 2000).

Previous studies reported that different species responded differently to the light spectra; therefore, light sources have high influences on cell growth rate, productivity, proximate composition (percentage of ash-free dry weight) and photosynthetic pigment formation (Singh et al., 2015; Saefurahman et al., 2013). To date, light emitting diodes (LEDs) are the prevalent light sources that have been employed for illumination of microalgae cultivation. Compared to classic cool white fluorescence lamps, LED lights have advantages over selectable wavelengths with low power consumption. These merits make it suitable for photobioreactors (PBR) that are designed for biomass production to optimize the photosynthesis efficiency and reduce costs (Pires et al., 2017). Singh and Singh (2015) indicated that green algae contain chlorophyll *a* and *b*, which are the main light harvesting pigments and sensitive to blue (407-488 nm) and red (621-700 nm) light. Xi et al. (2016) reported that *H. pluvialis* generated higher number of cells under red LED light while blue wavelength illumination developed cyst formation and increased astaxanthin biosynthesis. Another study performed on *H. pluvialis* by Sun et al. (2015) showed that a mixed light of blue and white, applied with a ratio of 3:1 at 7,000 xl (~95 μmol photons m^{-2} s^{-1}), expedited the morphologic changes from green vegetative cells to non-motile red cyst cells. Under normal

conditions, the appropriate irradiation for *H. pluvialis* cultivation is estimated to be between 40 to 50 μmol photons m^{-2} s^{-1}. Typically, different optimal values for cultivation were reported in each study as other parameters were involved in these studies, such as temperature, medium composition, and the strain of species (Shah et al., 2016). Besides the light source and intensity, the light/dark regime (photoperiod) is another important factor on algae growth and biomass production. In the natural environment, the light regimen, including light intensity and photoperiod, continuously changes with geographic location. Changes in frequencies of the photoperiods are considered to have essential impacts on productivity and photosynthesis efficiency (Grobbelaar, 2009), and individual microalgae species respond differently to the light regime. Krzemińska et al. (2014) investigated the influence of different photoperiods (24 h illumination and a 12: 12 h light/dark cycle) on the growth rate and biomass productivity of several green microalgae species. The results demonstrated that the highest biomass production obtained from *Botryococcus braunii* and *Scenedesmus obliquus* were under continuous 24 h illumination, whereas a better productivity from *Neochloris* species were observed under 12: 12h light/dark cycle. Recent studies on vegetative cultivation of *H. pluvialis* reported that light/dark cycles of 12: 12h and 16: 8h are common choices (Saha et al., 2013; Kang et al., 2006).

2.3.1.2 Temperature

Temperature is another critical parameter effecting biomass production due to seasonal and diurnal fluctuations in the real world (Wan et al., 2014). Many outdoor

microalgae cultivation operations have been conducted; however, previous studies reported that the greenhouse effects led to temperature fluctuations between 10 to 45ºC in these outdoor production systems (Ras et al., 2013). Li (1980) reported that optimal temperature conditions for most microalgae species are between 20 to 25ºC. A widely tolerable range for cellular division and photosynthesis has been confirmed to be approximately in the range of 15 to 35ºC. Obviously, an elevated temperature has a positive impact on cellular division and photosynthesis; however, once the provided temperature exceeds the optimal value, a dramatic decrease happens in the microalgae growth rate. This might be ascribed to heat stress, which leads to the degradation in enzyme (i.e. denaturation and inactivation) or alteration of proteins, which participate in the photosynthetic processes (Ras et al., 2013; Salvucci and Carfts-Brandner, 2004).

Several research papers have confirmed that microalgae chemical compositions showed different responses to high and low growth temperatures, depending on species. For instance, in some species, protein contents and secondary carotenoids (i.e., astaxanthin) contents increased with elevated growth temperature, resulting in a reduction of lipid and carbohydrate components (Renaud et al., 2002). Converti et al. (2009) studied the effects of temperature on the growth and lipid contents of *Chlorella vulgaris* (tested at 25, 30, and 35ºC) and *Nannochloropsis oculata* (tested at 15, 20, and 25ºC). Their findings indicated that the growth of *C. vulgaris* was not significantly impacted by temperature, but the lipid contents reduced from 14.71 to 5.90% as temperature rose from 25 to 30ºC. For *N. oculata*, the lipid content doubled (from 7.90

to 14.92%) when temperature increased from 20 to 25ºC, while growth rate declined. The optimal temperature for the *H. pluvialis* was estimated to be 20ºC in another study performed by Giannelli et al. (2015). In order to determine the influences of temperature on the growth of *H. pluvialis*, the study cultivated the strain under 20, 23.5, 27, and 30.5ºC. They observed that the growth rates gradually declined as temperature increased; and the final biomass harvested at 20ºC was approximately 2.8 times higher than that obtained at 30.5ºC. Furthermore, Han et al. (2013) suggested that the study on the temperature impacts on a light-dark cyclic cultivation should be performed separately because they observed different effects of daytime and nighttime temperatures on biomass concentration in a study performed using *Chlorella pyrenoidosa*. Then, Wan et al. (2014) conducted an experiment to investigated both daytime and nighttime temperature (tested at 8, 13, 18, 23, 28, and 33ºC) effects on cell growth and astaxanthin accumulation of *H. pluvialis* during a light-dark cyclic cultivation. The results showed that the optimal daytime temperature for cell growth and astaxanthin accumulation was 28ºC with highest net biomass production of 0.064 g $L^{-1}d^{-1}$ and astaxanthin production of 2.3 mg $L^{-1}d^{-1}$. All their results showed the tendency that both growth and astaxanthin contents increased with the increasing temperature, and the minimum interval change was observed between 23 and 28ºC, while cell death occurred at 33ºC, suggesting a suitable daytime cultivation temperature in a range of 23 to 28ºC. Meanwhile, biomass and astaxanthin accumulation were not significantly affected as nighttime temperature changed from 8

to 28ºC.

2.3.1.3 pH

pH control is another indispensable factor in microalgae cultivation because they are susceptible to pH changes. Photosynthesis process and carbon dioxide (CO_2) capture by microalgae increases the pH levels in mediums during the daytime while respiration reverses the process and lower the pH levels at night. Besides this daily cycle, pH usually increases at high microalgal cell densities (Pires et al, 2017; Bartley et al., 2014). As an essential inorganic carbon source, some researchers suggested to inject CO_2 into bioreactors through aeration to maintain the pH value in algae cultures at the desired levels. Other chemicals, such as sodium bicarbonate, HCl, are also used to regulate pH level by forming carbonic acids (Chi et al., 2011; Giordano et al., 2005). Further, pH may affect the biochemical compositions on microalgae cells. Bartley et al. (2014) examined the effects of pH on growth and lipid accumulation of *Nannochloropsis salina* and studied a pH range of 5-10. They found that the highest growth rate and lipid productivity were obtained at pH levels of 8 and 9. Another research reported that the optimal pH levels for biomass and lipid productivities of *Tetraselmis suecica* and *Chlorella sp.* were 7.5 and 7, respectively (Moheimani., 2013). To date, the optimal pH for *H. pluvialis* culture in terms of biomass and astaxanthin production was reported to be within the range of pH 7.00-7.85 (Shah et al., 2016; Hata et al., 2001). Sarada et al. (2002) adjusted the *H. pluvialis* culture medium pH to 5.0, 6.0, 7.0, 8.0, and 9.0. They observed that the maximum biomass was gained in

cultures growing at 7.0, and carotenoids contents were higher at pH 7.0-8.0 while cell number and carotenoids accumulation were lower at pH levels of 5.0, 6.0 and 9.0.

2.3.1.4 Growth mediums

Different culture medium composition effects on *H. pluvialis* growth have also been studied. Sufficient micro- and macronutrients are needed for optimal growing of microalgae. Growth medium recipes vary from species to species. Vegetative cultivation of *Haematococcus* strains is a challenge owing to its slow growth rate and susceptibility to contamination (Sipaúba-Tavares et al., 2015). In order to maintain the optimal growth conditions for the vegetative stage, multiple growth mediums have been attempted for *H. pluvialis* cultivation. Recently, the most commonly used medium were reported to be BBM, BG-11, OHM, and their modifications (Shah et al., 2016). Nitrogen (N) and carbon (C) supplies are necessary for the growth of microalgal cells. Nitrate and ammonium were mentioned to be the optimal inorganic nitrogen sources, while organic nitrogen sources, such as urea, uric acid, and peptone, are often used to culture *Haematococcus* strains (Sarada et al., 2002). Carbon sources can be provided in a number of ways including HCO_3^-, CO_3^{2-} (dissolved in culture medium), and CO_2 (provided through aeration or gas injection system) (Pires et al., 2017). Acetate is also recommended as a suitable carbon source that promotes the algal growth. An apparent increase in specific growth rate of *H. pluvialis* has been proved with acetate concentrations increasing from 10 mM to 30 mM (Jeon et al., 2006). Microalgae can capture and hold CO_2 up to 20% from air system to achieve the highest biomass

production and increase lipid contents. This approach provides a potential opportunity for mitigating greenhouse gases emissions, which aim to lessen the excessive atmospheric CO_2 (Moreno-Garcia et al., 2017). One study evaluated the biomass and specific growth rates of *H. pluvialis* growth under the conditions of a 1% CO_2 addition and without any CO_2 supplement, showing an approximately 9.5 times enhancement of biomass dry weight under 1% CO_2 cultures compared to the cultures without CO_2 addition (Kaewpintong et al., 2007). Besides the nutrients derived from CO_2 and mineral nutrients, vitamins such as B_1, B_{12}, and biotin were found essential for the growth of vegetative cells (Göksan et al., 2011).

2.3.2 Culture conditions for cysts stage

H. pluvialis has been investigated for its antioxidative mechanisms in both vegetative and cyst cells, while the latter is suggested to accumulate massive astaxanthin for defense against a long-term stressful environment (Li et al., 2008; Kobayashi, 2003). As mentioned earlier, astaxanthin has essential protective functions against ROS and UV irradiation. Astaxanthin synthesis is regarded as a multifunctional response caused by various stress factors, such as nutrient limitation, high light irradiation, C/N ratio and salt stress (Lemoine and Schoefs, 2010).

2.3.2.1 Nutrient starvation

Nitrogen is an important nutrient supplement, affecting the growth and accumulation of biomass in the microalgae, it is also able to significantly change the rates of cell metabolism via transformation among different forms (Chen and Chen,

2006). Increased carotenoid accumulations under N-deprived cultures in several microalgae species have been well studied. Nutrients get depleted as cells grow in cultures and eventually achieve a starved condition; therefore, the initial desired N concentration is important for the first stage cultivation process (Minhas et al., 2016). Normally, nitrogen starvation occurs in N depleted or N limited cultures. The difference between the two conditions is that there are available but insufficient N existent in N limited mediums, while N depleted mediums lack N sources (Bona et al., 2014). When N is exhausted by microalgae, photosynthesis is supposed to continue and the resultant N-rich components, energy-rich components, such as lipid and carbohydrates, would be enhanced due to absorption of N, as well as the accumulation of pigments as carotenoids would improve (Minhas et al., 2016). Chlorophyll as a N-rich compound can be utilized as an intracellular N supporter to sustain the growth of cells once N sources are limited in cultures. However, chlorophyll degradation may occur due to the reduced level of chlorophyll a and b with reutilizing of N for cells (Li et al., 2008). These strategies are also employed in the *H. pluvialis* cultures and a remarkable improvement in the astaxanthin production is achieved due to N deficiency. For instance, Fábregas et al. (2003) studied the combined effects of light intensity and nutrient deficiency on astaxanthin synthesis by *H. pluvialis*. Their findings indicated that the highest astaxanthin values were obtained under N-deficiency with high and low light intensities, concluding that N depletion has a greater effect on astaxanthin synthesis than light intensity by stronger blocking effects on cell division. A study

performed by Kozlowska-Szereno and Zieliński (2000) also indicated that P limitation has a significant effect on *Chlorella vulgaris* growth but has no effects on chlorophyll contents in comparison with controls. Furthermore, Boussiba (2000) reported that N starvation led to approximately twice the rate of astaxanthin accumulation as compared with P starvation. Also, the extension of astaxanthin production under P-deprived cultures is irrespective of light intensity (Boussiba, 2000).

2.3.2.2 Light intensity

High light intensity is one of the primary factors, which contribute to massive amounts of carotenoids accumulation. As mentioned in the carotenoids section earlier, chlorophyll will become saturated with high light energy and rise to an excited level as triplet chlorophyll and produce high concentrations of singlet oxygen. Consequently, photo-oxidative processes would occur and destroy the cells when more light energy remains after photosynthesis (Krieger-Liszkay, 2005). Therefore, carotenoids are generated to quench excessive singlet oxygen to lower the chlorophyll, by responding to the up-regulation of gene expression to resist stress (Couso et al., 2012). Effects of a wide range of light intensities on microalgae astaxanthin production have been well documented. In general, gradual increases in light intensity can result in enhanced astaxanthin contents. However, the specific optimum values of LSP are different in different studies, indicating that the optimum light intensity for induction process is uncertain. Moreover, the different optimum levels may be affected by the presence of other stress factors, so that the effective induction of carotenogenesis may not be

necessary when other factors are considered (Shah et al., 2016). Saha et al. (2013) reported that despite the various types of illumination, the highest carotenoid content was achieved under continuous lighting conditions.

2.3.2.3 Carbon/nitrogen ratio

Carbon/nitrogen (C/N) ratio is another important factor, which contributes to astaxanthin accumulation. Low C/N ratio increases total biomass due to more N being available for growth, but leads to a decrease in astaxanthin production (Kang et al., 2006). High C/N ratio induction by acetate addition, along with iron, demonstrated apparent improvement in cyst formation, thereby changing the resultant astaxanthin content (Kang et al., 2006; Kobayashi et al., 1993). Orosa et al. (2005) examined the effect of different acetate addition on carotenoids production. The results showed that the total carotenoids were almost doubled as acetate concentration increased from 0.25 to 0.5% (w/v). A combination effect of N-deficiency and acetate addition under high light irradiation showed a significant improvement on astaxanthin production (Choi et al., 2002).

2.3.2.4 Salinity

It has also been reported that salinity stress induces ROS accumulation; therefore, NaCl additions are frequently used to inhibit cell growth and stimulate the astaxanthin synthesis (Boussiba and Vonshak, 1991). Tam et al. (2012) tested different NaCl concentrations on both growth and astaxanthin accumulation on *H. pluvialis*. A reduced cell growth and increased carotenoid contents per cell were observed under

salinity stress. The highest astaxanthin content was obtained at 2.5% NaCl concentration additions, which achieved a 4.8 folds increase in astaxanthin after a 15-day induction period. Another study on *H. pluvialis* culturing indicated that a 2.5 to 4.4 folds increase in astaxanthin content obtained as NaCl concentrations increased from 0.25 to 0.5%, while the content decreased at 1% NaCl concentration (Sarada et al., 2002).

2.3.3 Production mechanisms

Microalgae can grow in different metabolic pathways, and there are three main methods for *H. pluvialis* biomass and astaxanthin production: photoautotrophic, heterotrophic, and mixotrophic cultivation. These typically occur in open raceway ponds or controlled environment PBRs.

2.3.2.1 Photoautotrophic growth

Microalgae can be cultured photoautotrophically directly using light as an energy source, assimilating atmospheric CO_2 as inorganic carbon source and absorbing other inorganic nutrients from the aquatic environment (Moreno-Garcia et al., 2017). Photoautotrophic cultivation of microalgae is mainly done in outdoor open ponds or PBRs. Photoautotrophic is usually preferred to heterotrophic conditions if aiming to produce by-products (i.e. lipids, proteins, carotenoids), due to the lower chance of contamination. It is obvious that the productivities of biomass and by-products under photoautotrophic conditions are highly dependent on lighting conditions, because the sunlight availability varies geographically (Moreno-Garcia et al., 2017). In a *H.*

pluvialis culture, photoautotrophic conditions can support both the vegetative cell phase (green stage) and the reddish inductive production phase (red stage). Previous studies have reported that the biomass productivities in green stage and red stage ranged from 0.01 to 0.5 g L^{-1} d^{-1} and 0.01 to 0.48 g L^{-1} d^{-1}, respectively (Shah et al., 2016).

2.3.2.2 Heterotrophic growth

Microalgae can also grow in the absence of light and use heterotrophic metabolism as an alternative pathway (Bhatnagar et al., 2011). Under this growth conditions, light is no longer needed as organic carbon substrates and energy sources are used for cell growth and synthesis of secondary metabolites (Martinez et al., 1991). Heterotrophic cultures can have a significant reduction in the cost for most cultivation steps (i.e., saving on the cost of illumination and reducing the cost associated with dewatering during harvesting steps), while it is possible to obtain a high biomass concentration (Moreno-Garcia et al., 2017; Perez-Garcia et al., 2011). Therefore, cost effectiveness and simplicity of daily maintenance and operations make heterotrophic culture system attractive for large-scale mass production of microalgae (Behrens, 2005). In some cases, compared with autotrophic cultures, Perez-Garcia et al. (2011) reported significantly higher growth rates, biomass accumulation, lipid content, and adenosine triphosphate (ATP) yield when microalgae were cultivated under heterotrophic cultures, depending on strain selection. In the case of *H. pluvialis*, many types of cheap organic carbon sources, such as glucose, acetate and glycerol, have been used for heterotrophic

cultivation, and the use of acetate for heterotrophic induction is reported to have great efficiency on stimulating encystment formation and initiating astaxanthin synthesis (Kang et al., 2005; Hata et al., 2001). Kang et al. (2005) conducted a comparative analysis between heterotrophic and photoautotrophic induction on astaxanthin production by *H. pluvialis*. Photoautotrophic induction was performed by continuous CO_2 supply or supplemented with bicarbonate (HCO_3^-), while acetate was used for heterotrophic induction. As a result, they found that more astaxanthin can be produced from bicarbonate-induced cells than in acetate-induced cells. The maximum astaxanthin concentration was achieved at 175.7 mg L^{-1} (approximately 7% of DW) under 5% CO_2 conditions, which was 3.4 times higher than that under heterotrophic induction. Consequently, they concluded that photoautotrophic induction performed better and is more efficient for astaxanthin synthesis than heterotrophic induction of *H. pluvialis.* There are several limitations of heterotrophic cultures, these include: (1) only a few microalgae species are able to grow heterotrophically; (2) the additional costs on organic substrates and the increased risks of bacterial and fungal contaminations; (3) excess organic substrates may inhibit cell growth and microalga is unable to generate light-induced metabolites (Perez-Garcia et al., 2011; Hata et al., 2001; Chen, 1996).

2.3.2.3 Mixotrophic growth

Microalgae can also be grown mixotrophically, under which microalgae utilize light for photosynthesis and organic carbon sources to support cell growth. Mixotrophy has the advantage of reducing photoinhibition, thereby increasing growth rates as

compared with autotrophic cultures. For many microalgae species, higher biomass productivities are usually obtained in mixotrophic cultures than those in heterotrophic cultures (Girard et al., 2014; Goksan et al., 2010). Since mixotrophic microalgae assimilate both organic and inorganic carbon sources and light simultaneously, it improves the flexibility of the cultivation processes and has become a successful strategy to generate higher biomass, lipid content, and by-products production (Praveenkumar et al., 2014). Mixotrophic growth conditions were employed for *H. pluvialis*, and the findings showed an enhancement of both biomass and astaxanthin production. The cell density ranged from 0.9 to 2.65 g L^{-1} and astaxanthin content reached 1 to 2% levels of DW when *H. pluvialis* grew under mixotrophic conditions (Zhang et al., 1999). Goksan et al. (2010) studied an alternative approach to the traditional mixotrophic culture of *H. pluvialis.* Sodium acetate was used as an organic carbon source, and added together with other inorganic nutrients prior to cultivation as traditional mixotrophy, while in another way it was added at the end of the log phase of the photoautotrophic mode as an alternative mixotrophy. The results showed that higher growth rate was obtained from the alternative mixotrophic approach than that in the traditional mixotrophic approach. However, mixotrophic cultures also bring risks of bacterial contamination in the organic materials input. And it still needs to be further understood before mixotrophic cultures can be scaled-up in mass culture and energy conversion analysis (Moreno-Garcia et al., 2017).

2.3.4 Cultivation systems

Most of the current microalgae cultivation systems can be categorized in three groups: open systems, closed systems and hybrid systems. The major difference among these systems is whether they are exposed to the surrounding natural environment or not. An open system usually refers to raceway or circular open ponds exposed to the surrounding environment. The closed systems can be classified into several types according to their reactor designs, including tubular, column, flat plate, membrane and attached cultivation system (Moreno-Garcia et al., 2017; Cai et al., 2013). While there is no doubt that cultivation systems play essential roles on biomass production, each type of system has their own advantages and disadvantage depending on the applications, as shown in Table 2.3 (Moreno-Garcia et al., 2017).

The raceway open pond is the most common open cultivation system in practice that has been extensively used since the 1950s. Raceway open ponds are shallow ponds (approximately 0.3 m deep) that are mixed and circulated by a paddle wheel, while being supported by sufficient sunlight and nutrients (Christenson and Sims, 2011). Raceway ponds are relatively inexpensive systems to build and operate, and they are often built on non-arable lands adjacent to power plants or wastewater treatment plants to access CO_2 from flue gas and wastewater as nutrient supplies (Cai et al., 2013). The major disadvantages are uncontrolled fluctuations in environmental conditions (i.e., temperature, light, photoperiod, rainfall, and seasonal variation) and biological and chemical parameters (i.e., pH, salinity, and the presence of other microorganisms),

which significantly affect biomass productivities (Kumar et al., 2015).

Compared with the open system, microalgae cultivated in a closed system or PBR typically require the additional costs of illumination, CO_2 dosing, and other control and maintenance devices. However, this reduces the risks of contamination, avoids water evaporation, and increases the photosynthetic efficiency. Tubular PBRs are widely used as large-scale production systems, which provide better control on environmental parameters, such as pH, temperature, and better protection from contamination (Shen et al., 2009; Carvalho et a., 2006). Although a higher biomass concentration can be harvested from this system, the expensive installation cost hinders the commercialization of closed systems. Closed systems are suggested for use in high-value algal bioproducts for the pharmaceutical and nutraceutical industries due to its strict management (Borowitzka, 1999). In some cases, hybrid systems are employed in a two-stage cultivation strategy, where inoculum is cultivated in PBRs at the first stage and then transferred in the second stage to open ponds for biomass accumulation. This approach combines the merits from both open and closed systems thereby minimizing contamination and obtaining high yields of biomass (Cai et al., 2013).

Table 2.3. Comparison of the characteristics of different microalgae cultivation systems.

Cultivation systems	**Advantages**	**Disadvantages**
Open system	Lower initial investment, cheaper maintenance and easier set-up compared to closed systems.	Low productivities, possible culture media contamination and evaporative losses. Large amounts of water needed.
Closed system (PBR)	High mass transfer and productivities; less contamination and more controlled conditions compared to open systems.	Higher cost and more complicated set-up compared to open systems.
Column PBR	Low shear forces, absence of wall growth, high efficiency of CO_2 use and the ability to use sunlight.	High energy requirement to attain the adequate lighting and mixing.
Tubular PBR	High light conversion efficiency.	Reduction in the photosynthetic efficiency. Very high energy consumption when compared with bubble column and flat plate PBRs.
Attached Cultivation	Improved productivities and increased water and energy savings as compared with the diluted systems.	Photoinhibition may occur.

2.4. Microalgae production integrated in wastewater treatment system

Microalgae grow rapidly and adapt in all ecosystems on earth. They are capable of surviving in all waters, such as freshwater, marine water and wastewater systems (Moreno-Garcia et al., 2017). Many microalgae species that are able to effectively accumulate biomass in various wastewater conditions have been investigated. They

have extraordinary capacities for assimilating the nutrients of wastewater, especially organic carbon, inorganic N and P. High concentrations of nutrients dissolved in wastewaters, in particular N and P, are required to be removed during wastewater treatment processes, otherwise they can trigger eutrophication if excessive nutrients are being discharged and accumulated in rivers and lakes (Pittman et al., 2011). Since microalgae have shown great capacity in removing N, P, trace elements, and toxic metals from wastewater, they have potential to be employed in the final (tertiary) treatment of wastewater. Compared with conventional chemical-based treatments, microalgae-based treatments have significant advantages in cost saving, and avoid the secondary contamination from chemicals, using relatively simple technologies (Hoffmann, 1998). Besides, microalgae-based treatments are ecologically safer and offer additional benefits from resource recycling (Christenson and Sims, 2011). Therefore, this approach has received considerable interest and is quite attractive in developing regions. Different types of wastewater sources have different compositions. And nutrient components have great effects on microalgae growth, biomass production and the yields of by-products (Cai et al., 2013). Table 2.4 shows the N and P concentrations and their ratios in different wastewater streams (Cai et al., 2013). The growth of microalgae under various wastewater resources, nutrient removal mechanisms, and the major benefits and challenges will be discussed in this section.

Table 2.4. Total nitrogen (TN) and total phosphorus (TP) contents of different wastewater sources.

Wastewater sources	Description	TN (mg L^{-1})	TP (mg L^{-1})	N/P
Municipal wastewater	Sewage	15-90	5-20	3.3
Animal wastewater	Dairy	185-2,636	30-727	3.2-7.6
	Poultry	802-1825	50-446	4-16
	Swine	1,110[a]-3,213	310-987	3.0-7.8
	Beef feedlot	63-4,165	14-1,195	2.0-4.5
Industrial wastewater	Textile	21-57[a]	1.0-9.7[b]	2.0-4.1
	Winery	110	52	2.1
	Paper mill	1.1-10.9	0.6-5.8	3.0-4.3
	Olive mill	532	182	2.9
	Tannery	273	21[b]	13.0

[a] Total Kjeldahl nitrogen

[b] Total orthophosphates (PO_4^3-P)

2.4.1 Microalgae growth in different wastewaters

2.4.1.1 Municipal wastewater

Increasing urbanization and population expansion have resulted in large quantities of municipal wastewaters produced every day. Pescod (1992) indicated that approximately 85,000 t d^{-1} of wastewater would be produced in a city with a population of 500,000. Municipal wastewaters have typically lower levels of N and P than livestock and industrial wastewaters, while they may contain amounts of heavy metals, such as lead, zinc and copper (Cai et al., 2013). Physical and chemical treatment methods are commonly used for removing buoyant, non-buoyant, and dissolved organic materials from wastewaters (Ruiz-Marin et al., 2010). Microalgae cultivation integrated into the municipal wastewater treatment systems for nutrient removal has been extensively studied. For instance, Pittman et al. (2011) reported that

Chlorella sp. and *Scenedesmus sp.* performed with very high efficiency on nutrient removal in sewage wastewater after secondary treated, particularly in the removal of ammonia, nitrate and total P, ranging from 80 to 100% removal rates in many cases. In another study, Lau et al. (1995) indicated that *C. vulgaris* could remove more than 90% of N and 80% of P from primary treated sewage wastewater. In our laboratory, β-carotene rich *Dunaliella salina* was investigated and the findings showed that their capacity for removing nitrate, ammonia and phosphorous from local municipal wastewater after a six-day cultivation was in the range of 45 to 88% (Liu and Yildiz, 2016).

2.4.1.2 Agricultural wastewater

Agriculture is another major wastewater producing sector. Agricultural wastewater is frequently derived from livestock production, and contains high levels of N and P, as shown in Table 2.4 (Wilkie and Mulbry, 2002). Generally, livestock manure is often treated and utilized as fertilizer. However, nutrients in manure may not be completely consumed due to the various ratios of N/P requiring by the crops. As a result, excess nutrients find their ways to the surrounding aquatic systems and cause eutrophication significantly reducing water quality (Cai et al., 2013). As in the case of municipal wastewaters, previous studies have also demonstrated that microalgae can significantly assimilate N and P from manure-based wastewaters. For example, An et al. (2003) reported that 80% of nitrate content was effectively removed from piggery wastewater by *Botryococcus braunii*. Moreover, compared with microalgae that were cultivated in

municipal wastewaters, Wilkie and Mulbry (2002) indicated that higher microalgae growth rates and equivalent nutrient removal efficiencies were observed in manure added recycling wastewater.

2.4.1.3 Industrial wastewater

Wastewaters discharged from industrial operations contain more heavy metal pollutants and relatively lower N and P, depending on the operation (Ahluwalia and Goyal, 2007). These types of wastewaters could affect the quality of the receiving waters because toxic materials are discharged. Therefore, microalgae used for industrial wastewater remediation is predominantly focused on heavy metals and organic chemical toxins, rather than N and P (Ahluwalia et al., 2007). Since industrial wastewaters contain lower N and P contents and high levels of toxic elements, most microalgae cannot grow well. It is necessary to select specific strains that have high metal absorption capacities to handle industrial wastewater remediation. So far, only a few strains have been explored for metal removal capacity research. One study using carpet mill wastewater, which has relatively lower toxins and higher N and P contents, reported that the freshwater microalgae *Botryococcus braunii* and *Chlorella saccharophila*, and the marine alga *Pleurochrysis carterae*, grew well in untreated wastewater with large amounts of biomass generated (Chinnasamy et al., 2010). Percent nitrogen and phosphorus removal efficiencies by various microalgae species in different wastewater resources are summarized in Table 2.5 (Cai et al., 2013).

Table 2.5. Nitrogen and phosphorus removal efficiencies (%) by various microalgae species in different wastewater resources.

Microalgae	Types of wastewater	Removal rates	
		TN (%)	TP (%)
Chlorella sp.	Brewery wastewater	87	80
	Municipal wastewater	62.5	90.6
C. vulgaris	Municipal wastewater	55-88	12-100
	Industrial wastewater	30-95[a]	20-55
	Domestic wastewater	-	97.8
C. kessleri	Artificial medium	8-19[b]	8-20[c]
C. pyrenoidosa	Industrial wastewater	87-89	70
C. reinhardtii	Artificial medium	42-84[a]	13-14[c]
Scenedesmus sp.	Artificial medium	30-100[a, b]	30-100[c]
S. obliquus	Municipal wastewater	79-100[a]	47-98

[a] Ammonia nitrogen (NH_4^+-N).

[b] Nitrate (NO_3-N), Nitrite(NO_2-N).

[c] Total orthophosphates (PO_4^3-P).

2.4.2 Minkery wastewater

In Nova Scotia, the mink industry is the largest agricultural industry, which has had tremendous growth with an increase of 415% since 1997. It has been reported that mink production in Nova Scotia accounted for about 54.3% of Canada's total mink production in 2012 (Statistics Canada census, 2012). Most of the mink farms are concentrated in or near the headwater of the Carleton River, NS. It has been estimated that the total market value of mink products was over $100 million in 2010 and reached $140 million in 2012 (Statistics Canada census, 2012). However, the Nova Scotia Department of Environment reported that the high concentration of mink farms poses negative environmental impacts on adjacent aquatic ecosystems, resulting in eutrophication and cyanobacterial blooms, thereby deteriorating water quality (Taylor, 2009). As with most wastewaters, minkery

wastewater contains high amounts of ammonia, nitrate, and phosphorus (Ferguson, 2002). Brylinsky (2012) published a report surveying the water quality survey in seven lakes located within the Carleton River Watershed Area. Findings showed that the most serious nutrient over-enrichment problems were located within the upper region close to high concentrations of mink farming. Moreover, they found that some of the phosphorus was from the cleaning operations or storage of mink, and a major source of phosphorus was likely due to the utilization of superphosphate during mink operation activities. Boon (2014) reported that mink farms annually produce approximately 18,000 tons of mink manure and 16 million liters of urine in Nova Scotia. Therefore, as a local wastewater and nutrients resource, minkery wastewater has the potential opportunity for microalgae biomass production, which would reduce the concentration of target pollutants before they are discharged into surrounding waters.

2.4.3 *H. pluvialis* growth in wastewater

To date, *H. pluvialis*, as a popular microalga candidate for astaxanthin production and wastewater remediation, has been extensively studied in recent years. For instance, the resultant astaxanthin contents obtained under primary-treated sewage and primary-treated piggery wastewater with dilution were in the range of about 5.1% and 5.9% dry weight of total biomass, respectively (Kang et al., 2006). Another study showed that over 90% of nitrogen and phosphorus were removed by *H. pluvialis* when it reached its highest growth rates under domestic secondary effluent. Furthermore, the lipid contents of *H. pluvialis* were improved from 25% to 43% in a 10-day nutrient

depletion cultivation (Wu et al., 2013). Therefore, *H. pluvialis* is considered as a promising candidate for integrated systems of wastewater treatment and microalgae cultivation, while producing high-value bioproducts. However, Kang et al. (2007) and Hata et al. (2001) indicated that high concentration of nutrients would cause inhibitory effects on *H. pluvialis* growth, and thus, the suitable concentration of wastewater must be determined.

2.4.4 Mechanisms of nutrient removal

It is important to understand the mechanisms of algal nutrient uptake so that microalgae are able to maximize removal rates from aforementioned wastewater resources by various microalgae strains. The metabolic pathways can be dictated by elemental constituents, such as nitrogen, phosphorus, and carbon (Cai et al., 2013). From stoichiometric equations, the nutrients involved in microalgae growth are shown below (Chisti, 2007).

$100\ CO_2 + 84.5\ H_2O + 11\ NO_3^- + 13\ H^+ + HPO_4^{2-} \leftrightarrow C_{100}O_{48}H_{183}N_{11}P + 136.75\ O_2$

$100\ CO_2 + 73.5\ H_2O + 11\ NH_4^+ + HPO_4^{2-} \leftrightarrow C_{100}O_{48}H_{183}N_{11}P + 114.75\ O_2 + 9\ H^+$

$100\ HCO_3^- + 11\ NO_3^- + 113\ H^+ + HPO_4^{2-} \leftrightarrow C_{100}O_{48}H_{183}N_{11}P + 136.75\ O_2 + 15.5\ H_2O$

$100\ HCO_3^- + 23.5\ H_2O + 11\ NH_4^+ + HPO_4^{2-} \leftrightarrow C_{100}O_{48}H_{183}N_{11}P + 139.75\ O_2 + 9\ H^+$

2.4.4.1 Nitrogen

Nitrogen is an essential nutrient that supports the growth of an organism. Microalgae can convert inorganic nitrogen, such as nitrate (NO_3^-), nitrite (NO_2^-), and ammonium (NH_4^+), to its organic form (i.e., proteins, peptides, enzymes, chlorophylls,

energy transfer molecules, and genetic material) via the assimilation process. Atmospheric nitrogen (N_2) can be also fixed and transformed into ammonia by microalgae (Barsanti and Gualtieri, 2014; Cai et al., 2013). Figure 2.5 displays the simplified assimilation process of inorganic nitrogen, which occurs across the plasma membrane (Cai et al., 2013). As shown, the nitrate reductase and nitrite reductase, nitrate and nitrite ultimately turn into ammonium prior to their incorporation into ammonium. Nitrate is reduced to nitrite when nitrate reductase assists nicotinamide adenine dinucleotide (NADH) transfer two electrons, further transformation of six electrons with involvement of nitrite reductase and ferredoxin (Fd) resulting in the conversion of nitrite to ammonium. Lastly, glutamine synthetase turns nitrite to amino acid glutamine by using glutamate (Glu) and ATP (Cai et al., 2013).

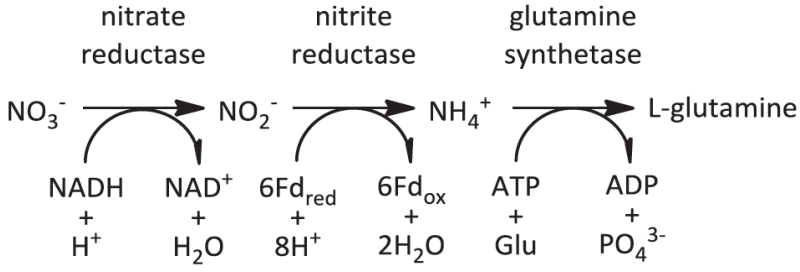


Figure 2.5. Simplified schematic of assimilation of inorganic nitrogen.

In natural conditions, nitrate is the most stable oxidized form of nitrogen that exits in an aquatic system. The presence of nitrate induces the nitrate reductase activities so that they play an essential role in microalgae growth (Barsanti and Gualtieri, 2014). However, Maestrini et al (1986) indicated that ammonium is a preferred form of

nitrogen in mediums, and microalgae tend to consume nitrate after ammonium is almost completely assimilated from culture medium. This suggests that wastewaters with high ammonium concentrations facilitate a rapid growth of microalgae. However, some studies determined that excess ammonium can have repressive effects on microalgae growth, depending on the tolerance of different microalgae species. Collos and Berges (2004) reported that the ammonia tolerance of different microalgae species ranges from 25 μmol L^{-1} to 1,000 μmol L^{-1}.

2.4.4.2 Phosphorus

Phosphorus is another crucial nutrient for microalgae cell growth and a key factor in their energy metabolism. Inorganic forms of phosphorus, frequently found as $H_2PO_4^-$ and HPO_4^{2-}, can be consumed and incorporated into organic compounds (found in nuclei, lipids, proteins, etc.) through phosphorylation with energy input from light and generation of ATP from adenosine diphosphate (ADP) (Martinez et al., 1999).

2.4.4.3 Carbon

Atmospheric carbon dioxide (CO_2) can be directly captured and fixed by microalgae for its cell growth in many autotrophic culture systems (Moreno-Garcia et al., 2017). Organic forms of carbon, such as soluble carbonates, can also be absorbed and converted in the process of heterotrophic or mixotrophic growth systems through carbonic anhydrase activities. This carbon fixation approach provides a great potential for effective mitigation of greenhouse gases, which could benefit biomass production and reduce environmental burdens.

2.4.4.4 Impact of environmental factors on nutrient uptake rates

It is obvious that environmental factors, such as temperature, pH, nutrient composition, and inoculation density, affect the efficiency and nutrient removal rates of microalgae. The composition of nutrients has a direct significant influence on nutrient uptake. Kunikane et al. (1984) indicated that an optimal initial nitrogen-phosphorous ratio can be found for the maximal nutrient removal efficiency. However, this ratio differs by species, therefore, the range of initial concentration of N and P for optimal cell growth and uptake mechanism has been extensively studied (Rhee, 1978). For instance, Laliberte et al. (1997) found that phosphorus could not be successfully taken up by *P. bohneri* when nitrogen was sufficient. Another study from Khan and Yoshida (2008) observed that the uptake rate of nitrogen increased when a carbon source was added. The appropriate N:P mass ratio for microalgae growth and consequent N removal was estimated to be 5:1 (Boelee et al., 2012). However, it was also found that the ratio of N:P in secondary municipal wastewater was over 30:1 due to limited P. Thus, N removal by microalgae was fast in the initial process, and then slowed down to a negligible level when P was completely depleted (Wang et al., 2017). However, in order to maintain growth-related N assimilation, some species are able to support N uptake mechanism by using intracellular P even when extracellular P is depleted (Wu et al., 2015). Furthermore, the solubility of ammonium and phosphates can be affected by pH. The elevated pH can accelerate volatilization of ammonium and P removal via alkaline precipitation, while the reduction of pH due to CO_2 enrichment may reduce the removal

mechanism (Ho et al., 2011; Garcia et al., 2000; Laliberte et al., 1997). In an extreme alkaline condition, microalgae metabolism would be inhibited, and the nutrient uptake would be reduced through related assimilation, which may not facilitate wastewater treatments (Wang et al., 2017). Light intensity and regime are other important environmental factors that can significantly affect the nutrient uptake rates of various microalgae species. Sukačová et al. (2015) reported that P removal rates declined to 26-38% when some of microalgae species were cultivated under continuous lighting. It has also been reported that nutrient removal rates can be reduced to half in lower temperatures such as 15ºC, compared with the rates at 25ºC, and the removal capacity ceased when temperature drops below 5ºC (Chevalier et al., 2000).

2.5. Production process

2.5.1 Two-stage cultivation strategy for astaxanthin production

The main steps from laboratory to pilot-scale process for *Haematococcus* derived astaxanthin production in PBRs has been extensively studied recently. Pérez-López et al. (2014) reported that the main stages in astaxanthin production from microalgae can be divided into five stages: 1) cleaning of the reactors; 2) culture preparation; 3) biomass and astaxanthin cultivation; 4) harvesting; and 5) extraction of the resultant astaxanthin.

Currently, there are two proposed strategies designed for the commercial production of astaxanthin from microalgae: the one-stage and the two-stage strategy. One-stage is an approach that performs under continuous cultures with limiting stress

at a steady-state, while the two-stage strategy separates the processes of biomass production (green stage) and astaxanthin production (red state) (Aflalo et al., 2007; Del Río et al., 2005). Most studies have achieved efficient astaxanthin productions in the two-stage strategy in *Haematococcus* cultures. For instance, Fábregas et al. (2001) used a two-stage culture system for the production of *Haematococcus* astaxanthin. A higher cell density was obtained after a 6-day cultivation period and the highest astaxanthin productivity was achieved as cultures were transferred to high irradiation conditions after 15 days. The feasibility of a one-stage astaxanthin production has been evaluated by Del Río et al. (2008). They used a continuous cultivation of *H. pluvialis* under a nitrogen limitation alternative to the conventional two-stage culture. The productivity results of the experiment supported the claim that high astaxanthin production was achieved in this new strategy and even superior to the two-stage strategy provided in the literature, with respect to the production efficiency. Aflalo et al. (2007) reported a comparison analysis between one-stage and two-stage strategy in terms of astaxanthin productivity, efficiencies, and yields. As a consequent, higher astaxanthin yields and productivities were obtained in the two-stage system. Moreover, individual parameters can be adjusted and optimized independently in each green and red stage, which provides a great improvement on production efficiency. Therefore, two-stage strategy is suggested as a better operational system that fits in massive commercial astaxanthin production.

2.5.2 Harvesting techniques

Harvesting is one of the most energy-intensive processes and challenging issues for commercial-scale microalgae biomass production. It has been reported that the harvesting process contributes approximately over one third of the total biomass production costs (Shah et al., 2016; Singh et al., 2013). In addition to the high costs of harvesting processes, most harvesting techniques have several other drawbacks, such as flocculant toxicity or unavailability for large-scale production (Li et al., 2011). Conventional harvesting techniques can be listed as centrifugation, foam fractionation, chemical flocculation, electro-flocculation, membrane filtration, and ultrasonic separation. Among these techniques, centrifugation is the most common method that is used for the production of high-value metabolites (Singh et al., 2013). This method is frequently used in the *Haematococcus* cultures as well, and usually combined with other techniques. Normally, the cells are separated from water by gravitational settling and subsequently concentrated with centrifugation (Han et al., 2013). Panis et al. (2016) indicated that more than 95% biomass recovery efficiency can be achieved when *H. pluvialis* is harvested via both flotation and disk-stack centrifugation.

2.5.3 Extraction and purification of astaxanthin

Extraction and purification are crucial steps in maximizing the yields of astaxanthin from *H. pluvialis*. Various types of organic solvents have been used for natural astaxanthin extraction (Ambati et al., 2014). Since *H. pluvialis* has a thick-wall in cyst cells, it always requires a pre-treatment of cyst cells prior to solvent extraction.

Kobayashi et al. (1997) reported that heat-acetone treatment followed by lytic enzymes treatment or lyophilisation eliminated chlorophyll of cyst cells, yielding 70% of astaxanthin from the cells. Furthermore, in order to reduce the loss of astaxanthin during the extraction steps, Sarada et al. (2006) proposed that pre-treatment of cyst cells with organic and mineral acid followed by acetone extraction could improve the extractability of astaxanthin. Currently, a number of novel extraction methods have been explored with increasing concerns related to environmental safety. For instance, supercritical fluids (SCF) as a green technology, has gained growing interest from food industry. Previous research found that SCF extraction has higher efficiency as compared to conventional methods (Ciftci, 2012). In particular, supercritical carbon dioxide (SC-CO_2) is the most commonly used SFC method for extraction, ascribed to its advantages in non-toxic, non-flammable, costly method, adjustable solvent properties, and significantly shorter extraction times (Cuellar-Bermudez et al., 2015). Moreover, integration of SC-CO_2 extraction and some co-solvent treatments, such as ethanol, acetone, HCl, and hexane are also used to extract astaxanthin from microalgae. Machmudah et al. (2006) reported that SC-CO_2 extraction with 5% ethanol achieved more than 80% astaxanthin extraction from *H. pluvialis* within 4 hours. Therefore, SCF methods are considered as effective extraction methods and therefore suggested for large-scale production of astaxanthin from microalgae. Further development of extraction methods is still anticipated prior to commercial use.

CHAPTER III

BIOMASS AND ASTAXANTHIN PRODUCTION BY *H. PLUVIALIS* IN MINKERY WASTEWATER

3.1 Introduction

With the increasing development of urbanization, industrialization and increased agricultural activities, the world will be faced with energy shortage challenges in the near future due to rapid depletion of non-renewable resources. It has been estimated that 50% more energy would be required globally by 2030 than its current use (Sani et al., 2013). The concerns about energy scarcity are forcing researchers to explore renewable and cost-effective energy alternatives. Fortunately, the extensive studies on microalgae-based production provide a number of innovative technologies and feasible strategies, relieving the stress on global energy market. *Haematococcus pluvialis*, a freshwater microalgae species, was selected to investigate in this study due to its extraordinary capacity of astaxanthin production, which is an essential antioxidant compound.

The increasing shortage of freshwater resources emphasizes the importance of wastewater reuse, recycling, and encourages advanced wastewater remediation technologies for sustainable development (Wang et al., 2017). Minkery wastewater (MW), obtained from the Dalhousie Fur Animal Research Centre in Bible Hill, Nova Scotia, was used to grow microalgae in this study. It contains high levels of nutrients, such as ammonia, nitrate, and phosphorus, which are able to support algal growth.

Due to the unique cellular characteristics of *H. pluvialis* strain, a two-stage

cultivation system was introduced in this study. Besides, there are several factors that can affect cell growth, biomass, and astaxanthin productivities. Therefore, multiple parameters in both stages were considered in order to optimize the biomass and astaxanthin production by *H. pluvialis*. In the first cultivation stage, the available MW concentrations, volume ratio of MW and microalgae concentrations, the initial concentration of *H. pluvialis*, cultivation time and nutrient removal capacity were evaluated. In the second induction stage, the comparative analysis of astaxanthin productivities among the two selected MW concentrations and traditional BBM was conducted. A comparison of culture medium in the induction period with distilled water was estimated as well. The specific objectives in this chapter were to determine the nutrient uptake efficiency of *H. pluvialis* in the cultivation stage, and to determine the optimal parameters in both stages and their relevant cultivation times.

3. 2. Methodology

3.2.1 Microalgae strain and culture conditions

Haematococcus pluvialis (CPCC 93) was obtained from the Canadian Phycological Culture Centre, Department of Biology, University of Waterloo, Ontario, Canada. 600 mL stock culture of *H. pluvialis* was cultivated photoautotrophically in a modified Bold's Basal Medium (BBM; chemicals supplied by Sigma-Aldrich, Table 3.1) in 1 liter Erlenmeyer flasks at room temperature (~20ºC) and continuously illuminated by a cool-white fluorescent light (32 W, 6,500K, 50 μmol photons $m^{-2}s^{-1}$). Aeration was provided by a gas exchange system with pure air and stirred at 100 rpm

by a mechanical stirrer (HI 190, Hanna Instruments, U.S.A., Figure 3.1).

Table 3.1. Nutrient concentrations in Bold's Basal Medium.

Nutrients	**Bold's Basal Medium (mg/L)**
$NaNO_3$	250
$MgSO_4 \cdot 7H_2O$	75
$CaCl_2 \cdot 2H_2O$	25
K_2HPO_4	75
$Na_2EDTA \cdot 2H_2O$	10
H_3BO_3	10.91
$MnCl_2 \cdot 4H_2O$	1.81
$ZnSO_4 \cdot 7H_2O$	0.222
$Na_2MoO_4 \cdot 2H_2O$	0.390
$CuSO_4 \cdot 5H_2O$	0.079
$Co(NO_3)_2 \cdot 6H_2O$	0.0494
KH_2PO_4	175
KOH	6.2
$FeSO_4 \cdot 7H_2O$	4.98
NaCl	25

Figure 3.1. 2.25L Tubular PBR culturing system of *Haematococcus pluvialis*.

3.2.2 Preparation of minkery wastewater

Minkery wastewater (cage-washing wastewater) was obtained from the Dalhousie Fur Animal Research Centre in Bible Hill, Nova Scotia. The samples were collected on the same day (March 2016) to reduce the experimental errors and maintain the homogeneity. Raw minkery wastewater samples were frozen in a refrigerator before they were pre-treated prior to use in cultivation.

Since MW contains extremely high levels of major nutrients, such as N and P, the dilution of raw MW was necessary to eliminate the inhibition effects on microalgae growth. A series of dilutions by using distilled water were used for preliminary tests to determine the optimum concentrations of MW for the strain in terms of growth rates and biomass productivities. Large suspended particles were removed through a glass microfiber filter with 1.5 μm pores (691, VWR, UK). After filtration, all wastewater samples were autoclaved (Brinkmann Tuttnauer 1730M-B/L, Figure 3.2) for sterilization at 121°C, under 15 psi for 20 minutes. This pre-treatment process helps to eliminate potential detrimental bacteria and other microorganisms. Lastly, all the pre-tested samples were stored in a refrigerator at 4°C until they were used in experiments.

Figure 3.2. Manual Autoclave Dental Steam Sterilizer (Brinkmann Tuttnauer 1730M-B/L) used in the study.

3.2.3 Cultivation system designed for the two-stage strategy

3.2.3.1 Vegetative green stage

An indoor closed system for the vegetative stage was constructed in this study. In the vegetative cultivation period, 200 ml of 8-day-old *H. pluvialis* cultures and 800 ml MW solutions that mixed in 1 L volume were transferred from Erlenmeyer flasks to 2.25-litre bench-scale vertical column controlled environment photobioreactors (Aqua Medic GmbH, Plankton Reactor, Bissendorf, Germany). Schematic diagram of the vertical column photobioreactor shown in Figure 3.3 (Yildiz et al., 2013). Each of the PBRs was attached to an independent cool-white fluorescent light (8 W, 6,700K) and kept at room temperature (~20ºC), which provided sufficient light source for microalgae to undergo photosynthesis. A gas exchange system was also attached to the PBR to continuously agitate the aquatic solution. The bubbles generated from aeration have benefits of maximizing CO_2 dispersion into solution and significantly avoiding cells sticking to the inner surface of the PBR. The effective capture of CO_2 capture by microalgae was facilitated, consequently increasing growth rates and biomass productivities in the PBR system. In addition, the temperature sensor and pH control

device were attached to the transparent tubular plastic cylinders. The pH was controlled *via* aeration due to formation of carbonic acid as air injected into the system, thereby lowering the pH level in the culture medium. The measurement error of the pH meter was ±0.2 pH. An attached gas dosing solenoid valve maintained the pH level by proving appropriate CO_2 content. Daily monitoring and maintenance of the pH control device and aeration were performed to sustain proper environment in the PBR cultivation system.

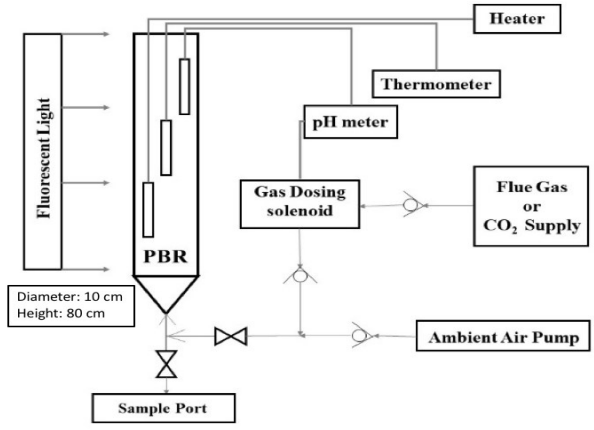


Figure 3.3. Schematic diagram of the vertical column photobioreactor.

3.2.3.2 Encystment red stage

Another independent closed system was designed for the second induction stage (shown in Figure 3.4). After the cultivation stage, a combination of high light intensity (200 µmol photons) and nutrient depleted conditions was provided to create stress conditions. For this stage, 40 mL samples were transferred from the vertical column PBRs to plastic culture dishes and placed under 200 µmol photon m^{-2} s^{-1} white LED lights for astaxanthin production.

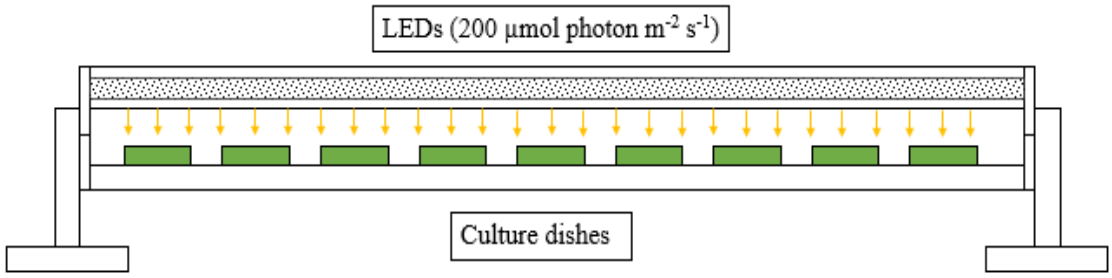


Figure 3.4. Schematic diagram of the designed closed system for the second induction stage.

3.2.4 Experiment design

3.2.4.1 Effect of different MW concentrations on the growth of *H. pluvialis*

8-day-old *H. pluvialis* cultures were transferred from Erlenmeyer flasks to vertical column PBRs. Light intensities on each PBR were maintained with continuous light at 50 µmol photon m^{-2} s^{-1}. Other conditions were sustained with stock culture. In the preliminary test, after filtration, MW was diluted at 1%, 2% and 4% concentrations. The findings indicated that *H. pluvialis* could not tolerate MW concentrations that were higher than 2%. In particular, *H. pluvialis* died after 2 days when cultivated in 4% MW concentration. A significantly higher growth rate of *H. pluvialis* was observed under 1% MW concentration as compared with 2% MW concentration. Therefore, a series of MW concentrations, using 1, 1.5 and 2% MW were conducted as parameter to determine a better culture medium for *H. pluvialis* growth.

3.2.4.2 Effect of different mixing ratios of MW concentration and microalgae inoculate culture

Since the composition of culture medium has important effect on microalgae growth, the volume ratio between MW concentration and microalgae concentration

needs to be determined as parameter. Generally, the ratio between inoculate culture and the new culture medium ranges from 1:4 to 1:9 (Stein, 1973). Thence, 100 and 200 ml microalgae concentrations, which were mixed with the corresponding 900 and 800 ml MW concentrations in 1 L volume, were tested in this section of the study.

3.2.4.3 Effect of the initial density of *H. pluvialis*

When time effectiveness of the biomass production is taken into consideration, the initial density of microalgae becomes another important parameter that may directly influence the viability and productivity of microalgae. A set of 0.09, 0.11, 0.13, and 0.16A initial densities, described by the optical density (OD) value, were used to determine the effect of initial density of *H. pluvialis* with proper MW concentration and culture ratios that had been decided before.

3.2.4.4 Testing nutrient removal rates, biomass and astaxanthin accumulation

H. pluvialis were inoculated into suitable MW concentrations, initial density and ratio of MW and microalgae concentration, which were already determined in the previous section of the study. Three nutrient parameters including nitrate, ammonia, and phosphorus were evaluated until they became deficient. At that point, vegetative cells would reach a stationary stage; and then they were transferred to a photoautotrophic induction period and unsynchronized, and remained under high light intensity (200 μmol photon m^{-2} s^{-1}) for 18 days. In the second induction period, samples were taken for analyses of astaxanthin contents. In this part of the study, the optimal minkery wastewater concentration, and the relevant cultivation and induction

days were determined. Nutrient removal capacity of *H. pluvialis* during the cultivation stage was evaluated as well. All experiments were performed with three replications (Figure 3.5).

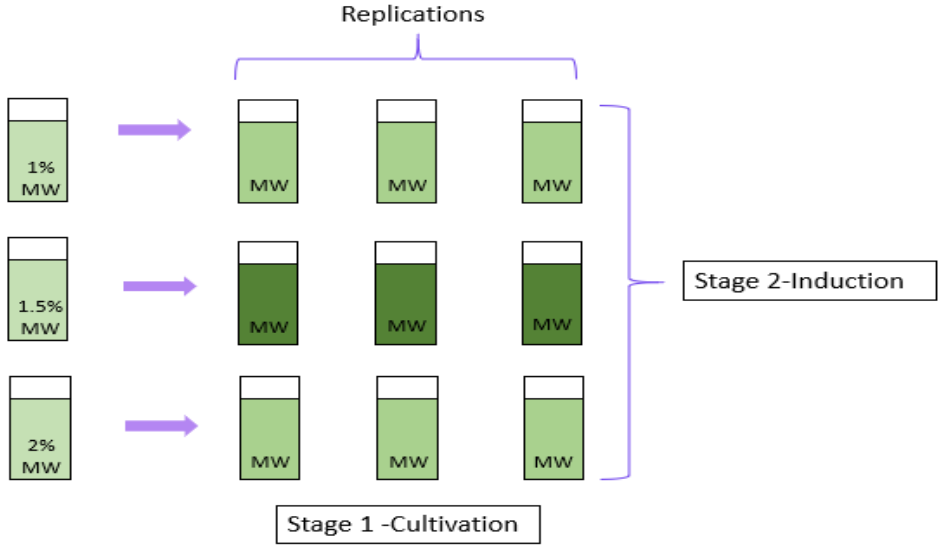


Figure 3.5. Flow diagram for determining the suitable minkery wastewater concentration (MW represents minkery wastewater).

3.2.4.5 Testing the culture mediums during the induction period

In this section of the study, *H. pluvialis* were first inoculated into the predetermined optimal MW concentration medium. Considering that small amounts of nutrients would still be available in the minkery wastewater cultivation mediums after the stationary stage, a parallel experiment was conducted to determine whether continuous culture could be used under stress conditions. Therefore, a certain amount of green-phase cells were quickly harvested by centrifugation and transferred to distilled water medium to start the induction period as a comparison, under the same conditions as in the previous part. Final astaxanthin contents were analyzed to

determine whether the minkery wastewater performs better on carotenoid accumulation than the distilled water during the induction period. All experiments were performed in three replications (Figure 3.6).

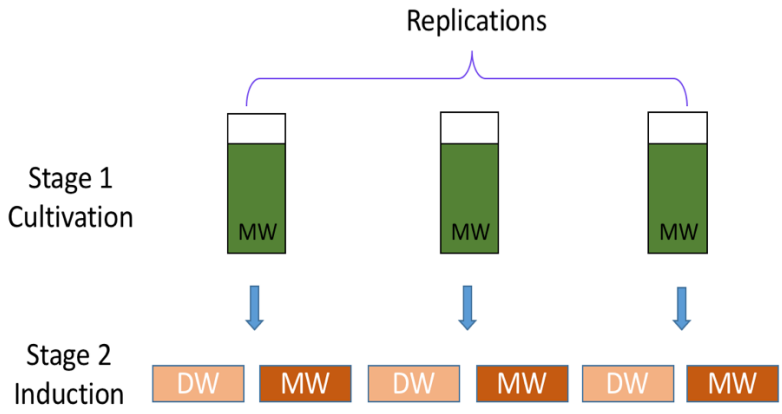


Figure 3.6. Flow diagram for determining the suitable culture medium for induction period (MW represents minkery wastewater; DW represents distilled water).

3.2.4.6 Statistical analysis

All experiments were performed with three replications. The ANOVA and Tukey's comparison method were performed for statistics analyses using MiniTab 18.

3.2.5 Analytical methods

In this part of the study, the microalgae growth kinetics, biomass productivity, chlorophyll contents, astaxanthin contents, and nutrient characteristics in solutions were analyzed to accomplish our objective. Optical density (OD) was used to determine the microalgae growth during the vegetative cultivation period, and was also used for measuring the chlorophyll contents during both stages and astaxanthin contents during the induction period. Measurement of OD values for growth rates and chlorophyll contents were carried out every 12 hours. The biomass productivity in the

vegetative stage was determined by dry weight (DW) measurements using the biomass samples collected in the initial and final day of each growth period.

3.2.5.1 Estimation of microalgae growth and biomass productivity

Optical density is a commonly used method for quantification of cell density. In this study, the OD values were measured by using a UV-Vis spectrophotometer (Cole-Parmer, US, Figure 3.7) with a light path of 10 mm at 680 nm to draw the growth curve during the vegetative cultivation period (Sun et al., 2015). The measurement error of spectrophotometer in terms of wavelength accuracy and photometric accuracy were ± 2nm and ±0.004 A at 0.5 A, respectively. Before reading the actual OD values, blanks for each sample were prepared to ensure the accuracy of measurement. The blanks consisted of a mixture solution of MW and BBM with proper ratios, depending on experimental design for each unit.

Figure 3.7 Cole-Parmer UV-Vis spectrophotometer used in the study.

Dry cell weights (DW) of each set were determined in the beginning and the end of vegetative cultivation period. 50 ml solution samples were collected from PBRs and measured after filtering through pre-weighted 0.6 μm Whatman GF/F glass microfiber filters (691, VWR, UK). Prior to filtration, the filters were first rinsed with distilled water, and then, placed in aluminum dishes and dried in a muffle furnace (Thermo scientific, US) (Figure 3.8 a) at 550°C for 15 min.

According to the Standard Methods for the Examination of Water and Wastewater, Method 2540 (APHA, 1998), the biomass estimation was obtained through the measurement of volatile suspended solids (VSS). After filtration of the samples, the aluminum dishes with filters were dried in an oven (VWR, UK) at 105ºC (Figure 3.8 b) for 15 min and weighted. Lastly, the aluminum dishes were transferred to the furnace and left there at 550°C for 15 min and weighed again.

Figure 3.8 The muffle furnace (a; left) and oven (b; right) used in the study.

The VSS (mg L^{-1}) was calculated by using Equation (1) below:

$$VSS = (W_b - W_a)/V \quad (1)$$

where:

W_b = the weight (filter plus aluminum dish and residues, mg) after dried at 105ºC for 1 h in the oven;

W_a = the weight (filter plus aluminum dish and residues, mg) after ashed at 550ºC for 15 min in the furnace; and

V = the known volume of aqueous samples through the filter.

The increased amount (mg L^{-1}) and percent increase (%) of biomass production were calculated using the following Equations (2) and (3), respectively:

$$\text{Increased amount of VSS} = VSS_{final} - VSS_{initial} \quad (2)$$

$$\text{Percentage increase of VSS} = [(VSS_{final} - VSS_{initial})/VSS_{initial}] \times 100 \quad (3)$$

where:

$VSS_{initial}$ = the DW measured on the initial day of the cultivation period; and

VSS_{final} = the DW measured on the final day of the cultivation period.

The biomass productivity (P, mg L^{-1} d^{-1}) was calculated by Equation (4):

$$P = (W_t - W_0)/t \quad (4)$$

where:

W_t = the final DW at the cultivation of t days; and

W_0 = the initial DW in the first cultivation day.

The specific growth rates of *H. pluvialis* were calculated using Equation (5)

(Göksan et al., 2011):

$$\mu = (\ln N_t - \ln N_0)/t \quad (5)$$

where:

N_t = the final DW at the cultivation of t days (mg L^{-1}); and

N_0= the initial DW in the first cultivation day (mg L^{-1}).

3.2.5.2 Estimation of nutrient removal capacity

The nutrient removal capacity of microalgae from MW was determined by a series of measurements during the vegetative cultivation period. The remaining filtrates after filtration through GF/F glass microfiber filter using a Millipore 47 mm vacuum filter assembly (Figure 3.9) were collected for nutrient analyses. A HANNA Multiparameter Photometer (HI 83200) (Figure 3.10) was used to analyze nutrient concentrations with relevant reagents, depending on nutrient parameters. Three major nutrients components were measured: nitrate, phosphorus, and ammonia. According to the Instruction Manual, the measurement error of nitrate, ammonia, and phosphorus were ±0.5 mg L^{-1}, ±0.05 mg L^{-1}, and ±0.3 mg L^{-1}, respectively. The measurements of MW characteristics prior to experiments were also performed by HANNA Multiparameter Photometer (HI 83200) and UV-Vis spectrophotometer (DR 6000™, HACH) (Figure 3.11).

Figure 3.9 Millipore filter assembly and the vacuum pump used in the study.

Figure 3.10 HANNA Multiparameter Photometer (HI 83200) used in the study.

Figure 3.11 UV-Vis spectrophotometer (DR 6000™, HACH, US) used in the study.

The removed amount (mg L^{-1}) and percent removal (%) of nutrients were calculated by using the following Equations (6) and (7), respectively:

$$\text{Reduced amount of } C_x = C_{final} - C_{initial} \quad (6)$$

$$\text{Percentage removal of } C_x = [(C_{final} - C_{initial})/C_{initial}]x100 \quad (7)$$

where:

C_x = nutrient concentration obtained for each parameter;

$C_{initial}$ = the nutrient concentration measured on the initial day; and

C_{final} = the nutrient concentration measured on the final day.

3.2.5.3 Estimation of pigment composition

Pigment concentration was determined by the following methods and Equations (9) and (10) according to Lichtenthaler, (1987). 2 ml of culture samples were taken and centrifuged using a Thermo Scientific centrifuge (CL 2 centrifuge, US, Figure 3.12) at 10,000 rpm for 5 min. Then, the supernatant was discarded, and 2 ml of 99.9% methanol was added to the pellet. The samples were then stored at 4^{o}C overnight under dark. After approximately 24 hours, samples were centrifuged at 10,000 rpm for 5 min and the supernatant was measured for pigment estimation by spectrophotometer at different absorbencies. The chlorophyll contents were calculated using the following Equations:

$$\text{Chlorophyll a (Chl-a, } \mu g/ml) = 16.72\ A_{665.2} - 9.16\ A_{652.4} \quad (9)$$

$$\text{Chlorophyll b (Chl-b, } \mu g/ml) = 34.09\ A_{652.4} - 15.28\ A_{665.2} \quad (10)$$

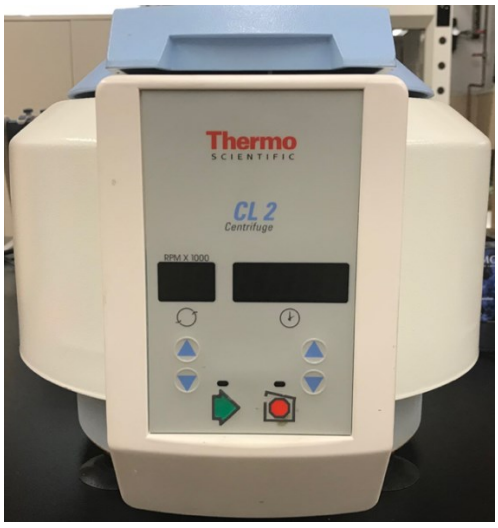


Figure 3.12 Thermo Scientific centrifuge (CL 2) used in the study.

The carotenoid content can be approximately calculated by recording the chlorophyll content Lichtenthaler, (1987). The carotenoids concentration (μg L^{-1}) was calculated by using Equation (11):

$$\text{Carotenoids } (\mu g\ L^{-1}) = (1000\ A_{470} - 1.63\ \text{Chl-a} - 104.9\ \text{Chl-b})/221 \quad (11)$$

Where: $A_{665.2}$, $A_{652.4}$, A_{470} = absorbencies at 665.2, 652.4 and 470 nm

The astaxanthin content was measured according to modified Boussiba method (Boussiba and Vonshak, 1991). For astaxanthin analysis, 5 ml culture samples after the induction period were collected and centrifuged for 10 min at 4000 xg, and a 5-ml solution of 5% KOH and 30% (v/v) methanol was prepared (1:1) and used to saponify the pellet at 70°C for 5 min to destroy the chlorophyll. Then, the supernatant was discarded, and the pellet was washed three times to remove the residual lye by using de-ionized water. The remaining pellet was extracted with 5 ml dimethyl sulfoxide (DMSO, > 99%) at 70°C for 10 min to recover the astaxanthin. The extraction

procedure was repeated at least three times until the cell debris was almost colorless. The absorbance of the extracts was measured at 490 nm.

The per unit volume astaxanthin concentration (mg L^{-1}) was calculated by using Equation (12) (Davies, 1976):

$$C_{astaxanthin}\ (mg\ L^{-1}) = 4.5 \times A_{490} \times V_a/V_b \quad (12)$$

where:

A_{490} = the absorbance of the extracts at 490 nm;

V_a = the volume of extracts; and

V_b = the volume of culture samples.

The astaxanthin productivity (mg L^{-1} d^{-1}) in column PBRs was calculated using Equation (13):

$$\text{Astaxanthin productivity} = (C_t - C_0)/t \quad (13)$$

where:

C_t = astaxanthin concentration (mg L^{-1}) in the PBR after t days induction;

C_0 = initial astaxanthin concentration (mg L^{-1}); and

t = induction time (days).

3.3. Results and discussion

3.3.1 Effect of minkery wastewater concentration on microalgae growth

3.3.1.1 Minkery wastewater characterization

The MW characteristics along with the major nutrient compositions were

summarized in Table 3.2. The raw MW sample has a high pH and very high turbidity with a dark brown color. After a series of proper dilutions of MW and pre-treatment at 1, 1.5 and 2% MW concentrations, the pH values were significantly dropped from 8.84 to 7.05, 7.16 and 7.23, respectively. Besides, the turbidity was removed by filtration. Compared with the traditional modified BBM culture mediums mentioned in the previous section, it was remarkable to observe that the majority of N source in BBM was supplied by nitrate, while in the MW were contributed by ammonium. Previous studies have indicated that wastewaters that contain high level of ammonium may accelerate the growth rates of microalgae, or inversely, has inhibitory effect on microalgae growth, depending on species' preferences and tolerances (Collos and Berges, 2004).

Table 3.2. The nutrient contents of the raw MW, 1%, 1.5% and 2% pre-treated minkery wastewater.

	Raw MW ($g\ L^{-1}$)	**1.0% MW ($mg\ L^{-1}$)**	**1.5% MW ($mg\ L^{-1}$)**	**2.0% MW ($mg\ L^{-1}$)**
Ammonium (NH_4^+)	4.17	41.7	58.3	72
Nitrate (NO_3^-)	0.22	2.15	3.76	5.11
Nitrite (NO_2^-)	0.20	1.14	1.98	2.78
Total phosphorus (P)	1.4	14	21.8	26.8
Sulfate (SO_4^{2-})	1	10	15	19
Chemical oxygen demand (COD)	12	120	175	210

3.3.1.2 Effect of wastewater dilutions and volume ratios on microalgae growth

H. pluvialis grew well in all the dilutions tested in MW ranging 1% to 2%. When a 1:9 volume ratio of microalgae and MW was used, Figure 3.13 shows that the growth of microalgae increased with dilution factors, changed from 2% MW to 1.5% MW, while a decline in growth took place when *H. pluvialis* was cultivated in 1% MW. Maximum cell density was observed at 1.5% MW with an OD value of 1.922A on the 9th day, followed by 1.872 and 1.692A in 2 and 1% MW, respectively. When the volume ratio of microalgae and MW was adjusted to 1:4, the maximum cell density still occurred at 1.5% MW, with the OD value reaching to 2.568A. Minimum cell density of 2.057A was recorded at 2% MW (Figure 3.14), implying that high nutrient content (>1.5% MW in this study) of culture medium hinders the growth of *H. pluvialis*.

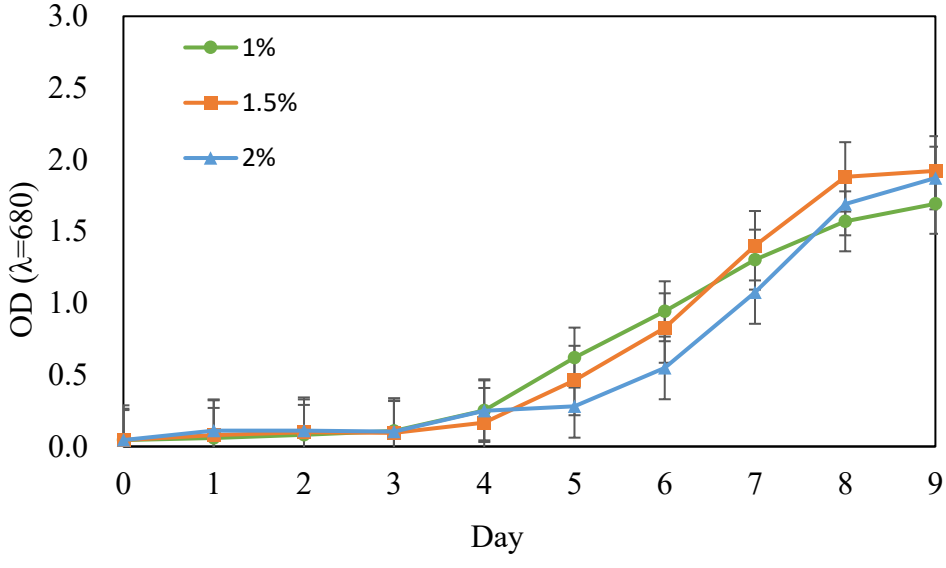


Figure 3.13 *H. pluvialis* growth curves at different MW concentrations (1, 1.5, 2%) under 1:9 volume ratio after 9-day cultivation.

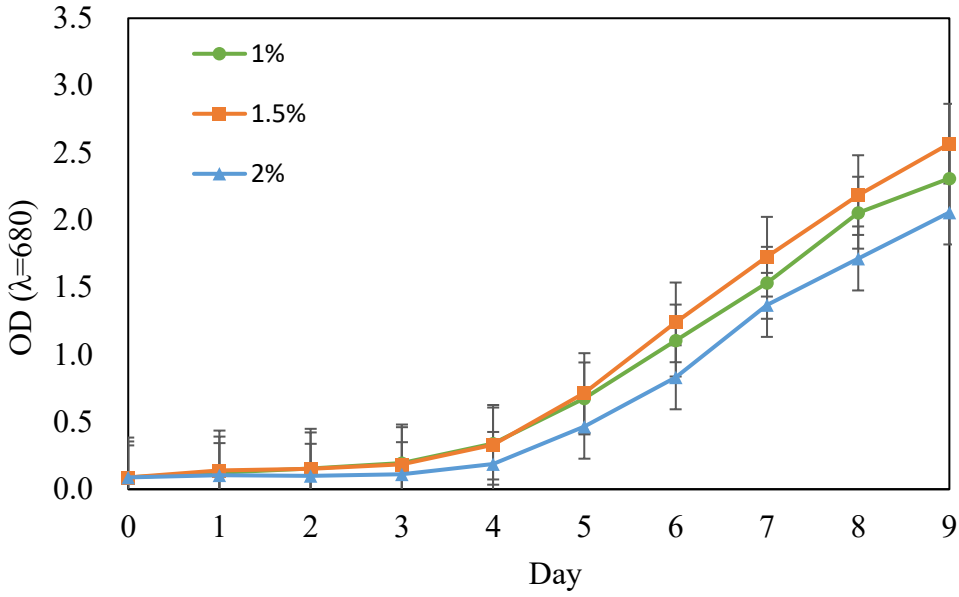


Figure 3.14 *H. pluvialis* growth curves at different MW concentrations (1, 1.5, 2%) under 1:4 volume ratio after 9-day cultivation.

Figure 3.15 shows the growth curves at different MW concentrations under two different volume ratios of solution after a 9-day cultivation period. The results indicated that the growth of microalgae performed better in a 1:4 volume ratios of solution, which represents a combination of 200 ml microalgae concentration (inoculum) (AC) and 800ml MW mixture solution in 1 liter PBRs. A lower ratio showed reduced microalgae growth where the maximum cell densities dramatically dropped from 2.437A to 1.807A. This can be ascribed to high initial cell density in 1:4 volume ratio solution that improves the viability of microalgae and make it better adapt to a new culture condition (Wang et al., 2013). Moreover, 200 ml AC provided more nutrient contents, especially nitrate as N source supplement, resulting in an improved N assimilation by microalgae and thus, stimulating biomass accumulation. In some studies, the wastewater feedstock used for microalgae growth was collected from

nitrate-rich stage at the treatment plant (Kang et al., 2006). Proper concentrations of nitrate may need to be added into culture medium accompanying with wastewater to ensure the optimal growth of microalgae (Kang et al., 2006). Therefore, in this study, a 1:4 volume ratio of AC and MW concentration (1 L in total) was used for subsequent experiments. As mentioned before, cell growth rate was low in 2% MW due to high nutrient contents, therefore excluded from MW tests in the sequential cultivation experiments.

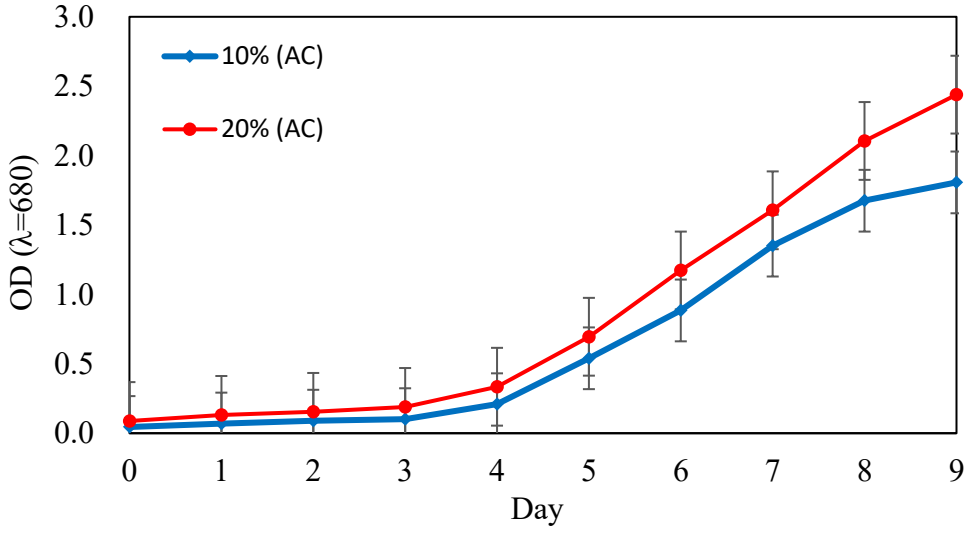


Figure 3.15 Mean *H. pluvialis* growth curves at different MW concentrations with 1:9 and 1:4 volume ratio of microalgae concentration (inoculum) and minkery wastewater after 9-day cultivation.

3.3.1.3 Effect of initial cell density on growth

The effect of initial cell density (ICD) on cell growth of *H. pluvialis* in a 6-day cultivation period was explored in this section of the study, and the results were presented in Figure 3.16. All the experimental units were cultivated under 1.5% MW with a 1:4 volume ratio of mixture solution. It is obvious that the growth rates were

dramatically increased as the ICD elevated from 0.09A to 0.13A. Then a slight downfall occurred as the value continued to increase to 0.160A. After 6 days cultivation, the optimal ICD was found at 0.13A, which resulted in the highest biomass accumulation - approximately 5 times higher than that obtained at 0.09A. However, it was close to the initial OD value at 0.11A (Figure 3.16). This result is suggesting a higher viability of *H. pluvialis* as the ICD surpasses 0.11A and optimal obtainable cell productivity when the ICD ranges from 0.11 to 0.13A. However, cell death frequently happens during the cultivation stage or transferring from the green stage to the red stage, with cell mortality rates of 20 to 80%, depending on strains and culture systems (Li et al., 2010). Wang et al. (2013) studied the effect of initial cell density on growth and biomass productivity of *H. pluvialis*. Seven levels of ICD were tested, including 0.1, 0.5, 0.8, 1.5, 2.7, 3.5, and 5.0 g L^{-1}. As a result, they found that the maximum biomass accumulations were observed at 0.8 and 1.5 g L^{-1} during the 10-day cultivations. They concluded that the identification of an appropriate ICD is important to an effective cultivation strategy that sustains maximum biomass productivity and thus astaxanthin production, while reducing the cell mortality rate.

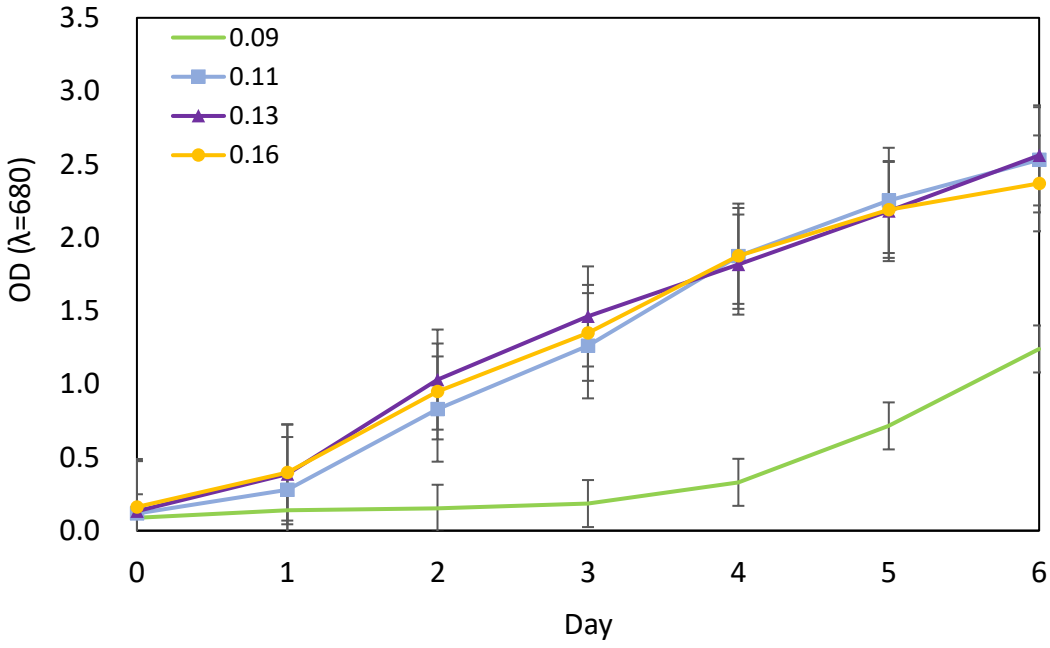


Figure 3.16 *H. pluvialis* growth curves at 1.5% MW (1:4 volume raio) with different ICD (described in OD value).

3.3.2 Nutrient removal capacities from different cultivation mediums and biomass accumulation by *H. pluvialis* during cultivation stage

3.3.2.1 Growth rates

The mean increased cell density was measured by the optical density of *H. pluvialis* in traditional medium (BBM) and two different MW concentrations as determined before. According to the growth curve of *H. pluvialis* displayed in the green vegetative stage, exponential growth rates in cell density occurred in first 2 days and then gradually flattened due to the depletion of N and P (Figure 3.17). The maximum cell density in terms of OD value was obtained at 1.5% MW with 2.487A, followed by BBM and 1% MW with 2.214A and 1.869A, respectively. Normally, the

measurement of chlorophyll is frequently used to estimate the biomass yields. In this study, the accumulation on total chlorophyll contents (chlorophyll a and chlorophyll b) in *H. pluvialis* cells were measured alongside cell growth. Consequently, the mean of maximum total chlorophyll content in BBM, 1% MW, and 1.5% MW reached 16.9 μg L^{-1}, 15.7 μg L^{-1} and 13.1 μg L^{-1}, respectively (Figure 3.18).

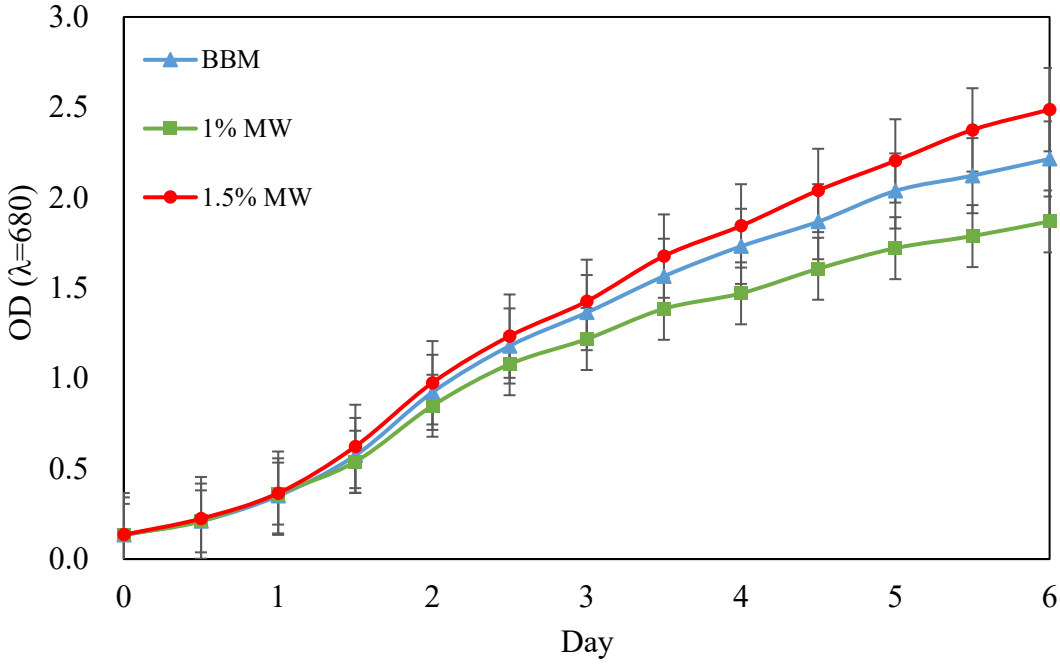


Figure 3.17 Growth curves of *H. pluvialis* in BBM, 1% MW, and 1.5% MW mediums after 6-day cultivation.

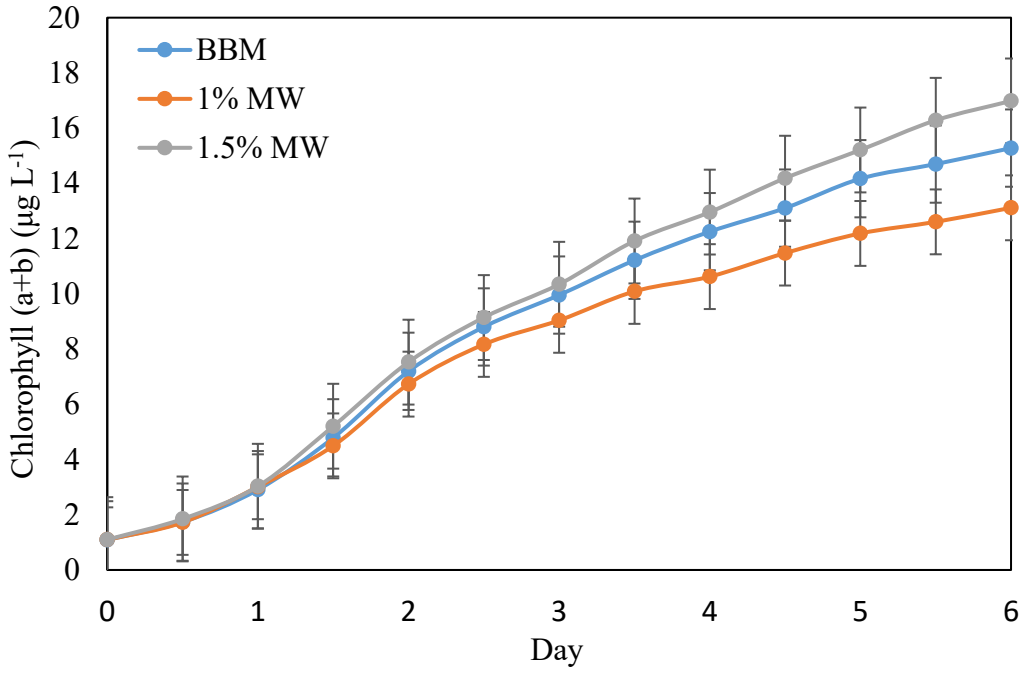


Figure 3.18 Total chlorophyll contents of *H. pluvialis* in BBM, 1% MW, and 1.5% MW mediums after 6-day cultivation.

3.3.2.2 Biomass and total carotenoid production

The findings showed that *H. pluvialis* grew well in BBM, 1% MW, and 1.5% MW with specific growth rates of 0.446 ± 0.01 mg L^{-1} day^{-1}, 0.399 ± 0.01 mg L^{-1} day^{-1}, and 0.451 ± 0.02 mg L^{-1} day^{-1}, respectively. The mean of increased biomass obtained from different culture mediums were shown in Figure 3.19 Based on the results, *H. pluvialis* achieved its highest biomass in 1.5% MW, which yielded 906.33 ± 34.0 mg L^{-1} biomass with the optimal biomass productivity of 151.1 ± 5.7 mg L^{-1} d^{-1} over the 6-day cultivation period. A Tukey comparison method (with 95% confidence) indicated that the biomass obtained from three culture mediums were significantly different from each other and categorized by letter grouping (see Table 3.3). The 1.5% MW produced significantly higher biomass than BBM and 1% MW ($p<0.05$), and the lowest biomass

production was observed in 1% MW with an average yield of 681 ± 21.5 mg L^{-1} and a biomass productivity of 113.5 ± 3.6 mg L^{-1} d^{-1}. However, the Tukey's test determined that there was no significant difference between the mean specific growth rates when microalgae grew under BBM and 1.5% MW ($p>0.05$), while both mediums showed significantly higher specific growth rates than that in 1% MW ($p<0.05$). Since there are totally different nutrient compositions and N sources between traditional medium and MW cultures, our findings indicated that BBM can comfortably be replaced by proper MW concentrations (1.5% in this study) while achieving high biomass production of *H. pluvialis*.

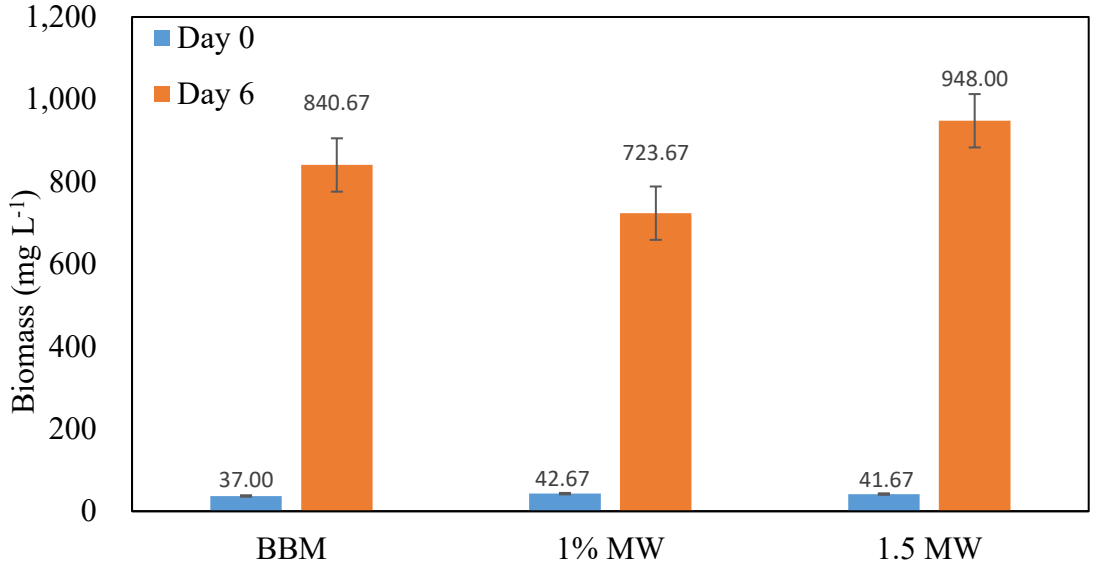


Figure 3.19 Biomass productions of *H. pluvialis* in BBM, 1% MW, and 1.5% MW mediums after 6-day cultivation period.

The increased biomass in percentage was also shown in Table 3.3. Another microalgae biomass production study using culture mediums of 1% MW and BBM was executed in our laboratory. In that study, Ji (2017) cultivated *Chlorella vulgaris*

under four different light cycles during a 6-day cultivation period: continuous light, 48-h light and 24-h dark, 24-h light and 48-h dark, and continuous dark. The findings showed that *Chlorella vulgaris* grew well under 1% MW and there was no significant difference in biomass accumulation between the continuous light or the 48-h light and 24-h dark regimes. However, it was remarkable to observe that the biomass increase in all light cycles under 1% MW were significantly higher than the biomass increases in BBM. Under the continuous light cycle, the highest increased biomass in percentage obtained from *Chlorella vulgaris* in 1% MW achieving 834% increase, which is close to but lower than what was observed in our study.

Table 3.3. The increased amount (mg L^{-1}) and percent increase (%) of biomass production in BBM, 1% MW, and 1.5% MW mediums after 6-day cultivation periods.

Medium	**Biomass (mg L^{-1})**	**Biomass increase (%)**
BBM	803.67 ± 32.2^{b}	1356 ± 113^{b}
1% MW	681.00 ± 21.5^{c}	993 ± 132^{c}
1.5% MW	906.33 ± 34.0^{a}	1412 ± 193^{a}

Means that do not share a letter are significantly different.

The means of increased chlorophyll contents in each culture medium were summarized in Table 3.4. In addition, the increased total carotenoids were calculated from the absorbance associated with chlorophyll measurement (see Table 3.4). In the green vegetative stage, the major carotenoids in *H. pluvialis* consisted of primary carotenes, such as lutein and β-carotene. The statistical analysis demonstrated that 1.5% MW had the highest carotenoid contents in the group, which was significantly higher

than that in lower MW concentrations and close to those obtained in BBM. The changes in chlorophyll and carotenoid contents are important considerations that can be used to evaluate the physiological state of the cells (Göksan et al., 2011). Besides, the accumulation of carotenoids in cells is often considered as an indicator of stress conditions in the culture, such as the depletion of essential nutrients during the cultivation period. Consequently, the total carotenoid contents obtained from *H. pluvialis* at the end of cultivation stage were estimated as 4.4 ± 0.4 (BBM), 3.6 ± 0.4 (1% MW), and 5.1 ± 0.7 (1.5% MW), respectively.

Table 3.4. The statistical analysis of specific growth rates (mg L^{-1} day^{-1}), biomass productivities (mg L^{-1} d^{-1}), increased total chlorophyll content (μg mL^{-1}) and increased total carotenoid content (μg mL^{-1}) in different culture mediums after 6-day cultivation.

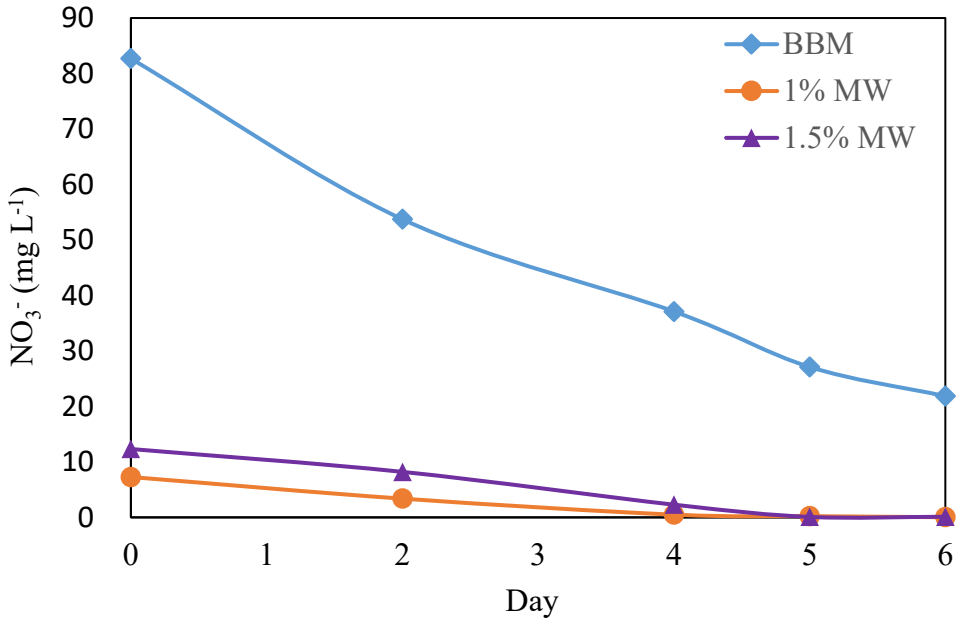
Medium	**Specific growth rate (mg $L^{-1}day^{-1}$)**	**Biomass Productivity (mg L^{-1} d^{-1})**	**Chl (a+b) (μg mL^{-1})**	**Carotenoids (μg mL^{-1})**
BBM	0.446 ± 0.01^{a}	133.9 ± 2.9^{b}	14.2 ± 1.7^{ab}	4.4 ± 0.4^{ab}
1% MW	0.399 ± 0.01^{b}	113.5 ± 3.6^{c}	12.0 ± 0.8^{b}	3.6 ± 0.4^{b}
1.5% MW	0.451 ± 0.02^{a}	151.1 ± 5.7^{a}	15.9 ± 2.1^{a}	5.1 ± 0.7^{a}

Means that do not share a letter are significantly different.

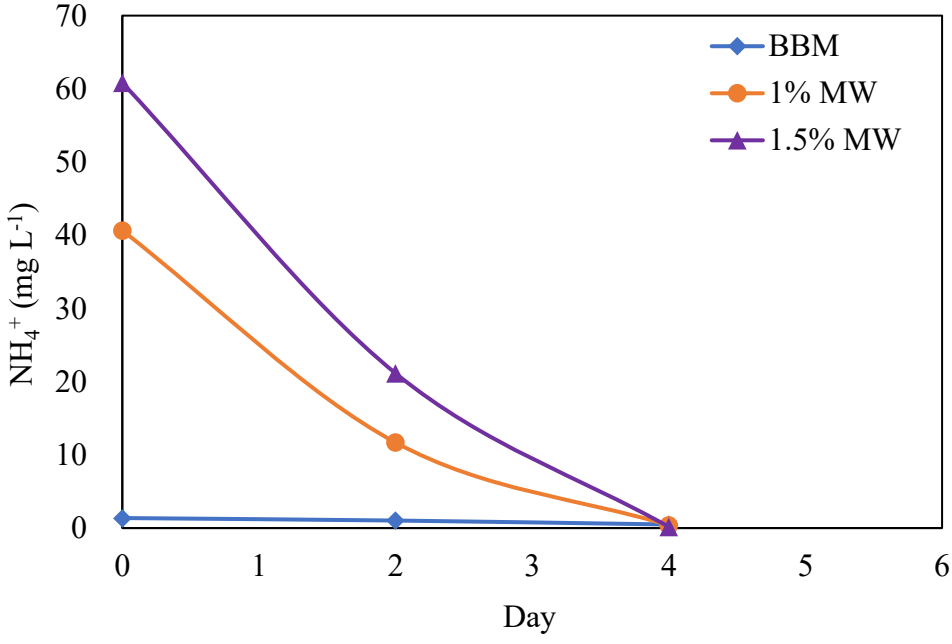
As compared with the growth characteristics of *H. pluvialis* under traditional BBM and MW, *H. pluvialis* showed a great adaption to MW culture. The rapid growth rates and biomass production suggest that minkery wastewater can be used as a nutrient source for this species.

3.3.2.3 Nutrient removal capacity

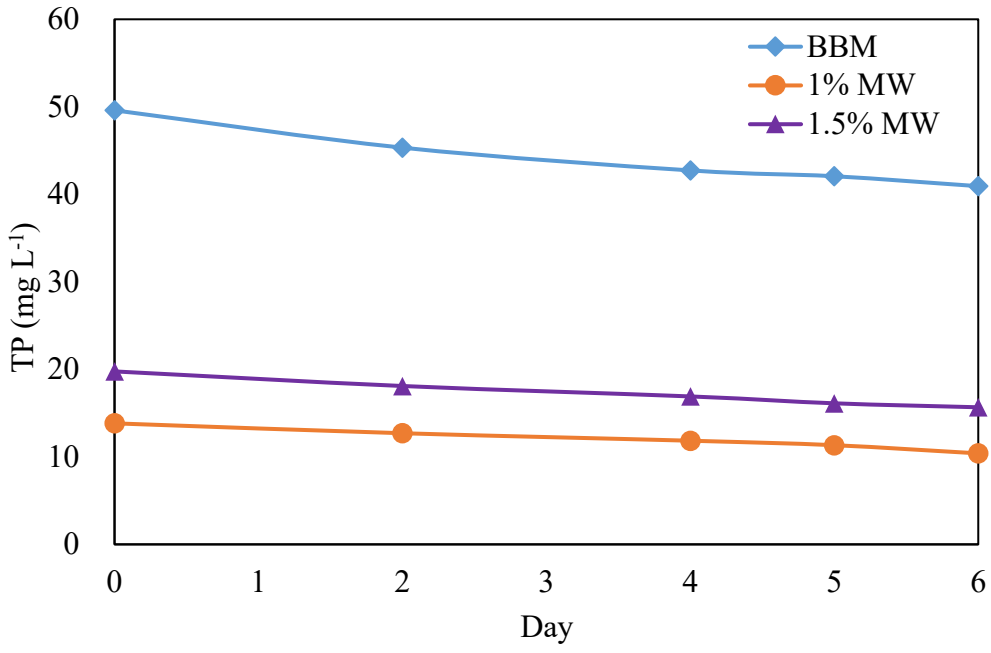
The removal of three important nutrients content, including NO_3^-, NH_4^+, and TP were provided in the Figure 3.20. Based on the findings, all the N sources were almost consumed completely by *H. pluvialis* in 4 days, while the removal of TP was relatively slower. The mean of initial concentrations of NO_3^-, which is an important N source for microalgae growth, in BBM, 1% MW and 1.5% MW mediums were estimated at 82.7 mg L^{-1}, 7.3 mg L^{-1}, and 12.3 mg L^{-1}, respectively. After a 6-day cultivation period, the resultant concentration declined to 21.9 mg L^{-1} in BBM, and down to zero in both MW cultures. The fastest uptake was observed for NH_4^+, the major N source in MW, which was completely removed from the all the three culture mediums within a 4-day cultivation period. It was interesting to observe that the high initial nitrogen concentrations led to higher removal capacities, regardless of the form of N source (see Figure 3.20 a and b. Findings also demonstrate the microalgae preference in nutrients uptake, clearly showing that NH_4^+ was the preferred form of nitrogen.


90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Day
NO3- (mg L-1)
BBM
1% MW
1.5% MW

(a)


70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
Day
NH4+ (mg L-1)
BBM
1% MW
1.5% MW

(b)



(c)

Figure 3.20 Removal of NO_3^- (a), NH_4^+(b), and TP (c) from BBM, 1% MW, and 1.5% MW mediums by *H. pluvialis.*

Total phosphorus (TP), which represents total orthophosphates (PO_4^3-P), was studied. Unlike N, the removal capacities of P were much lower in all cultures. As mentioned in the previous chapter, the capacity of nutrient uptake may be affected by the initial N:P ratio in the specific culture medium. The N:P ratio in BBM was about 8:5, with the removal capacities estimated at 5:1, which corresponded with the optimal N:P ratio for microalgae growth (Boelee et al., 2012). By contrast, the initial ratio of N:P in 1% MW and 1.5% MW mediums were approximately at 7:2 and 3:1, respectively, much higher than that in BBM. This implies that the relatively higher N:P ratios in MW medium resulted in a lower uptake of TP by *H. pluvialis*. The same situation happened in the case of *P. bohneri* that TP could not be successfully uptaken

when N concentration was relatively high (Laliberte et al., 1997). In comparison with other wastewater sources that are limited in P, such as municipal wastewater, the N removal capacities were fast in the first stage and then eased once P was depleted (Wang et al., 2017). Therefore, although the P levels in this study were still high in three cultures, the productivities of biomass were supposed to drop down due to the depletion of N sources and the cell density was adequate for astaxanthin production. Thus, after 5 days of cultivation process for N assimilation and 1 day sustain period in stationary stage in all cultures, green vegetative cells were transferred to the secondary stage for the astaxanthin production process. The mean removal capacity of *H. pluvialis* in total nitrogen (TN), which represents N derived from NO_3^- and NH_4^+ in solution, and TP from BBM, 1% MW, and 1.5% MW mediums in terms of the removed amounts (mg L^{-1}) and percentages (%) were provided in Table 3.5. About 75.1 ± 4.1% of TN was removed from BBM, while almost 100% removal capacities were achieved in both 1% and 1.5% MW mediums in 6-day cultivation periods. As for TP, the removal capacity (as described in percent) dropped down to a range from 17.5% to 24.8%. Another biomass production study on *Chlorella vulgaris* in 1% MW medium showed that the means of TN and TP removal percent reached to 96.2% and 29.7%, respectively, under continued light cycles after 6-day cultivation periods (Ji, 2017). Compared to *C. vulgaris*, *H. pluvialis* performed better in N assimilation from MW cultures and simultaneously had a lower uptake capacity for TP removal. When compared with the TN removal capacities by various other microalgae species

mentioned in the previous chapter, *H. pluvialis* showed an extremely high capacity in N consumption and great adaption to high ammonium concentration cultures without any inhibitory effects in the short-term cultivation period. Wang et al. (2010) reported that 85.6% of TP was removed from a municipal wastewater in 9 days. It should be noted that the wastewater used in this study contained much higher initial TP concentration than those in most municipal wastewaters. However, the removed amount of TP from those municipal wastewaters was much closer to our result.

Table 3.5. The means of TN and TP removal capacities (in amount and percent) from BBM, 1% MW, and 1.5% MW mediums by *H. pluvialis* after 6-day cultivation.

Medium	**Removed amount (mg L^{-1})**		**Removal percent (%)**	
	TN	**TP**	**TN**	**TP**
BBM	15.2 ± 0.9	8.7 ± 1.1	75.1 ± 4.1	17.5 ± 0.9
1% MW	32.9 ± 0.9	4.4 ± 0.4	99.7 ± 0.3	24.8 ± 1.8
1.5% MW	49.7 ± 1.2	4.1 ± 0.4	99.8 ± 0.2	20.7 ± 2.1

Means that do not share a letter are significantly different.

Considering the different N sources available in traditional and MW mediums, the means of NO_3^- and NH_4^+ removal capacities from BBM and 1.5% MW cultures by *H. pluvialis* are summarized in Table 3.6. The initial concentration of NO_3^- in BBM (82.7 ± 4.7 mg L^{-1}) was much higher than NH_4^+ in 1.5% MW (60.82 ± 1.6 mg L^{-1}); however, there was no significant difference between the resultant removed amounts (mg L^{-1}) of these two N sources ($p>0.05$). However, a significantly higher removal rate (percent) of NH_4^+ from MW than that of NO_3^- from BBM was observed ($p<0.05$). In many

studies, excess ammonium led to a reduction in microalgae biomass, while our findings suggested that *H. pluvialis* had a higher tolerance for ammonium and they were able to survive in various types of N source culture mediums. This suggests that the cultivation cost can be dramatically reduced if MW is used instead of the traditional culture medium, thereby achieving a cost-effective microalgae cultivation process.

Table 3.6. The means of NO_3^- and NH_4^+ removal capacities (in amount and percent) from BBM and 1.5% MW mediums by *H. pluvialis* after 6-day cultivation.

Medium	**N source**	**Initial concentration (mg L^{-1})**	**Final concentration (mg L^{-1})**	**Removed amount (mg L^{-1})**	**Removal percent (%)**
BBM	NO3-	82.7 ± 4.7	21.9 ± 0.4	60.8 ± 4.9[a]	73.5 ± 4.1[b]
1.5% MW	NH4+	60.8 ± 1.6	0.07 ± 0.02	60.8 ± 1.5[a]	99.8 ± 0.2[a]

Means that do not share a letter are significantly different.

According to Nova Scotia's Fur Industry Act (approved on January 11, 2013), the surface water concentration limits for NH_4^+, NO_3^- and TP are reported as 1 mg L^{-1}, 3 mg L^{-1}, and 2 μg L^{-1}, respectively (Fur Industry Act of 2010). The concentrations allowed for groundwater regarding these parameters were restricted at 4 mg L^{-1}, 13 mg L^{-1}, and 0.1 mg L^{-1}, respectively. This study showed that the concentration levels reached after a 6-day cultivation can meet the NH_4^+ and NO_3^- concentration limits for both surface water and groundwater in Nova Scotia. However, the resultant TP concentrations are still far from permissible discharge levels. If this becomes an issue, further nutrient recycling and treatment are required to remove the excess TP from

minkery wastewater.

3.3.3 Astaxanthin production during photoautotrophic induction

3.3.3.1 Astaxanthin production in BBM and MW cultures

After a 6-day cultivaiton period, 40 ml samples containing green vegetative cells from three different mediums were transferred to induction process for astaxanthin accumulation under continuous light at 200 μmol photon m^{-2} s^{-1} without any additions to the cultures. The encystment of the cells was triggered immediately under nutrient depleted conditions and high light intensity cultures. The accumulation of astaxanthin continued to increase in the first 9 days of induction period, and then, reduced rates were observed, gradually reaching to a stationary level after 12 days (see Figure 3.21). Microscopic examination confirmed that green vegetative cells were transformed into red cyst cells accompanied with cell size increases.

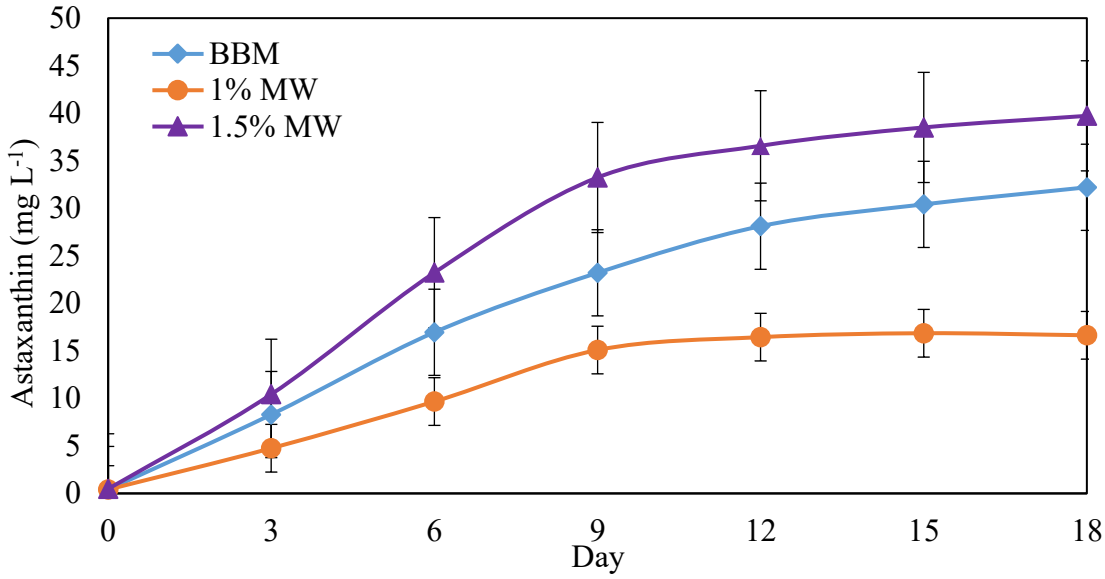


Figure 3.21 Astaxanthin productions in BBM, 1% MW, and 1.5% MW mediums by *H. pluvialis* during photoautotrophic induction (light at 200 μmol photon m^{-2} s^{-1}).

Table 3.7 shows that, after an 18-day induction period of *H. pluvialis*, the resultant astaxanthin concentraon reached 32.21 ± 1.58 mg L^{-1}, 16.64 ± 0.57 mg L^{-1}, and 39.72 ± 1.69 mg L^{-1} in BBM, 1% MW, and 1.5% MW cultures, respectively, with corresponding astaxanthin productivities of 1.78 ± 0.08 mg L^{-1} day^{-1}, 0.92 ± 0.03 mg L^{-1} day^{-1} 2.21 ± 0.09 mg L^{-1} day^{-1}, respectively. The astaxanthin production in the BBM medium was significantly lower than that in the 1.5% MW medium because not only it had less cell density in the initial day of the induction period but also there were still available N in the BBM cultures and differences in residual TP in the two cultures. Imamoglu et al. (2009) reported that under the same stress conditions, a higher yield of astaxanthin was obtained in N-deprived cultures compared with N-P-deprived cultures, indicating that N deprivation has a bigger effect on astaxanthin production compared to P deprivation. In our study, during the same cultivation and induction times, *H.*

pluvialis achieved its highest biomass and astaxanthin production in the MW medium rather than in the traditional culture medium of BBM, which shows that our findings were in agreement with the previous study.

Although *H. pluvialis* spent the same time for N assimilation in the two MW mediums, astaxanthin productivity in 1% MW medium was significantly lower than that in 1.5% MW medium due to the fewer initial cells obtained during the first cultivation period to synthesize astaxanthin ($p<0.05$). Based on these findings associated with a 12-day induction period, 1.5% MW mediums were selected for the subsequent experiment.

Table 3.7 Astaxanthin concentrations (mg L^{-1}) and productivities (mg L^{-1} d^{-1}) in BBM, 1% MW, and 1.5% MW mediums during the induction period by *H. pluvialis*.

Medium	**Astaxanthin (Day 0) (mg L^{-1})**	**Astaxanthin (Day 18) (mg L^{-1})**	**Astaxanthin Productivity (mg L^{-1} d^{-1})**
BBM	0.41 ± 0.05	32.21 ± 1.58	1.78 ± 0.08 [b]
1% MW	0.40 ± 0.08	16.64 ± 0.57	0.92 ± 0.03 [c]
1.5% MW	0.48 ± 0.05	39.72 ± 1.69	2.21 ± 0.09 [a]

Means that do not share a letter are significantly different.

Kang et al. (2006) studied the biomass and astaxanthin productivity from *H. pluvialis* in a primary-treated sewage (PTS) and a primary-treated piggery wastewater (PPW) with serial dilutions. They observed higher biomass in four-fold diluted PPW with successful removals of N and P after an 8-day cultivation period; and the resulting

astaxanthin concentrations reached to 83.9 and 42.3 mg L^{-1} in PTS and PPW cultures, respectively. The major N sources from both of these wastewaters were nitrate, which is different from MW. Similarly, N assimilation finished within 5 days of cultivation in the fourfold diluted PPW, and within 7 days in the eightfold diluted PPW. Besides, the TP concentrations in diluted PTS and PPW were relatively lower than those in the MW used in this study. Consequently, TP was depleted after only 5 days and 8 days of cultivation periods, respectively. In the study of Kang et al. (2006), it should be noted that the light intensity used for induction process was 200 μmol photon m^{-1} s^{-2}, which was the same light intensity used in our study. However, since *H. pluvialis* can be induced under both N-deprived and P-deprived conditions, higher astaxanthin production were achieved in all wastewater cultures in their study as compared to ours. In another research, Haque et al. (2016) studied the astaxanthin production from *H. pluvialis* by using wastewater collected from local bioethanol plants. They found that the optimum dilution factor for microalgae growth was 60 times with a 10.5% improvement on astaxanthin production over the standard mediums. These studies demonstrated that culturing *H. pluvialis* in wastewaters with proper dilutions can be a potential alternative to conventional mediums, for not only using wastewater sources to replace chemical nutrient inputs, but also reaching the goal of massive production of a commercial by-product derived from microalgae. So far, the wastewaters tested for *H. pluvialis* culturing are limited. Future work on culture optimization with respect to dilution factors, cultivation parameters, and PBR designs are needed to be explored to

eventually develop a large-scale astaxanthin production system.

3.3.3.2 Astaxanthin production in DW cultures

As mentioned in the previous section, there still existed some amounts of nutrients, which were available in MW cultures after the stationary stage. A parallel design that used DW for culture induction was conducted to determine whether continuous culture could achieve a significantly higher astaxanthin productivity under stress conditions. Other parameters were sustained as in the earlier experiment. It was apparent that astaxanthin could not successfully be synthesized in the DW cultures. Cell death was observed after only a 6-day induction period, during which green vegetative cells transformed into white color, and cytoclasis was observed under microscope. The study performed on *C. vulgaris* by Ji (2017) reported that using DW as cultivation mediums did support microalgae growth, and reached a 677% biomass increase. Imamoglu et al. (2009) utilized distilled water as a culture medium during the induction process with CO_2 injection under 546 μmol photon m^{-2} s^{-1}. The resultant astaxanthin concentration was non-significant in the N-deprived culture medium. However, it fails to apply in the induction process for *H. pluvialis* in this study without additional aeration. Therefore, the group comparison between MW and DW cultures was inconclusive due to high cell mortality in DW cultures.

3.4 Conclusion

In this section of the study, the best MW concentration, volume ratio, and initial density on green vegetative cultivation stage were determined. In particular, the

suitable MW concentrations were 1 and 1.5%, and the highest OD value was performed at 1.5%. The optimal volume ratio of AC and MW in a total volume of 1 L PBR column was 1:4 (200 ml AC versus 800 ml MW). The optimum ICD as represented in OD values were obtained at 0.13A (λ=680). The statistical analysis performed over biomass accumulation in the cultivation stage showed that the highest biomass was obtained in 1.5% MW among the three cultures, yielding a biomass production of 906.3 ± 34.0 mg L^{-1}. During the 6-day cultivation period, the mean removal percent of TN in BBM and two MW cultures were 75.1% and almost 100%, respectively, while the TP removal percent ranged from 17.5 to 20.7%. In the induction stage, the highest astaxanthin was also obtained in the 1.5% MW cultures at 39.72 ± 1.69 mg L^{-1}, which was higher than the one observed in BBM culture.

CHAPTER IV

EFFECTS OF ACETATE AND NACL CONCENTRATIONS ON ASTAXANTHIN PRODUCTION BY *H. PLUVIALIS*

4.1 Introduction

The influence of stress factors on astaxanthin production by *H. pluvialis* under various culture mediums were studied in previous studies. In Chapter 3, a photoautotrophic induction model was introduced and employed, where N-deficiency and high light intensity were executed for astaxanthin synthesis using MW cultures. In this part of the study, two more stress factors, carbon content and salinity were involved. A series of sodium acetate (NaAc) and sodium chloride (NaCl) concentrations were applied for mixotrophic induction process. The specific objective in this section was to determine the effects of acetate and NaCl on astaxanthin production by *H. pluvialis* under the 1.5% MW cultures.

4.2 Methodology

H. pluvialis cultures were grown in 1.5 % MW for 6-day cultivation periods in PBRs under continuous light. The other parameters, including pH, light intensity, and aeration levels were sustained at the same levels as before. Then, a 40 ml sample containg the green vegetative cells were transferred to a 12-day induction period for astaxanthin production. The employed concentrations of acetate were 20, 40, 60, and 80 Mm; while the NaCl concentrations were 0.4, 0.6, 0.8 and 1% (w/v). Continuous high light intensity at 200 μmol photon m^{-2} s^{-1} was provided by LEDs without aeration. One-way ANOVA and Tukey's comparison method was conducted by using Minitab

18 for statistical analyses. The resultant astaxanthin concentrations and astaxanthin productivities were determined using the equations described in Chapter 3. Three replications were run in this part of the experiments.

4.3 Results and discussion

4.3.1 Effects of acetate

The resultant astaxanthin productions of *H. pluvialis* under different acetate concentrations and untreated control cultures in the second induction stage after a 12-day growth period are shown in Figure 4.1. Compared with the control, astaxanthin content was stimulated significantly by the increased acetate concentration. The astaxanthin concentrations reached the maximum level of 52.80 ± 2.36 mg L^{-1} with an astaxanthin productivity of 4.40 ± 0.09 mg L^{-1} d^{-1} as the acetate concentration increased to 40 mM in growth mediums. However, reduced levels of astaxanthin production were observed when the acetate concentration exceeded 40 mM.

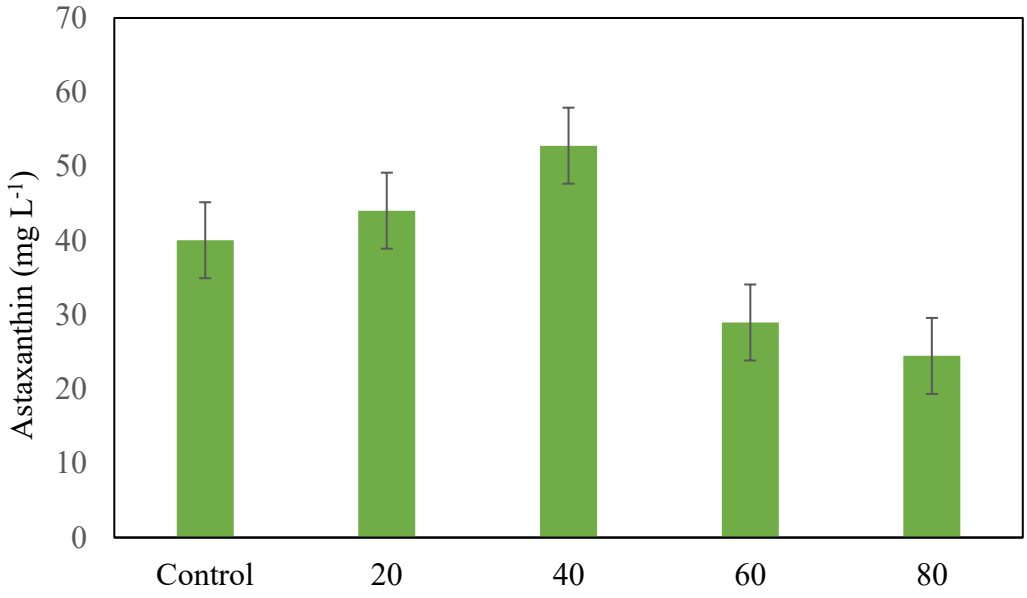


Figure 4.1 Effects of different acetate concentrations on astaxanthin production by *H. pluvialis* after 12-day induction period in MW mediums.

Table 4.1 demonstrates the Tukey's comparison test results among groups, indicating that the astaxanthin concentration obtained at 40 mM was significantly higher than those obtained at the other acetate concentrations ($p<0.05$). There were no significant differences in the resultant astaxanthin concentrations between the 20 mM and untreated acetated added control cultures, and also between the 60 and 80 mM cultures ($p>0.05$). A dramatic decline in astaxanthin synthesis occurred as acetate was continually added to cultures. Consequently, the lowest astaxanthin concentration was observed at the 80 mM acetate concentration, which accumulated approximately half the astaxanthin as compared to the control. This implies that a high acetate concentration hinders the biosynthesis of astaxanthin by *H. pluvialis*. In this experiment, the findings suggest an acetate concentration ranging from 20 to 40 mM. Kobayashi et al. (1997) reported that a higher C/N ratio was achieved in 45 mM

acetate cultures, resulting in a rapid cyst formation in about 4 days. In another study, Su et al. (2014) indicated that a high astaxanthin concentration was obtained in 45 mM acetate added cultures, which was provided with high light intensity after a 2-day induction duration. To date, not enough the research has been performed on the effects of acetate on *H. pluvialis* astaxanthin production. *H. pluvialis* culturing in most studies have used standard growth medium rather than wastewater cultures or other. However, this study is able to provide an appropriate acetate concentration that is close to the commonly used levels in previous studies.

Table 4.1. The means of astaxanthin concentrations (mg L^{-1}) and astaxanthin productivities (mg L^{-1} d^{-1}) under different acetate concentrations and untreated control.

Acetate (mM)	**Astaxanthin concentration (mg L^{-1})**	**Astaxanthin productivity (mg L^{-1} d^{-1})**
20	44.03 ± 2.79^{b}	3.67 ± 0.06^{b}
40	52.80 ± 2.36^{a}	4.40 ± 0.09^{a}
60	28.97 ± 1.82^{c}	2.41 ± 0.06^{c}
80	24.47 ± 2.29^{c}	2.04 ± 0.19^{c}
Control	40.06 ± 3.27^{b}	3.34 ± 0.28^{b}

Means that do not share a letter are significantly different.

4.3.2 Effects of salinity

In order to investigate the effect of salinity on astaxanthin production of *Haematococcus* cultures, NaCl was added to N-deprived 1.5% MW cultures from 0.4% to 1.0% (w/v) and compared with an untreated control. As shown in Figure 4.2,

astaxanthin concentration increased dramatically from 41.77 ± 3.70 mg L^{-1} to 57.45 ± 2.63 mg L^{-1} as NaCl concentration increased from 0.4 to 0.6%. However, astaxanthin production in the 0.8% NaCl cultures dropped down to 20.30 ± 1.17 mg L^{-1}, and 1% NaCl cultures had much lower astaxanthin concentration than the control.

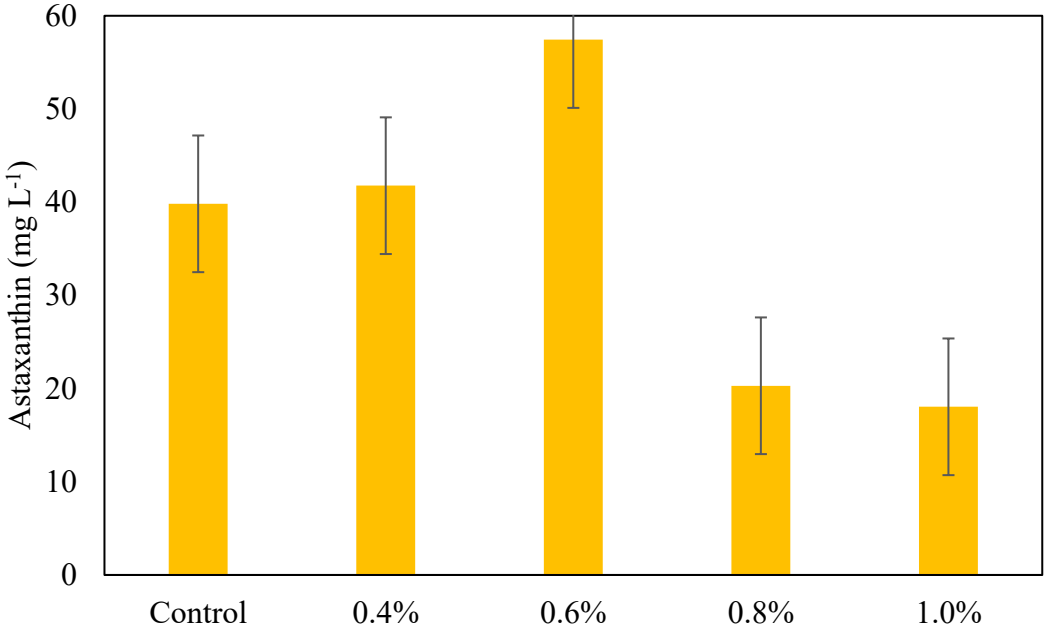


Figure 4.2. Effects of different NaCl concentrations on astaxanthin production by *H. pluvialis* after 12-day induction period in MW mediums.

Table 4.2 indicates that cultures with 0.4% NaCl showed non-significant astaxanthin production in comparison to the control ($p>0.05$). NaCl concentrations at 0.4, 0.6 and 0.8% did not share the same letter with each other in the Tukey's comparison test, indicating that they were significantly different from each other ($p<0.05$). As a result, the maximum astaxanthin production was harvested at the 0.6% NaCl added cultures with an astaxanthin productivity of 4.79 ± 0.22 mg L^{-1} d^{-1}, which was approximately 1.4 times higher than control. The trend in the astaxanthin contents

reflected that increased NaCl concentration levels in culture had an adverse effect on astaxanthin synthesis. In the range of NaCl concentration, a notable enhancement in astaxanthin production by *H. pluvialis* in MW cultures was realized. Lower astaxanthin production in 1% NaCl added cultures was in accordance with the observation of Sarada et al. (2002). Another study performed by Kobayashi et al. (1997) reported an optimal NaCl concentration of 0.1% in *Haematococcus* standard cultures with the addition of other stress factors. Furthermore, a successful astaxanthin production was achieved by the addition of NaCl from 0.25 to 0.5% to the mediums (Shah et al., 2016). In conclusion, high salinity concentration inhibits the encystment formation and therefore astaxanthin synthesis due to the death of many cells.

Table 4.2. The means of astaxanthin concentrations (mg L^{-1}) and astaxanthin productivities (mg L^{-1} d^{-1}) under different NaCl concentration and untreated control.

NaCl (%, w/v)	**Astaxanthin concentration (mg L^{-1})**	**Astaxanthin productivity (mg L^{-1} d^{-1})**
0.4%	41.77 ± 3.70^{b}	3.48 ± 0.31^{b}
0.6%	57.45 ± 2.63^{a}	4.79 ± 0.22^{a}
0.8%	20.30 ± 1.17^{c}	1.69 ± 0.10^{c}
1.0%	18.04 ± 3.07^{c}	1.50 ± 0.26^{c}
Control	39.82 ± 2.58^{b}	3.32 ± 0.21^{b}

Means that do not share a letter are significantly different.

4.4 Conclusion

The efforts in this part of the study increased the resultant astaxanthin production by *H. pluvialis* under MW mediums by adding a proper concentration of acetate or NaCl. Compared to the untreated control, the results indicated that the maximum astaxanthin productions were observed at 40 mM and 0.6% NaCl, with astaxanthin productions of 52.80 ± 2.36 mg L^{-1} and 57.45 ± 2.63 mg $L^{-}1$, respectively. These findings facilitate the further efforts on the selection of optimal combined conditions of acetate and NaCl by using response surface methodology.

CHAPTER V

OPTIMIZATION STUDY OF ASTAXANTHIN PRODUCTION *BY H. PLUVIALIS* IN MINKERY WASTEWATER CULTURES USING RESPONSE SURFACE METHODOLOGY

5.1 Introduction

The effects of individual variables on the astaxanthin production of *H. pluvialis* have been quantitatively studied in the previous Chapters. In order to optimize the astaxanthin production and make it possible for large-scale commercial astaxanthin production, it is important to quantify the effects of the combined stress conditions that maximize the astaxanthin synthesis by *H. pluvialis*. Response surface methodology (RSM) was used for this optimization study, which is a collection of mathematical and statistical techniques to model the relationship with a response variable and quantitative factors. It can also make statistical predictions by determining the levels of factors that optimize the response variable, based on the fit of a polynomial equation of the experimental data (Montgomery, 2017). This experimental design has the advantages of significantly reducing the number of experiments, time and cost of experimentation, and simultaneously realizing optimization of the variables (Bezerra et al., 2008). In this study, two independent variables, acetate and NaCl concentrations, were investigated. The screening levels of each variable were determined according to the results obtained in Chapter 4. In this Chapter, the effects of concentrations of sodium acetate and NaCl during the induction period of astaxanthin production were evaluated by RSM, and the maximum yields were determined under the optimal

combined concentrations.

5.2 Methodology

5.2.1 Cultivation and induction conditions

H. pluvialis cultures were grown in 1.5 % MW cultures for biomass production in PBRs under continuous light. Other parameters, such as pH, light intensity, and aeration were sustained at the same levels as before. After a 6-day cultivation period, the green vegetative cells were transferred to a 12-day induction process for astaxanthin synthesis. In the induction process, light intensity was provided at 200 μmol photon m^{-2} s^{-1}.

5.2.2 Experimental design and statistical analysis

Central composite design (CCD), a second-order design in RSM, was used to develop mathematical models, which were applied to study the effects of individual variables, their interaction effects on the response, and to determine the optimum levels in each variable for the process. Two independent variables selected for the astaxanthin production were acetate concentration (X_1) and NaCl concentration (X_2). The levels of each variable were determined by the preliminary experiments in Chapter 3 and the relevant studies documented in previous literature. According to this design, 13 experimental combinations were performed, including 5 center points, 4 axial points and 4 fact points. Five coded levels of each variable (-1.414, -1, 0, 1, 1.414) were presented. The alpha value was calculated by using Equation (14), which depends on the number of variables (n) in the factorial design (Table 5.1). The experimental design

in the coded and actual levels is shown in Table 5.2.

$$\alpha = (2^n)^{1/4} \quad (14)$$

The response (Y) was the resultant astaxanthin concentration after the 12-day induction process. A second-order polynomial equation provided in Equation (2) with linear, quadratic and interaction effects was developed to test the relationship and interrelationships of the variables.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<} \sum_{j} \beta_{ij} x_i x_i \quad (15)$$

where Y was the response variable, x_i and x_j were the independent variables, β_0 was the constant intercept coefficient, β_i represented the linear coefficient, β_{ii} represented the quadratic coefficient, and β_{ij} represented the interaction coefficient. The experimental results were analyzed by employing the analysis of variance (ANOVA) with a 95% confidence level using Minitab 18. In order to confirm the optimum combination of the two independent variables, a verification of the optimum condition suggested by the model was tested in the lab afterwards.

Table 5.1. Independent variables and levels used for the CCD.

Factors	**Coded variables**	**Levels**				
		-1.41421 (-α)	**-1**	**0**	**1**	**1.41421 (+α)**
Acetate (mM)	X_1	26	30	40	50	54
NaCl (%)	X_2	0.46	0.50	0.60	0.70	0.74

5.3 Results and discussion

5.3.1 Regression model and ANOVA analysis

5.3.1.1Model fitting

The resulting *Haematococcus*-derived astaxanthin concentrations are affected by a number of variables, such as high light intensity, high C/N ratio, elevated salinity, and their combinations. A 5-level-2-factor CCD design was developed, and the completed experimental designs (13 runs) and corresponding response data are listed in Table 5. 2. The resultant astaxanthin concentrations varied between 19.19 mg L^{-1} and 68.54 mg L^{-1}. The regression model for the yield of astaxanthin in the actual levels of variables developed the second order-polynomial equation as shown below:

$$Y = 65.26 - 3.73\ X_1 - 4.30\ X_2 - 15.39\ X_1^2 - 19.19\ X_2^2 + 12.14\ X_1X_2 \quad (16)$$

where Y was the resultant astaxanthin concentration (mg L^{-1}), X_1 was the acetate concentration (mM), and X_2 was the NaCl concentration (%, w/v).

Table 5.2 The central composite design of two independent variables, and experimental values for the astaxanthin production by *H. pluvialis*.

Run	Coded values		Real values		Astaxanthin concentration (mg L^{-1})	
	X_1	X_2	Acetate (mM)	NaCl (%)	Actual	Predicted
1	-1	-1	30	0.50	46.68	50.86
2	1	-1	50	0.50	19.19	19.11
3	-1	1	30	0.70	18.11	17.98
4	1	1	50	0.70	39.18	34.79
5	-1.41421	0	26	0.60	42.68	39.76
6	1.414214	0	54	0.60	26.10	29.21
7	0	-1.41421	40	0.46	35.92	32.97

Run	Coded values		Real values		Astaxanthin concentration (mg L^{-1})	
	X_1	X_2	Acetate (mM)	NaCl (%)	Actual	Predicted
8	0	1.414214	40	0.74	17.65	20.81
9	0	0	40	0.60	62.61	65.26
10	0	0	40	0.60	64.17	65.26
11	0	0	40	0.60	64.67	65.26
12	0	0	40	0.60	66.32	65.26
13	0	0	40	0.60	68.54	65.26

Table 5.3 lists the fit summary of the regression model, which indicates how well the regression model fitted the response and the suitability level of the polynomial equation to predict the yields of astaxanthin. A very good correlation was obtained in this model ($R^2 = 0.9799$, $p<0.05$), indicating a strong relationship between the response and the variables. The lack of fit of the astaxanthin yield was not significant ($0.082>0.05$), which confirms the validity of the model. Figure 5.1 compares the actual experimental data and the predicted values provided by the quadratic model. The linear regression with an R^2 value of 0.9799 demonstrated that the fitted model sufficiently covered the experimental data. The summary of ANOVA implies that the regression model is well fitted and is highly reliable.

Table 5.3. The fit summary of the regression model.

Model	F-value	P-value	Lack of fit	R-square	R-square (adj)
Astaxanthin yield	68.27	0.000	0.082	0. 9799	0. 9656

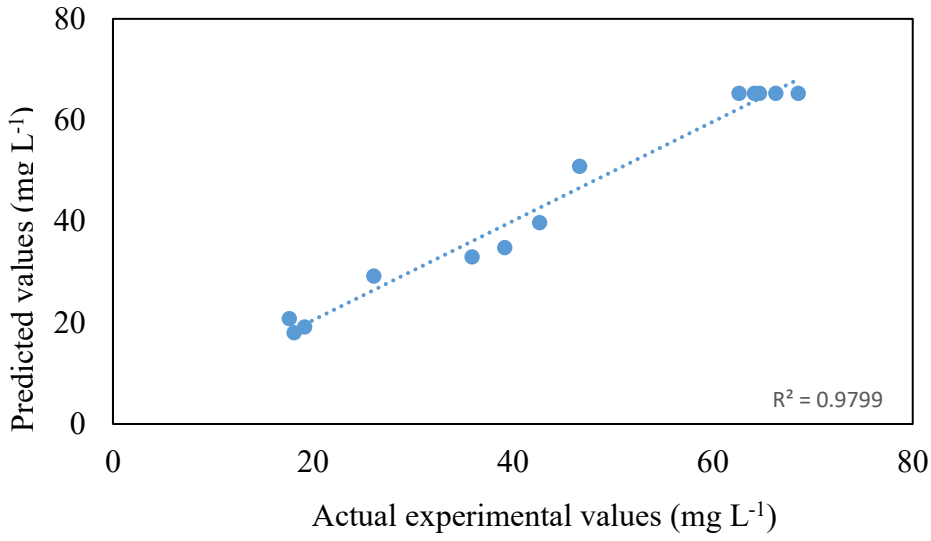


Figure 5.1. Actual experimental values versus predicted values for astaxanthin production.

5.3.1.2 Effect of linear and quadratic models

The yield of astaxanthin based on the effect of the two stress factors on the response function is summarized by the ANOVA analysis in the coded level of variables in Table 5.4. The statistical results showed that the total linear effects, total quadratic effects, and total interaction effect were significant with p-values less than 0.05. In the linear model, all the individual variables had positive effects on the yield of astaxanthin. However, the linear effect of X_2 (NaCl) is more significant than that of X_1 (acetate) with a higher F-value and a lower P-value, suggesting a more influence on the response. In the quadratic model, both acetate and NaCl concentrations showed significant effects on the yield of astaxanthin ($p<0.05$).

Table 5.4. The analysis of variance (ANOVA) in coded level of variables for the regression model.

Source of variation	Coefficient of regression equation	F-values	P-values
Constant	65.26	-	-
Linear	-	9.66	0.010
X_1	-3.73	8.31	0.024
X_2	-4.30	11.02	0.010
Quadratic	-	139.07	0.000
X_1^2	-15.39	122.63	0.000
X_2^2	-19.19	190.67	0.000
Interaction	-	43.89	0.000
X_1 X_2	12.14	43.89	0.000

The main effects plots shown in Figure 5.2 indicate how the levels of each variable affected the response. It shows that the resultant astaxanthin concentration significantly increased to 54.27 mg L^{-1} as acetate concentration increased from 26 mM to 40 mM, which was likely due to a relatively higher C:N ratio achieved under a lower range of acetate addition, thereby accelerating the biosynthesis of astaxanthin. However, further increases of acetate concentrations significantly reduced the yield of astaxanthin to 26.10 mg L^{-1} at 54 mM acetate concentration, suggesting an inhibition effect of high acetate concentrations on astaxanthin production. This result is in agreement with the conclusion of the previous Chapter and within the optimal range suggested by the

literature. Compared to the acetate, the effect of NaCl concentration on astaxanthin was significantly higher. Similarly, the resultant astaxanthin concentration increased from 35.92 mg L^{-1} to 56.44 mg L^{-1} when the NaCl concentration elevated from 0.46% to 0.60%, and then, dramatically decreased to 17.65 mg L^{-1} with a further increase in salinity level. Cordero et al. (1996) reported that cell death was observed at 0.8% NaCl concentration, while in this study, the negative effect on astaxanthin production occurred when NaCl concentration was higher than 0.6%.

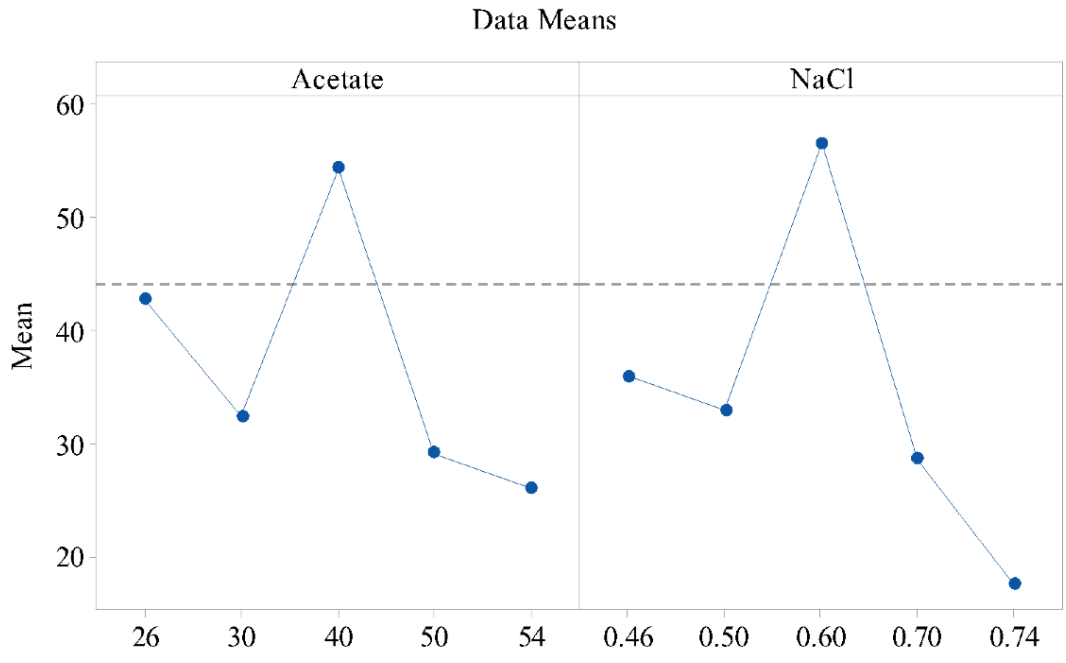
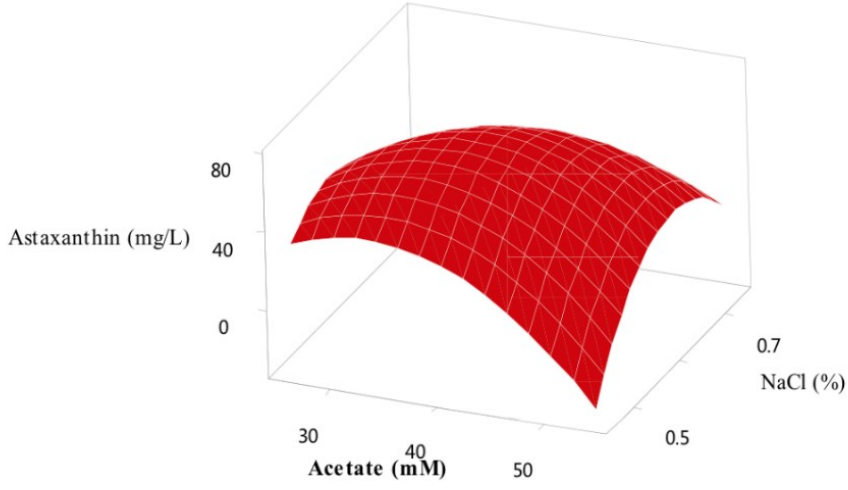


Figure 5.2. Main effect plots of two independent variables on response (mg L^{-1}).

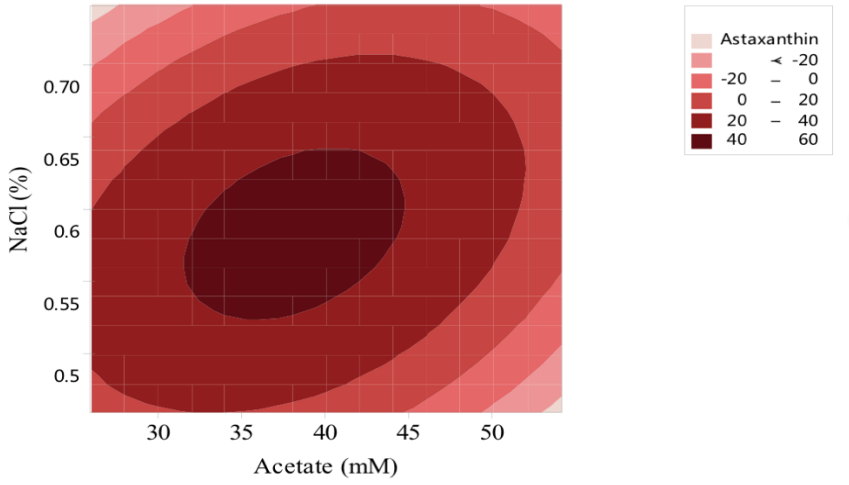
5.3.1.3 The interaction effect on the yield of astaxanthin

The ANOVA indicated that the interaction effect was significant with a p-value less than 0.05. As shown in Figure 5.3, the interaction effect of the two variables was

performed in the response surface and contour plots. Figure 5.3 (a) shows that when the salinity level increased with an increase in acetate concentration, the yield of astaxanthin production increased significantly at first and then decreased gradually after the optimum condition was reached. Figure 5.3 (b) demonstrates an ellipsoid counter plot, which indicated that the maximum yield of astaxanthin can be achieved when NaCl concentrations range from 0.525 to 0.64% with an increase in acetate concentration from 32 to 45 mM.



(a)



(b)

Figure 5.3 Surface (a) and contour (b) plots of astaxanthin contents.

5.3.2 Process optimization

The optimal conditions of acetate and NaCl concentrations for maximizing the resultant astaxanthin yield was determined by the optimizer (Minitab 18) according to the mathematical model. The optimal conditions provided by the model were 38.14 mM in acetate concentration and 0.58% in NaCl concentration. The theoretical value of the astaxanthin concentration was predicted at 65.98 mg L^{-1} with the 95% confidence interval, and ranged from 62.14 to 69.82 mg L^{-1} (Table 5.5). Three verification experiments were performed based on the suggested optimal conditions. As a result, a 67.95 ± 3.93 mg L^{-1} of astaxanthin concentration was achieved, which is slightly higher than the predicted value but still within the range of the 95% confidence interval. This implies that the developed model for the astaxanthin production was adequate and reliable.

Table 5.5 Actual values versus predicted values from optimization process.

	Acetate (mM)	NaCl (%)
Coded level	-0.185705	-0.157135
Real value	38.14	0.58
Predicted value	65.98 ± 1.62	
Actual value	67.95 ± 3.93	

5.3.3. RSM used for astaxanthin production by *Haematococcus* in other cultures

The response surface methodology has been widely used to analyze the biomass and astaxanthin production by *H. pluvialis*. Sarada et al. (2002) studied the effect of sodium acetate, sodium chloride and the culture age of the *H. pluvialis* on biomass, astaxanthin content and astaxanthin production under a one-stage cultivation system using RSM. As a result, the optimal conditions of acetate and NaCl concentrations were recommended between 31 to 50 mM, and 0.55 to 0.63% (w/v), respectively, where the resultant astaxanthin was found over 13 mg L^{-1}. The astaxanthin yield in our two-stage system was much higher than the yield obtained by Sarada et al. (2002), which was mostly due to the higher biomass yield in the initial point of the induction process. However, the optimal conditions of the two stress factors in this study fall in the recommended range of the former study. A number of previous studies showed that the optimal levels of acetate and salinity widely differ. For instance, Cordero et al. (1996) induced the *Haematococcus* cells with salinity levels varying from 0.1 to 0.4% (w/v) and the sodium acetate from 0.025 to 0.1 g L^{-1}. The maximum astaxanthin

production was observed in 0.2% of NaCl and 0.05 g L^{-1} of acetate. The optimal levels of NaCl in other studies were found to be as high as 0.8 and 1% (w/v) (Harker et al., 1996; Borowitzka et al., 1991). The difference in the optimal conditions among the studies is highly influenced by other environmental conditions, such as culture mediums, temperature, light intensity and regime. The effect of acetate in astaxanthin production may also be increased with a proper addition of Fe^{2+} in the cultures (Su et al., 2014). Many other factors have also been extensively studied using RSM. Fabregas et al. (2000) studied the optimization process of the culture medium in the growth of *H. pluvialis* and Thana et al. (2008) used RSM to study the optimal conditions of supercritical carbon dioxide extraction method for *Haematococcus*-derived astaxanthin. The quality of the response surface approximation is highly dependent on the selection of data points (i.e. range of levels), therefore, the screening of these factors is important to improve the veracity of the predicted response.

5.4 Conclusion

The optimization process of astaxanthin production from *H. pluvialis* in MW cultures was evaluated by RSM. The optimum acetate and NaCl concentrations were determined to be 38 mM and 0.58%, respectively. The yield of astaxanthin reached 67.95 ± 3.93 mg L^{-1}, which was approximately 1.7 times higher than the astaxanthin production under the photoautotrophic induction process presented in Chapter 2.

CHAPTER VI

CONCLUSION

6.1 Experimental conclusion

H. pluvialis grew well in the appropriately diluted MW mediums and a higher biomass production was realized as compared with conventional culture medium under optimal growth condition. The findings support the claim that integration of wastewater into microalgae cultivation has the advantages of reducing cultivation costs, natural resources inputs, and simultaneously obtaining high-value bioproducts. Minkery wastewater contains extremely high level of ammonia, which is a different N source from BBM. Results showed that *H. pluvialis* achieved maximum biomass at 1.5% MW cultures, yielding 906.03 ± 34.0 mg L^{-1}, with a successful removal of total nitrogen and total phosphorus in a 6-day cultivation. The optimal initial cell density and volume ratio between microalgae and MW were also determined to have great help on maximizing the biomass yield.

Our study confirmed that astaxanthin can be successful produced in MW mediums when *H. pluvialis* was exposed to stress conditions, including nutrient depletion, increased light intensities, enhanced salinity, and combined stress conditions. In a photoautotrophic induction process, nutrient-deprived vegetative cells were exposed to a high light intensity (200 μmol photons m^{-2} s^{-1}) for astaxanthin production. The resultant astaxanthin production achieved 39.72±1.69 mg L^{-1} in 1.5% MW mediums. While in a mixotrophic induction process, the addition of acetate and NaCl to the

induction in MW cultures significantly improved the resultant astaxanthin production. The highest resultant astaxanthin was obtained at 40 mM acetate, and 0.6% (w/v) NaCl, respectively. The final optimization step was realized by using response surface methodology, and the results indicated that the optimal combination of acetate and NaCl for astaxanthin production was determined to be at 38.5 mM and 0.58%, respectively. The resultant optimal astaxanthin concentration in MW mediums reached 67.95 ± 3.93 mg L^{-1} after a 12-day induction period.

6.2 Future research

Microalgae–derived bioproducts requires a large amount of water and chemical nutrients for cell growth, which has a conflict with water scarcity and water pollution issues in many regions. This study provides a novel approach that utilizes minkery wastewater to grow microalgae. It should be noted that the raw wastewaters have been diluted with 98.5% distilled water to allow microalgae to survive in the cultures. In order to achieve a water efficient production, recycling of liquid waste after extraction is suggested in the future works or in a commercial application.

Since *H. pluvialis* has complex life-cycle, it is important to maximize the biomass yield in the green vegetative stage in the first step, thereby improving astaxanthin production in the red cystment stage. Therefore, various cultivation parameters, such as culture mediums, PBRs, environmental factors, and stress factors are needed to be explored to improve the biomass and astaxanthin productivities, while lowering the consumption of water and energy and reducing the risks of contaminations in the

resultant products. Other wastewater types are encouraged to be investigated in *Haematococcus* cultures. Moreover, the relevant optimal production routes and advances in technologies are needed. The improvements in integration processes, harvesting and extraction technology will contribute to accelerate the speed of the *Haematococcus*-derived astaxanthin from laboratory scale to commercial scale. Further study in these areas can have a profound influence on the market of natural astaxanthin from *H. pluvialis*.

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