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Investigation of lutein production and recovery from Chlorella vulgaris using phototrophic cultivation

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Abstract

Microalgae have been recognized as a natural reservoir of the valuable commercial carotenoid lutein due to its high lutein content and fast growth cycle. However, the production of lutein from microalgal sources is not yet sufficiently cost-effective to compete with traditional marigold flower-based processing (Park et al., 2015). This thesis aims to investigate the factors affecting lutein production and recovery from microalgae using a phototrophic cultivation mode.

The closed photobioreactors can offer controlled conditions for faster microalgae growth. A coiled tubular tree photobioreactor (CTPBR) design was first investigated for cultivating the cold tolerant microalgae, *Chlorella vulgaris* UTEX 265, under various conditions for lutein production. The response surface method by central composite design was used to measure the interaction of light irradiance, photoperiod cycle and temperature on microalgae growth and lutein production. The results demonstrated that the CTPBR was an effective reactor configuration, and 14 h day-light, 120 µmol photons m^{-2} s^{-1} and 10^{o}C was the optimal condition for lutein production in experimental ranges.

The lutein extraction from wet microalgae was next investigated. The lutein production was monitored throughout the micro-algal growth phase, and the biomass drying method and the cell disruption method were investigated. The simultaneous lipid and lutein extraction was also studied. The performance of solvent polarity on extraction was compared quantitatively using Nile Red as a solvatochromic polarity probe. An 80% (v/v) ethanol in hexane was recognized as the optimal solvent for lutein and lipid co-extraction, which contributed to a 13.03 mg/g lutein and 7% (w/w) lipid yield.

A single-step simultaneous extraction, saponification and primary purification process for free lutein production from wet microalgae biomass was then investigated using binary solvent mixtures. The extraction kinetics were modeled using Fick's second law of diffusion. The optimized conditions for the apparent mass transfer rate and yield was found to be pre-treatment with ultrasonication at 0.5s working cycle per second, react 0.5 hour in 160 mL final solvent volume of 1:3 ether/ethanol (v/v) with 2 mL 10% (w/v) ethanolic KOH.

Overall, an effective method was developed for high yield lutein production from wet microalgae.

Keywords

Chlorella vulgaris, solvent extraction, low temperature, lutein, kinetics, microalgae, photobioreactor, saponification

Co-Authorship Statement

Contents of chapter 2 to 5 were published or submitted to refereed journals and were co-authored by Dr. Amarjeet Bassi, who provided editorial and technical advice.

Xinyi Li provided experimental assistance to part of the experiments performed in chapter 4 and revised the drafts of manuscript, so is listed as a co-author.

Yuruihan Wang carried out part of the extraction experiments, and is listed as a co-author in chapter 5

Dedication

To my mother Min Su and father Yan Gong for their unconditional and endless love.

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Nomenclature

Abbreviation	Description	Units
AARD	Average absolute relative deviation	
adj-R^2	Adjusted regression coefficients	
ANOVA	Analysis of variance	
BASF	Badische Anilin & Soda-Fabrik	
BBM	Bold's Basal Medium	
B & D	Bligh & Dyer	
BHT	Hydroxytoluene	
BKT	β-carotene ketolase	
C_a	Concentration of alkali in solvent	g KOH/L solvent
CCD	Central Composite Design	
Chl	Chlorophyll	
CPBR	Closed photobioreactors	
CTPBR	Coiled tubular tree photobioreactor	
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/associated protein-9 nuclease	
D	Diffusion coefficients	cm^2/s
DHA	Docosahexaenoic acid	
DMAPP	Dimethylallyl diphosphate	
DNA	Deoxyribonucleic acid	
EPA	Eicosapentaenoic acid	
E_N^T	Relative polarity index	
FAME	Fatty acid methyl ester	
f_1	Fraction of the solute extracted in fast stage	
f_2	Fraction of the solute extracted in slow stage	

HPH	High pressure homogenizer	
HPLC	High-performance liquid chromatography	
IPP	Isopentenyl pyrophosphate	
LHC	Light Harvesting Complexes	
MAE	Microwave-assisted extraction	
MEP	Methylerythritol 4-phosphate	
MN	Meganuclease	
MVA	Mevalonic acid	
NPQ	Non-photosynthetic quenching	
PDS	Phytoene desaturase	
PSY	Phytoene synthase	
$\mathbf{R_a}$	Ratio of alkali to dried algae biomass	L 10%(w/v)KOH/kg
RNAi	RNA interference	
RSM	Response surface method	
$\mathbf{R^2}$	Regression coefficients	
SFE	Supercritical fluid extraction	
STDEV	Standard deviation	
TALEN	Transcription activator-like effector nuclease	
TE	Transcriptional engineering	
TF	Transcription factors	
UAE	Ultrasound-assisted extraction	
UTEX	University of Texas	
Greek letters		
$\boldsymbol{\lambda_{max}}$	Maximum absorbance wavelength	nm
μ	Specific growth rate	d^{-1}

Chapter 1

1 Introduction

Lutein is a commercial carotenoid that can be marketed for the nutraceutical market as an important dietary source for human health. Microalgae represent a more attractive alternative for lutein production compared to the traditional source, i.e. marigold flowers (Cordero et al., 2010). Various methods are being employed currently for the extraction of lutein from microalgae (Park et al., 2015); however, a more effective process still needs to be developed for cost-effective large scale production.

While microalgae are being investigated for many applications, optimization of their growth rates and product yields are vital factors for the industrial application of microalgae for lutein and biodiesel production (Briassoulis et al., 2010). Currently, closed PBRs or open ponds are two common alternatives for large scale microalgae production (Briassoulis et al., 2010). Closed photobioreactors (CPBR) have higher efficiency than open systems (Morita et al., 2002); however, to counterbalance the higher capital and operational cost of CPBRs, either higher efficiency photobioreactors need to be developed at controllable cost or higher value-added product like lutein need to be produced.

This study proposes that if a successful process for lutein production is desired; both the microalgae cultivation and the lutein extraction process should be considered. The cold weather of the Canadian context should also be taken into consideration for microalgae cultivation. An interesting question which can be addressed is the understanding of lutein production mechanisms, and the optimization of environmental variables for lutein production at lower temperature conditions (0 - 15^{o}C) common in the Canadian context. Further, due to the sensitivity of lutein, a suitable extraction method with minimum lutein degradation needs to be developed. Thus, both an effective lutein extraction method and a photobioreactor adaptable to cold weather with high lutein productivity are highly desired.

In this study, a holistic strategy to investigate the lutein recovery from microalgae is applied. The culture conditions for microalgal growth were manipulated to understand the effect of process variables on lutein production from microalgae. A new extraction process

which combines several steps was investigated. To better understand the course of this research, the objectives and the sequence of experiments conducted are summarized in the next sections.

1.1 Structure of the thesis

The research is divided into 3 main stages as shown in fig. **1-1**: the first step was to optimize the lutein production using a novel photobioreactor and various cultivation conditions (Chapter 3). The second step was to optimize the lutein extraction method and get valuable by-products (Chapter 4); and finally, a simplified extraction procedure was developed and kinetics modeling was carried out (Chapter 5).

In the first stage, a coiled tubular tree photobioreactor (CTPBR) was utilized at different irradiance, temperature and CO_2 conditions. The determination of values of the variables is based on the literature data and operation limitations. A response surface method (RSM) was applied to the low temperature condition microalgae growth experiment design.

Once the model was obtained, the operational points of irradiance, temperature and photoperiod that give the highest lutein productivity were applied to the second stage experiments, mainly for the accumulation of microalgae biomass. The extraction method was studied based on those conditions. Different pre-treatment methods (freeze dried cells, frozen cells and untreated wet cells) and disruption methods (ultrasound, bead beater, solvent, etc.) were evaluated with different solvent type, solvent polarity, treatment times and solvent/solid ratio. The simultaneous lutein and lipid extraction from microalgae is also attempted at different solvent polarities.

In the third stage, the single-step binary solvent extraction of free lutein was investigated. The solvent type, usage, and extraction times were the focus of this study. The kinetics of extraction were modeled by Fick's law of diffusion. The fitted models were established to find the optimal conditions that allow the highest lutein yield.

In addition to the Chapter 3-5 above mentioned, Chapter 1 is an introduction to the thesis structure and research objectives, Chapter 2 is the literature review offering the background

knowledge for carotenoid production from microalgae, while Chapter 6 concludes the thesis and provides some future recommendations to further expand this area.

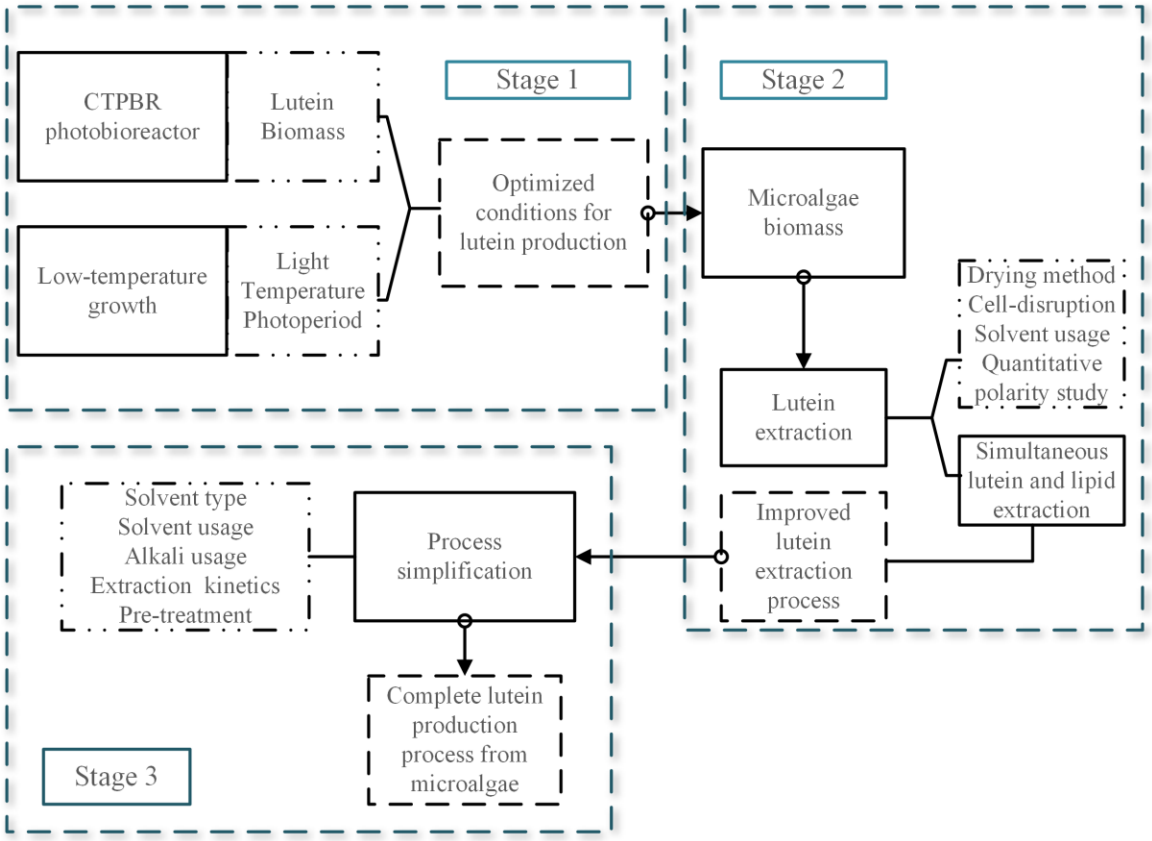

Figure 1-1 General scheme of research structure (CTPBR stands for coiled tubular tree photobioreactor, the solid dashed boxes stand for study name, the dot dashed boxes stand for study parameters, and the dashed boxes stand for study outcome)

1.2 Research objectives

1.2.1 Overall objective

The overall goal of this study was to investigate the lutein production and recovery from *Chlorella vulgaris* using phototrophic cultivation. Several variables such as environmental conditions, and photobioreactor configuration were investigated for optimizing lutein production. The extraction efficiency of lutein from the microalgae was also compared and modelled for different methods.

1.2.2 Specific Objectives

Specific-objective 1: To investigate microalgae cultivation in a small scale of a coiled tubular tree photobioreactor (CTPBR). The CTPBR was employed to cultivate *Chlorella vulgaris* UTEX 265 in comparison with those in Erlenmeyer flasks under various temperature and light conditions for lutein production. The effect CTPBR of was shown in the form of algal specific growth rate.

Specific-objective 2: To model microalgae cultivation under light and low temperature stressed conditions for lutein production. *C. vulgaris* was cultivated in CTPBR and Erlenmeyer flasks to show the effect of stress conditions on lutein productivity. Both the lutein content in microalgae and the algal specific growth rate were studied. The influence and interaction of light irradiance strength, lighting cycle and temperature on microalgae and lutein production efficiency at low temperature range were also studied in flasks via response surface method (RSM).

Specific-objective 3: To identify the optimal harvesting time for lutein production. The cellular lutein content and microalgal growth was monitored throughout the entire cell growth cycle. The kinetics of lutein accumulation gave the information to determine the time for either fastest lutein production or highest lutein content in biomass.

Specific-objective 4: To investigate the development of a suitable lutein extraction method from wet microalgae. Several extraction parameters such as the biomass to solvent ratio, drying method, cell disruption method were investigated. The performance of solvents on lutein extraction was compared quantitatively using Nile Red as a solvatochromic polarity probe. Finally, the simultaneous lipid and lutein extraction was also studied for different polarities using an ethanol-hexane binary solvent.

Specific-objective 5: To develop a one-step lutein extraction process from wet microalgae. A single-step simultaneous extraction, saponification and primary purification process for free lutein production from wet microalgae biomass was established by using

binary solvent with alkali addition. The kinetics of this process was modeled by equations derived from the Fick's second law.

Specific-objective 6: To model and optimize the single-step free lutein production from wet microalgae. The effects of type of organic solvent, solvent polarity, presence of cell disruption, alkali and solvent usage, and the interaction of alkali usage and solvent volume on lutein yields were examined.

1.3 Novelty and contributions

The literature review fills in the gap of few review papers focused on the downstream processing of carotenoids extraction from microalgae. Here, we summarized the recent biotechnological advances in microalgal carotenoid production; and identified the challenging aspects of carotenoids production from microalgae and proposed some possible future directions.

In the photobioreactor study, a photobioreactor using conical configuration in a coiled manner is designed. This is a first study of cultivation of microalgae at low temperatures stressed conditions, and for lutein production. An effective photobioreactor configuration for microalgae cultivation and lutein production is developed.

- Demonstrated the influence and interaction of light irradiance strength, lighting cycle and temperature on microalgae and lutein production efficiency at low to moderate temperature ranges.

- Found optimum conditions for lutein production from microalgae.

The lutein extraction study applies Nile red to quantitatively study the effects of mixture ratio and solvent polarity on lutein extraction and integrated lutein and lipid extraction.

- First determined the optimal harvesting time point of microalgae for lutein production.

- Validated the wet extraction method of lutein from microalgae is ideal.

- Identified the best extraction parameters for lutein extraction from microalgae.
- First established a quantitative method to study the effect of solvent polarity on lutein extraction, and simultaneous lipid and lutein extraction.

The modeling of single-step free lutein extraction investigates the extraction kinetics of lutein extraction from microalgae, and developed a new lutein extraction method that skips drying, and combines extraction, saponification and purification.

- Established a single-step simultaneous extraction, saponification and primary purification process for free lutein production from wet microalgae biomass.
- First modeled the kinetics of lutein extraction from microalgae, and proved the extraction rate was found to be controlled by the intra-particle diffusion.
- Demonstrated the effects of type of organic solvent, solvent polarity, method of cell disruption, alkali and solvent usage on lutein extraction rates and yields, and found the optimal operating area for extraction.

Chapter 2

2 Literature review: Carotenoids Production from Microalgae

The information in section 2 has been slightly changed to fulfill formatting requirements. It is substantially as it appears in the paper **"Carotenoids from Microalgae: A Review of Recent Developments", published in Biotechnology Advances, December 2016, Vol. 34, No. 8, pages 1396-1412.**

2.1 Abstract

Carotenoids have been receiving increased attention due to their potential health benefits (Manayi et al., 2015). Microalgae have been recognized as a fast-growing natural reservoir of various biologically active compounds including as a source of a high content of carotenoids (Ho et al., 2014). However, the production of micro-algal carotenoids is not yet sufficiently cost-effective to compete with traditional chemical synthetic methods and flower-based processing (Li et al., 2011; Nguyen, 2013). This review aims to summarize the recent biotechnological advances in microalgal carotenoid production. The current technologies involved in their bioprocessing including cultivation, harvesting, extraction, and purification are discussed with a specific focus on downstream processing. The recent developments in chemical and biochemical synthesis of carotenoids are also reviewed for a better understanding of suitable and economically feasible biotechnological strategies. Some possible future directions are also proposed.

2.2 Introduction

Microalgae play a fundamental role in ecosystems (Guedes et al. 2011). Microalgae are gaining attention as they can produce a wide variety of valuable products similar to those in higher plants but with a faster cultivation time. Microalgae also have less environmental limitations owing to their short life cycle and high adaptability. Examples of bio-products from microalgae include pigments, polyunsaturated fatty acids, vitamins, lipids and proteins, some of which have already been successfully commercialized (Halim et al.,

2012a). Of these, carotenoids represent an important category of useful products derived from microalgae.

The major carotenoids of commercial value found in microalgae include the anti-oxidants astaxanthin, β-carotene, lutein, lycopene, and canthaxanthin. The large-scale manufacture of the carotenoids from algae is currently quite challenging in terms of their cost-effective production, extraction and purification. An integrated bioprocessing approach using microalgae needs to consider both the upstream production of microalgae and the downstream harvesting and extraction of carotenoids. The existence of rigid cell walls in many algal species poses difficulties as this prevents full recovery of bioactive compounds. This is, therefore, a significant bottleneck in the overall bioprocess.

Many recent reviews have previously discussed microalgae and their products and applications (A Catarina Guedes et al., 2011; Markou and Nerantzis, 2013; Mata et al., 2010); however, there has been less focus on the downstream processing aspects. In this review, an attempt has been made to emphasize the extraction and downstream processing steps as a critical component for the overall bioprocessing. First the chemistry and biochemistry is described for a better understanding of the carotenoid production. Second the biotechnology, engineering and downstream approaches are discussed.

2.3 Chemistry and biochemistry of carotenoids

Carotenoids are lipophilic compounds that are usually colored yellow, orange or red. With over 750 types recognized, carotenoids are the most diverse and wide-spread pigments in nature (Sasso et al., 2012; Varela et al., 2015). Most carotenoid share a common C40 backbone structure of isoprene units (termed terpenoid), and are classified into two groups: carotenes and xanthophylls. Some common carotenoid structure can be found in Figure **2-1**. Each of the carotenoids consists of different *trans* and *cis* isomers. Xanthophylls, the oxygenated derivatives of carotenes (which are hydrocarbon only), are relatively hydrophilic compounds due to the presence of hydroxyl groups and keto-groups at the end rings. As antioxidants, carotenoids are in general sensitive to light, oxygen and heat, which made them difficult to handle.

In spite of the diversity of the carotenoid family, less than 30 carotenoids play important roles in photosynthesis (Varela et al., 2015). Most of these are located in the thylakoid membranes, and are bound with the Light Harvesting Complexes (LHCs) (Nisar et al., 2015). The carotenoids function to absorb light and quench excess energy in photosynthetic metabolism. Some primary carotenoids like lutein serve as accessory pigments, which can transfer absorbed energy to chlorophylls (Ye et al., 2008), therefore expanding the light absorbing spectrum of algae or plants.

Secondary carotenoids like astaxanthin and canthaxanthin play a role in cell protective mechanisms. Unlike primary carotenoids which are tightly associated with structural and functional components in the cellular photosynthetic apparatus, the secondary carotenoids are produced to high levels and are exported in oily droplets to form a protective layer when the cells are exposed to stressed conditions, and provide the pink/red color of the stressed algae (Begum et al., 2015; Wang et al., 2015). Most carotenoids are found in ester or di-ester form, therefore saponification is needed after the extraction of pigments (Rebecca et al., 2011).

Due to their anti-oxidant property, carotenoids can protect cells from reactive radicals, prevent lipid peroxidation, and promote the stability and functionality of the photosynthetic apparatus (Grossman et al., 2004). The integrity of membranes, which is essential for cell survival, can also be promoted by carotenoids. In particular, they improve the cell membrane fluidity under high temperature or high light conditions (Camejo et al., 2006). Similar stabilization effects were reported for low temperature as well when the lipids became more unsaturated (Ramel et al., 2012). In addition, the excess energy generated inside the cell can be dissipated as heat by non-photosynthetic quenching (NPQ). The energy dissipation is to protect cell damage from chemical reactive species ($^1O_2^+$, ^{3}Chl*), and is achieved by intersystem crossing from triplet state carotenoids to the ground state (Musser et al., 2015; Niyogi et al., 1997; Velikova et al., 2005).

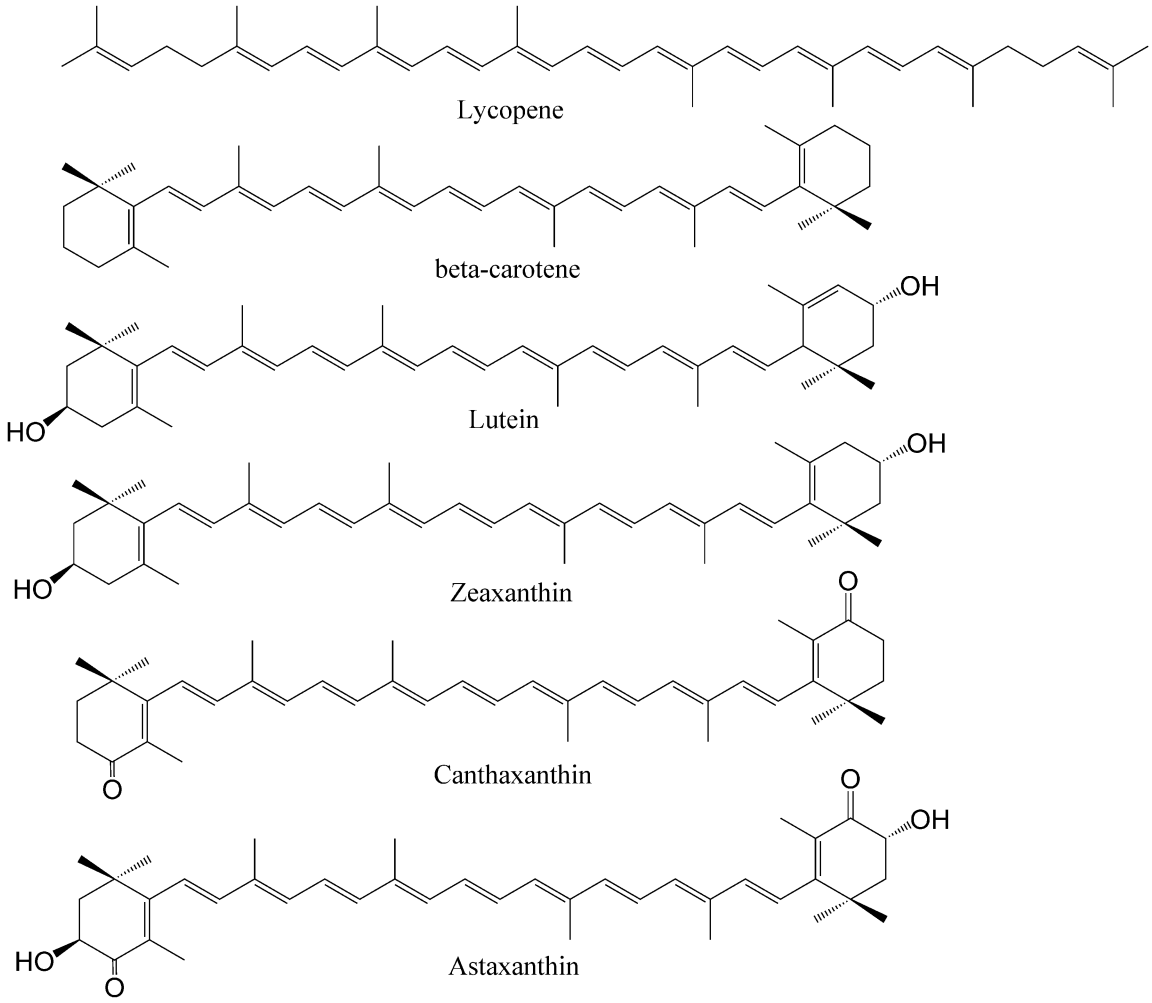

Figure 2-1 Chemical structure of some common carotenoids found in microalgae

2.3.1 Biosynthesis of carotenoids

The biosynthesis of carotenoids differs from species to species; however, almost all photosynthetic microalgae or plant species share the common primary metabolic pathway as shown in figure **2-2**.

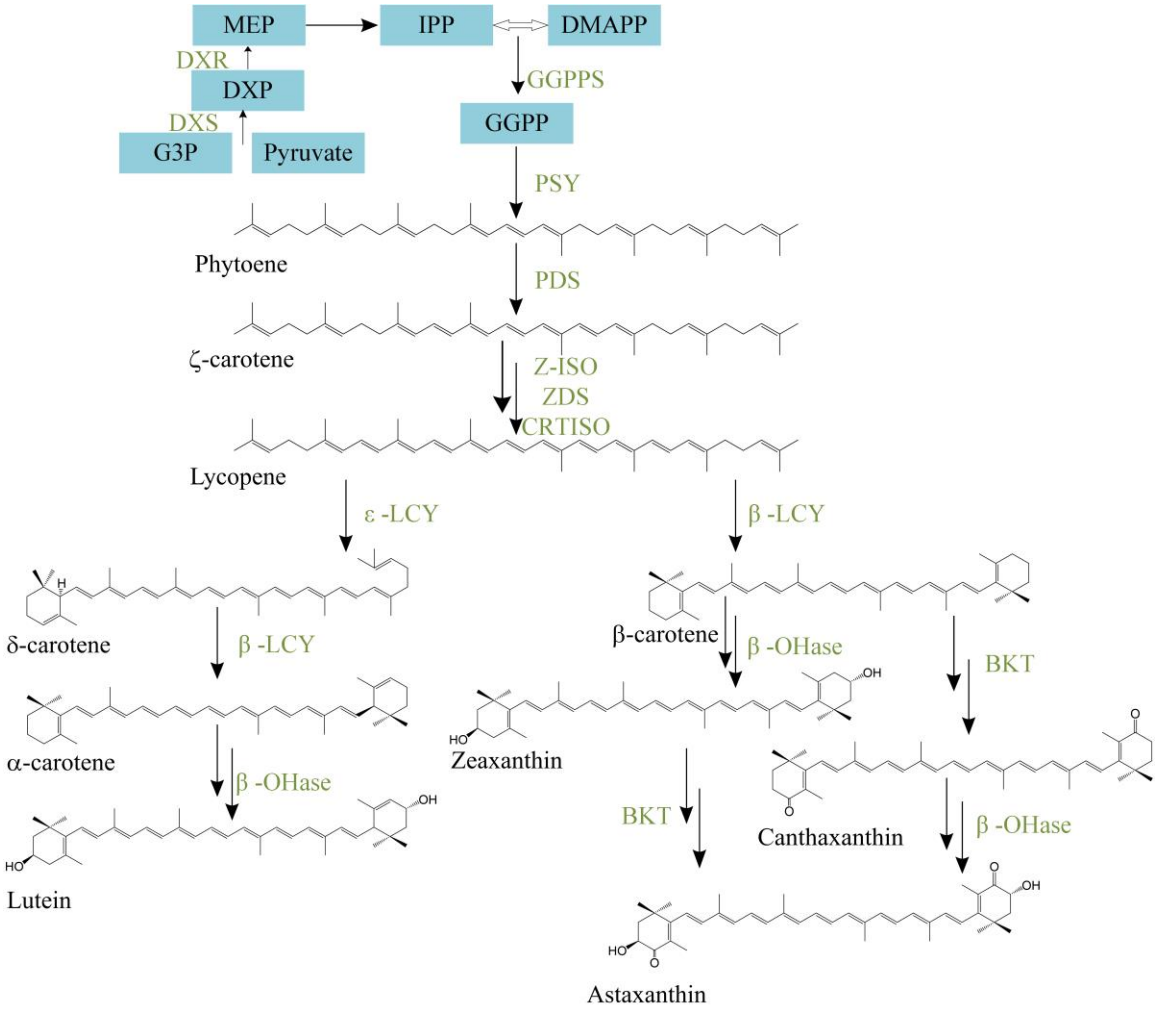

Figure 2-2 Primary steps of biosynthetic pathway of carotenoids in most green microalgae species, and higher plants share almost the same steps except the biosynthesis of astaxanthin which is only found in limited species of microalgae. Isopental pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are the building blocks of all carotenoids, the oxygenated xanthophylls are derived from α- or β-carotene. The enzymes involved are shown: β-LCY, β-cyclase; β-OHase, β-carotene hydroxylase; CRTISO, carotenoid isomerase; DMAPP, dimethylallyl diphosphate; DXP, deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; ε-LCY, ε-cyclase; ε-OHase, ε-carotene hydroxylase; G3P, glyceraldehyde-3-phosphate; GGPP, geranylgeranyl diphosphate; GGPPS, GGPP synthase; IPP, isopentenyl pyrophosphate; MEP, methylerythritol 4-phosphate; PDS, phytoene desaturase; PSY, phytoene synthase; ZDS, ζ-carotene desaturase; Z-ISO, ζ- carotene isomerase. (adapted from Nisar et al. 2015)

All pathways initiate from the same C5 building block, isopentenyl pyrophosphate (IPP) or its isomer, dimethylallyl diphosphate (DMAPP), produced from either Acetyl-CoA (the cytosolic mevalonic acid pathway (MVA) pathway) or pyruvate and G3P (the plastidic methylerythritol 4-phosphate (MEP) pathway). Although both pathways lead to the same end-product, it was suggested that the carotenoid synthesis uses IPP or DMAPP derived from the MEP pathway (Barredo, 2012). Then the intermediate C15 farnesyl diphosphate (FPP) or C20 geranylgeranyl diphosphate (GGPP) is synthesized by successive chain elongation in the head to tail style in the presence of enzymes. This step is followed by head to head condensation as in Figure **2-2**, which forms the C40 carotenoid, phytoene. In the presence of desaturase, ζ-carotene can be formed in algae or higher plants (the metabolic pathways in bacteria or fungi would slightly differ). Then the first colored carotenoid, lycopene is formed (Varela et al., 2015). Further, by two types of cyclization reactions, the commonly recognized α-carotene or β-carotene structures are produced. Additional chain transformations, including hydroxylation, epoxidation, ketolation, glycosylation and oxygen cleavage then can lead to the highly diverse carotenoid family (Barredo, 2012). Astaxanthin however is not found in many higher plants, it is more commonly synthesized from canthaxanthin or zeaxanthin by photosynthetic microalgae (Mann et al., 2000).

The biosynthesis of carotenoids takes place in the chloroplast, with some specific steps located in the cytoplasm. Phytoene synthase (*PSY*) is among the key enzymes for carotenoid biosynthesis in photosynthetic organisms since it carries out a rate limiting step. The expression for *PSY* or other synthase genes can be up-regulated by environmental stresses. Several reviews on enzymes in the biosynthesis pathway of carotenoids are available (Bertrand 2010; Nisar et al. 2015).

2.3.2 Chemical synthesis of carotenoids

The total chemical synthesis of carotenoids (starting with β-carotene synthesis), was developed by three teams (Karrer and Eugster; Inhoffen et al., and Milas et al.) independently in 1950 (Britton et al., 1996). Typical total synthesis examples are shown in Figure **2-3**. Currently, many synthesis pathways are available. The first scaled up method

was the Roche synthesis (C19+C2+C19, Grignard coupling, elimination, then partial hydrogenation) by F. Hoffman-La Roche & Co. Ltd in 1954. Later in 1960, the higher yield Badische Anilin & Soda-Fabrik (BASF) pathway emerged based on the Wittig condensation from the original synthesis of Inhoffen et al., C20+C20; and many other pathways emerged later (Ribeiro et al., 2011). Synthetic large scale production of astaxanthin became available in the 1990s, also from the Roche group (Higuera-Ciapara et al., 2006). More recently, the fermentative reduction method for industrial-scale total synthesis of (3R,3'R)-zeaxanthin led to a new direction of carotenoid synthesis (Ito et al., 2009).

Although the chemical synthesis of carotenoids is a well-established market, the use of these products in direct human consumption is limited due to the safety concerns. The natural carotenoids are usually a complex mixture of various isomers, and are usually found mixed with other bioactive compounds. Synthetic carotenoids, however, are predominantly mixed by all-*trans* compounds. Due to the competitive inhibition among carotenoids for human absorption, the intake of certain carotenoid isomers is considered not as safe as the intake of the natural occurring mixtures (Patrick, 2000). Thus the applications of synthetic carotenoids are limited to animal feed, colorants, preservants etc. The natural carotenoids have the advantage of lower toxicity and higher customer preference for medicine or supplements (Praveenkumar et al., 2015). Therefore, with the high cost of chemical synthesis, natural carotenoids are gaining more attention. However, due to current production technology limitations, only ca. 2% beta-carotene of the global market is from natural sources (Dufossé et al., 2005).

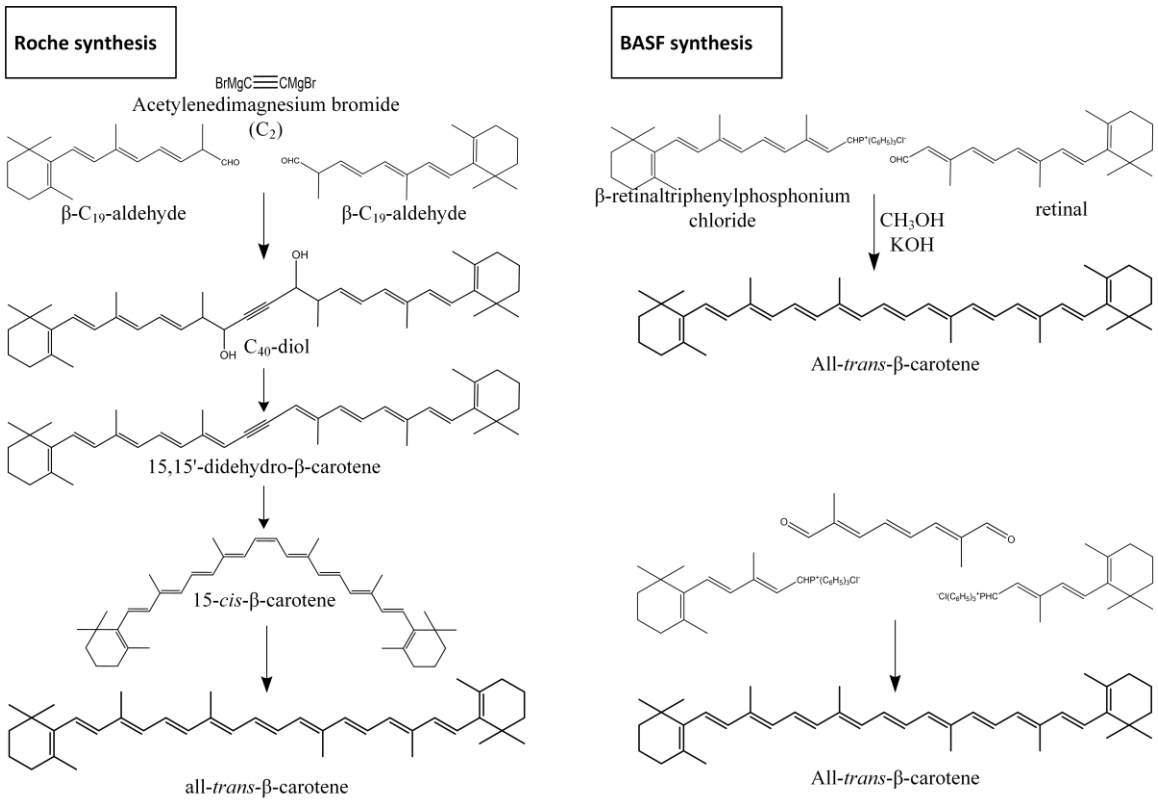

Figure 2-3 Typical pathways of carotenoids synthesis

2.4 Significance of carotenoids to human health

Even though carotenoid have widespread applications as food colorants, cosmetics and feed additives (Ye et al., 2008); it was not until recently that the benefits of carotenoids for human health were better understood. Similar to the protective roles carotenoids played in microalgae and plants, these pigments provide a protective role for humans. Many studies have reviewed the health benefits of carotenoids, which are usually related to anti-oxidant activities or as pro-vitamin A (Britton, 1995; Chuyen and Eun, 2015; Fiedor and Burda, 2014; Manayi et al., 2015; J. Zhang et al., 2014). The anti-oxidant property in general mediates the harmful effects of free radicals, which in turn can potentially protect humans from compromised immune response, premature aging, certain cancers, cardiovascular diseases, and/or arthritis. The carotenoids are also frequently reported to reduce the risks of AIDS, diabetes, cataract, macular degeneration, and neurodegeneration (Dufossé et al., 2005; Varela et al., 2015). Deficiency in these pigments may result in exophthalmia, night blindness, and in severe cases keratinization of the conjunctiva and cornea (Britton, 1995).

Today, the major carotenoids of market interest are β-carotene, astaxanthin, lutein (with zeaxanthin), lycopene, and canthaxanthin. Fucoxanthin is another carotenoid that can be produced by microalgae. Although not a major market sharer, fucoxanthin has been marketed as an anti-obesity functional food, anti-cancer and potential anti-inflammatory agent (Heo et al., 2010; Nanba and Toyooka, 2008). The benefits of these six common carotenoids to human health are summarized in Table **2-1**.

Astaxanthin and β-carotene are the two most recognized carotenoids in the global market, and make-up almost half of the carotenoid market (Business Communications Company, 2015). Astaxanthin is best recognized for the pinkish color in aquatic fish and shrimps. Being the strongest anti-oxidant in carotenoids, astaxanthin exhibits several-fold stronger anti-oxidant activity than vitamin E and β-carotene. As reported by some authors, it has the potential to enhance antibody production, anti-aging, sun-proofing, and it also demonstrates anti-inflammatory effects when administered with aspirin (Li et al., 2011). Guerin et al. (2003) have reviewed the benefits of astaxanthin for human health. Another carotenoid, β-carotene, is responsible for the prevention of toxin build-up in liver, potentially improves the immune system, and may have a preventative role in eye diseases like night blindness and cataract (Dufossé et al., 2005). However, some recent studies have related long-term β-carotene intake with increased risk of cancer as well as increased cancer death rate (Liu, 2013; Virtamo et al., 2014).

Two other bio-products, i.e., lutein and zeaxanthin are also becoming increasingly important in the nutraceutical market since they are now understood to play a significant role in eye health (Manayi et al., 2015). As the predominant pigments in the macula, lutein is clinically proven to prevent cataract and macular degeneration. These compounds also may function as strong anti-oxidants to decrease around 60 chronic disease risks (Ye et al., 2008). In general, these two xanthophylls are not considered toxic, and are relatively safe for human consumption.

Lycopene was marketed as an anti-oxidant and was proposed for treatment of cardiovascular diseases and prostate cancer; however, insufficient scientific evidence is present at this time to support this. Canthaxanthin may protect people from some blood

disorder diseases. However, it was reported to be possibly unsafe in daily consumption, and may potentially cause blindness or aplastic anemia when consumed in large quantities for the purpose of tanning the skin (Clinton, 1998; J. Zhang et al., 2014). Fucoxanthin is another carotenoid that can be produced by microalgae, and it is attracting increasing attention for its potential anti-obesity, anti-cancer and anti-inflammatory activities (Heo et al., 2010; Nanba and Toyooka, 2008). It is also considered a safe compound for human health and some authors reported that it did not exhibit toxicity and mutagenicity at low dosages (Beppu et al., 2009; Peng et al., 2011).

Table 2-1 Health benefits of six carotenoids confirmed by human studies

	Health benefits	Reference
Astaxanthin	Strong anti-oxidant property	(Fasano et al., 2014)
		(Chew et al., 1999)
	Anti-inflammatory effects	(Li et al., 2011)
	Anti-cancer	(Park et al., 2010)
	Cardiovascular health	(Pashkow et al., 2008)
Lutein	Prevent cataract and age-related macular degeneration	(Manayi et al., 2015)
		(Granado et al., 2003)
	Anti-oxidant property	(Bone and Landrum, 2003)
	Anti-cancer	(Cha et al., 2008)
	Prevent cardiovascular diseases	(Vijayapadma et al., 2014)
		(Alves-Rodrigues and Shao, 2004)

β-carotene	Prevent night blindness	(Dufossé et al., 2005)
	Anti-oxidant property	(Virtamo et al., 2014)
	Prevent liver fibrosis	(Shaish et al., 2006)
		(Virtamo et al., 2014)
Lycopene	Anti-cancer	(Viuda-Martos et al., 2014)
	Prevent cardiovascular diseases	(Srinivasan et al., 2009)
		(Devasagayam et al., 2004)
	Radiation protector	
	Anti-oxidant property	
Canthaxanthin	Create tan color	(Zhang et al., 2014)
	Anti-oxidant property	
Fucoxanthin	Anti-obesity	(Abidov et al., 2010)
	Anti-oxidant property	(Nanba and Toyooka, 2008)

2.5 Advantages and Disadvantages of microalgae as a carotenoid source

Microalgae have potential to serve as natural pools of biochemicals with various health potential. Compared to higher plants, microalgae have a faster growth rate. Lin *et al.* (2015) reviewed the technological aspects and productivity of lutein using microalgae vs. marigold flowers. Microalgae, especially those strains belonging to chlorophyta, such as *Dunaliella salina, Haematococcus pluvialis, Chlorella vulgaris, Chlorella zofingiensis* and *Chlorella pyrenoidosa* have been successfully developed in the mass production of β-carotene, astaxanthin, canthaxanthin, lutein and other carotenoids (Kyriakopoulou et al., 2015; Mendes-Pinto et al., 2001; Prommuak et al., 2013; J. Zhang et al., 2014).

Compared to plants, microalgae usually have a higher specific carotenoid content (mg/g). The lutein content in marigold flowers is commonly reported to be 0.3 mg/g, while for microalgae, the content is usually over 4 mg/g (Ho et al., 2014). Both astaxanthin and β-carotene were reported to be over 50 mg/g under specific stress conditions of cultivation for the microalgae (Kyriakopoulou et al., 2015; Suh et al., 2006). Microalgae also have higher carotenoid content than macroalgae, e.g. diatoms, as the alternative source, have up to 15 times higher fucoxanthin (18.23 mg/g) than the predominant producer, seaweed (Gómez-Loredo et al., 2015; Kim et al., 2012).

Due to their versatility in adapting to a wide range of growth conditions and climates, (e.g., glacial to tropic, fresh water to hyper-saline), and varied pH, microalgae show a clear advantage over plants. These microalgae can be produced year-round, this eliminates the requirements of long-term storage and subsequent potential degradation of the stored carotenoids. Meanwhile, wastewater can be used as a nutrient source. Therefore, the micro-algal process helps to reduce the pressure on both the carbon and the water footprint.

Microalgae production of carotenoids is less labor-intensive compared to higher plants as it does not require cutting, drying and many other common farming operations. In addition, some pigments like astaxanthin are rarely found in higher plants, which makes microalgae a more versatile carotenoid source.

Chemically synthesized carotenoids are generally cheaper than natural pigments, but many undefined diseases have been related to the use of synthetic products (Göçer et al., 2006), e.g. synthetic β-carotene has been related with some increasing risk of lung cancer and cardiovascular diseases in smokers or asbestos workers (Omenn et al., 1996). The biological functions and food safety concerns have increased the recent market on natural pigments in particular for human consumption (Li et al., 2011). However, although many studies have been carried out, the cost of production of most carotenoids using microalgae is still prohibitive. Many challenges still exist in downstream processing, especially the harvesting and extraction processes. These aspects are considered below.

2.6 Current technology for carotenoid production

Synthetic astaxanthin and β-carotene have occupied the majority of the market. While the production of carotenoids from microalgae is of increasing interest, only natural astaxanthin is approved by FDA for direct human consumption. The synthetic astaxanthin costs ca. $1000~2000/kg, and sells ca. $2500/kg; while the estimated production cost of natural astaxanthin can be reduced to $700 using microalgae as a source (Li et al., 2011; Nguyen, 2013).

In the photosynthetic metabolism of secondary carotenoids, mainly astaxanthin and canthaxanthin, the pigment content can be increased by introducing environmental stresses such as elevated light, low nitrogen, or salt-stress. *Haematococcus pluvialis* is able to accumulate over 50 mg/g astaxanthin, therefore it is recognized as one of the major sources of astaxanthin since the late 1990s. Genetic modification for higher astaxanthin content has been successfully developed for this strain. Some companies have been established based on this production line, e.g. Cyanotech (Hawaii, USA), Algatechnologies (Israel), and Astareal (Japan). The switch of some companies like Algacan (Canada) from biofuel production to carotenoids production also showed the feasibility of this process.

One other mature bio-product line is β-carotene from microalgae *Dunaliella salina,* which shares the similar bioprocessing operations as for astaxanthin production. The largest production processes are reported to be in Austria (Curtain, 2000) and Israel (Ben-Amotz, 2004). A two-stage cultivation strategy is commonly applied due to the contradiction of growth and pigmentation. The first stage is a “green” phase where the most suitable conditions for micro-algae growth are provided. When the cell concentration reaches a certain level, stress conditions (such as low nutrients or high light) are applied to force cells accumulate more carotenoids, this is called the “red” phase since the cells turn a red or pink color (Wichuk et al., 2014).

Lutein produced from marigold flowers (*Tagetes erecta* and *Tagetes patula*) has a more competitive price than synthetic methods. Therefore, the biological process is dominant in this industry. Marigold flowers are rich in xanthophylls and have the advantage of their simple xanthophyll component in the petals: no significance level of other pigments exist

other than lutein and zeaxanthin esters. Therefore, the extract is easier for further separation and purification processes. Usually, the milled dry flower petals undergo a solvent extraction process (typically n-hexane) for the oleoresin, and if necessary, KOH can be added for the release of free lutein. The production areas are mainly located at developing countries such as China, India and some African countries due to the labor-intensive process. Companies like Super Lutein (Japan) sells lutein as eye health promoting products. Microalgae have been proposed as an alternative lutein source, extensive research has been conducted on process development due to the higher lutein content. A comparison of the two processes can be seen in figure **2-4**. The processing of lutein from microalgae requires fewer operational steps than that from marigold flowers to produce crystalline form of lutein.

Apart from the processes discussed above, a yeast (*Phaffia rhodozyma*) has been reported to have potential to produce astaxanthin, yielding higher biomass concentration and less heavy metal content. The microalgae *C. zofingiensis* is also able to produce canthaxanthin at a level of 8.5 mg/g under salt stress and light limiting conditions. Bacteria such as *Xanthophyllomyces dendrorhous, Saccharomyces cerevisiae, Blakeslea trispora* and *E. coli* have been genetically modified to produce astaxanthin, β-carotene, lycopene, and canthaxanthin (Nanou and Roukas, 2016; Scaife et al., 2012). Although satisfactory carotenoids content can be achieved (over 10 mg/g) (Alper et al., 2005; Q. Li et al., 2013), the cost of production still remains high in both biomass production and the downstream processing.

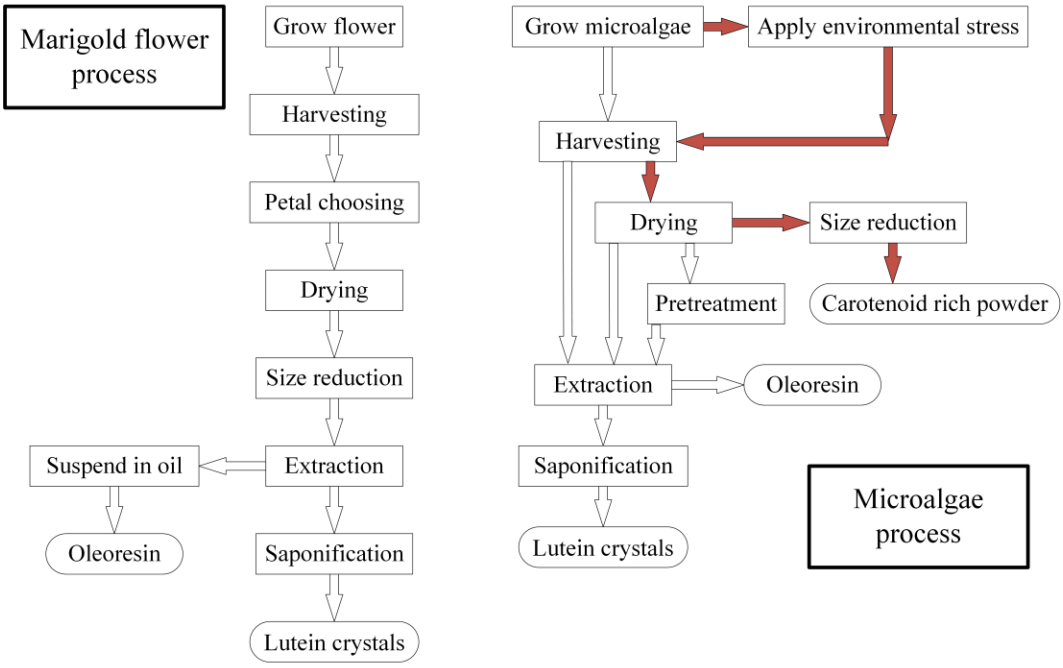

Figure 2-4 The process diagram of carotenoids from microalgae and from marigold flowers. The hollowed arrow implies the production of xanthophylls like lutein. The filled arrow indicates the process of commercialized astaxanthin production, the same process also applies to β-carotene.

2.7 Technologies of microalgae cultivation for carotenoids production

In this section, the technologies used to cultivate microalgae are discussed briefly. To acquire high productivity of carotenoids, both the microalgae production rate and carotenoid content in microalgae need to be optimized. First, the strategies for microalgae production are described.

2.7.1 Cultivation systems

At this time, the two most commonly applied technologies of the microalgae cultivation for carotenoid production are the open pond systems or closed photobioreactors (PBRs). The cost of open ponds is reported to be much lower than that for closed PBRs. Raceway ponds are the most commercially employed methods as they are the cheapest to construct and maintain (Borowitzka and Mohemani, 2012). Paddle-wheels usually give a flow rate

and suspend cells more uniformly, providing better mass transfer (Singh and Sharma, 2012). The liner is typically the most expensive capital cost, with mechanical mixing as the major operational cost. Other open pond systems including shallow lagoons and ponds, inclined systems, circular central-pivot ponds, and mixed ponds are also available, but much less attention has been paid to them due to the low productivity. Rogers et al. (2013) have estimated the economic requirements for open ponds, concluding that water loss, CO_2 and nutrient requirements would be the major concerns for large scale algae production. The drawbacks of using open pond systems are obvious: uneven light intensity, poor mass transfer, bad weather resistance aside from tropical areas, and contamination from other algal/bacterial strains (Singh and Sharma, 2012). Therefore, the closed PBRs seem preferred.

Wichuk et al. (2014) stated that light-driven photosynthesis efficiency is the bottleneck for large scale microalgal carotenoids production. PBRs represent the most successful approach in harvesting light, optimizing fluid dynamics, mass transfer, and minimizing water loss. Flat or tubular PBRs are the basic design structures; various modifications and extensions can be added based on them. As PBRs are much more expensive to build and operate, to scale up in an inexpensive way is the major challenge now facing the researchers and industry. However, since a variety of parameters can be adjusted, higher biomass quality is possible in addition to higher productivity. Gupta has reviewed PBRs for large-scale algal production (Gupta et al., 2015). Olivieri et al. (2014) also summarized the advances in PBR design for microalgae production and modeling. An immobilized film method (attached cultivation) was studied as well by Zhang et al. (2014), however, it is not as efficient as suspended PBRs due to poor light penetration (Zhang et al., 2016).

Being a mature process for fermentation, the stirred tank is suitable for heterotrophic microalgae cultivation as it has low light penetration but can increase mass transfer due to enhanced mixing. For autotrophic growth, vertical tubular or airlift PBRs are easy to build, and have relatively satisfactory biomass production considering the cost. One limitation for long tubular PBRs is O_2 inhibition due to O_2 accumulation in the tube. Flat panel PBRs (FBR) are available for high-density algal production under autotrophic growth conditions. However, the FBR is difficult to scale up owing to its configuration. Acién-Fernández et

al. (2013) concluded that most current PBR technologies are available for large-scale production, with companies established in Europe and all over the world. The cost of cultivating microalgae may be reduced to $5/kg in a horizontal PBR when operated in a 100-hectare scale, cheaper than raceway ponds at a commercial scale (Kleinegris et al., 2011). In another study, Li et al. (2011) evaluated the economics of a two-stage large scale microalgae production in (a) 1000 to 8000 L airlift PBR, (b) 100 m^2 raceway pond, getting an estimated astaxanthin cost of $718/kg, and an algae production cost of $18/kg.

2.7.2 Cultivation strategies

Among the microalgae species, the following were most frequently documented for carotenoids production: *Chlorella vulgaris, Chlorella protothecoides, Scenedesmus almeriensis, Dunaliella salina, Haematococcus pluvialis, Porphyridium cruentum* (Rhodophyta), and *Haslea ostrearia* (Diatom) (Pignolet et al. 2013). Stress conditions are often applied for high carotenoid content in microalgae, but different carotenoids have varied responses to stress conditions (Hodgson et al., 2016). The pigment content of some carotenoids like astaxanthin can be elevated from a few mg/g to over 50 mg/g, while the contents of carotenoids like lutein changes in much smaller scale and the microalgae growth rate is more essential in this case.

2.7.2.1 Stress-driven adaptive evolution

Unfavorable environmental conditions can be used for adaptive evolution. This represents a most adopted growth strategy to enhance carotenoid production. Investigation of the parameters involved in this process can help to obtain high carotenoid productivity. The limited production of biomass under stress could be countered by applying a multi-stage growth strategy (Hodgson et al., 2016).

2.7.2.1.1 Primary carotenoids

Primary carotenoids are growth-coupled metabolites. Lutein is a typical primary carotenoid that suffers to degradation under stress. Located in the chloroplast and mitochondria membranes (Collins et al., 2011), the pigmentation of lutein occurs at the center region of the algal cell. Many variables affect lutein productivity; the most common ones being the

type of algal species, temperature, light intensity, photoperiod, pH, nutrient availability, and salinity.

Temperature controls the enzymes involved in carotenoids biosynthesis, and also controls the growth rate. Low temperature decreases the nutrient uptake rate, and slows lutein accumulation (Bhosale, 2004). Higher temperature is favorable for cell growth and lutein accumulation. Fernández-Sevilla et al. (2010) showed that 28^{o}C is the optimum temperature for the lutein production considering the cell growth rate, while the inhibition from temperature starts at 32^{o}C, where the cellular lutein content decreases to half of that at 28^{o}C.

Light is a critical factor affecting carotenoid pigmentation. High light intensity increases the lutein content among the pigment pool (Maxwell et al., 1994), while decreasing the cellular lutein content. However, due to the benefits of abundant light to the microalgae growth (Xie et al., 2013), the lutein productivity increases as light intensity increases from 186 to 460 μmol photon m^{-2} s^{-1} (Cordero et al., 2010). Solovchenko *et al.* (2008) stated that the irradiance tolerance is a strain specific characteristic as a 6-8% decrease in total lutein content was observed for *Parietochloris incise,* whereas *S. almeriensis* exhibited good light tolerance till 1625 μmol photon m^{-2} s^{-1}. In addition, the full white light spectrum is more favorable than a monochromatic LED light source for lutein production (Ho et al., 2014). Microalgae also accumulate lutein under heterotrophic conditions. With 40 g/L glucose, a lutein productivity of 83.8 mg/L can be reached (Shi et al., 2000).

Nutrients also influence the lutein accumulation with the nitrogen source being the most essential for lutein production. When present in sufficient quantity, nitrate does not show significant effect on the lutein content, but as nitrate content decreases, the lutein content also decreases dramatically in *C. zofingiensis* (Cordero et al., 2010). The interaction between nitrate concentration and salinity is most significant for lutein biosynthesis from *D. salina* (Fu et al., 2014). Therefore, nutrient rich conditions favor the growth (Xie et al., 2013), while the nitrogen source does not influence the lutein production in *Muriellopsis sp.* (Jin et al., 2003).

The addition of oxidizing substances can slightly introduce oxidative stress, hence increase the cellular lutein content (A Catarina Guedes et al. 2011). Salinity itself also does not influence the lutein content, while combined with light or proper trace metals, it can improve the lutein production rate by 80% to 260 mg/L in *C. vulgaris* and remain stable when scaled up to a 25,000 L fermenter (Jeon et al., 2014).

The pH is important as it influences the CO_2 availability via the chemical conversions between CO_2, HCO_3^- and H_2CO_3. The shifting of *C. onubensis* growth from air to CO_2 provides increased cell growth; however, the accumulation of lutein at high cell density (5-6 g/L) does not depend on CO_2 concentration (Vaquero et al., 2014). Unlike the carotenoids that could be over-produced by the stress condition, natural over-production of primary carotenoids like lutein is much more difficult. Genetic modification might be the potential solution for this challenge (Mulders et al., 2014).

2.7.2.1.2 Secondary carotenoids

Some of the carotenoids, mainly secondary carotenoids like astaxanthin, can be produced under extreme conditions of microalgal stress to achieve greater cellular content levels. However, several primary carotenoids like β-carotene, can act as secondary metabolites under stress conditions, and therefore are discussed together (Hodgson et al., 2016). Under stress conditions, these carotenoids can be found in the cytoplasmic lipid globules rather than in the chloroplast (Collins et al., 2011). Two-stage cultivation has been successfully adopted for these kinds of carotenoid production (Wan et al., 2014). In the first stage, optimal conditions for cell growth allowed for cell accumulation (green phase, flagellate cell), while in the following stage (red phase, cysts), stress conditions are introduced for the pigmentation. This strategy is most commonly used for astaxanthin production. Continuous growth in the single stage under limited stress is easier in terms of operation, but is less used nowadays due to poor astaxanthin accumulation rates. Aflalo et al. (2007) compared the difference of two strategies for astaxanthin production, concluded that 2-stage operation was easier to scale up as well. Suh et al. (2006) developed a double layer reactor combining both green and red growth phases in a single reactor for simultaneous cell growth and astaxanthin production, and obtained an astaxanthin content of 57.9 mg/g.

The reduction of nitrogen, phosphate, and introduction of NaCl, especially under strong light conditions are also effective strategies for astaxanthin accumulation (Harker et al., 1996; Orosa et al., 2000). The stress condition requirement is similar for β-carotene (Bhosale, 2004).

Light also plays significant roles in carotenoids pigmentation at a wide range of intensity, from 50 to over 1250 µmol photon m^{-2} s^{-1}. Under strong light, cell division slows down and cell lysis increases (Bhosale, 2004). An increase in β-carotene content can be observed in *D. salina* under strong light (Ribeiro et al., 2011). Continuous lighting might also be favorable in terms of stressing the microalgae (Bhosale, 2004). Zhang et al. (2016) modeled the light attenuation, temperature, and nitrogen sources, concluded that 27°C, 4.4 mM NO_3^- would be optimal for astaxanthin production. Similarly, through another modeling study, a pH = 9, 20% NaCl, and 48 kerg cm^{-2} s^{-1} were found to benefit β-carotene production (Çelekli et al., 2014). The fucoxanthin accumulation by *Cyclotella cryptica* (diatom) is promoted by controlling light and nitrate (Guo et al., 2016). However, the best fucoxanthin production is not at the best growth conditions for *Isochrysis galbana* (diatom). More studies are required to reveal the effect of culture conditions on fucoxanthin production (Gómez-Loredo et al., 2015). Heterotrophic growth is not desirable for the carotenoid production, as the highest β-carotene content was around 1.01 mg/g (Ip and Chen, 2005), much less than that of autotrophic conditions where it can reach 40mg/g (Aflalo et al., 2007). Therefore little work has been done for heterotrophic algal carotenoids production (Lowrey et al., 2015).

Temperature has strong effect on carotenoid accumulation. Considering the growth rate and cell density, 24 to 29°C would be a suitable range for carotene production from *D. salina* (Bhosale, 2004). Ras et al. (2013) studied the ability of microalgae to withstand temperatures above the optimal range, which would be of particular interest to tubular PBRs, since the heat might accumulate as light is focused in the center, especially during outdoor cultivation. The effects of day and night time temperature were studied by Wan et al. (2014) to improve outdoor astaxanthin production rate. Daytime temperature at 28°C and slightly lower temperature in the night is optimal for both growth and astaxanthin

accumulation. Employing this method, 40-45 mg/g astaxanthin can be obtained (Margalith, 1999).

Ferrous salts also generate oxidative stress through the formation of hydroxyl radicals, thus can be used as an alternative energy saving strategy in place of strong light. This has been investigated for canthaxanthin production in *C. zofingiensis* but not for astaxanthin production (Pelah et al., 2004). The combination effect of strong light and low nitrate was examined by Cordero et al. (2010). Other heavy metals can also introduce oxidative stresses, but may not be suitable for human consumption.

Wang et al. (2013) indicated that initial cell density has an impact on growth, and 0.8 g L^{-1} was best for astaxanthin production. Astaxanthin productivity of 38 mg/g or 16 mg L^{-1} d^{-1} was possible under outdoor cultivation conditions.

2.7.3 Metabolic engineering

Microalgae serve as an excellent model host for metabolic pathway regulation or genetic engineering since they present the advantage of simplicity of culture and fast growth rates compared with plants. In addition, microalgae physiological and genetic analogies with plant cells, therefore could potentially reveal the gene coding for carotenoids biosynthesis in plant (Gimpel et al., 2015; Leu and Boussiba, 2014; Shah et al., 2016; Varela et al., 2015).

Mutagenesis using UV radiation or other methods have been applied to wild strains of microalgae for strain improvement. Jin et al. (2001) used mutagenesis to enhance zeaxanthin production in *D. salina*, and successfully generated two zeaxanthin-overproducing strains. A zeaxanthin epoxidase mutant was recognized in the study (Jin and Melis, 2003); analogous mutations exist in other strains like *S. obliquus* and *C. reinhardtii* (Ghosh et al., 2016). The zeaxanthin content (per cell) is 15-fold higher than the wild type under non-stressed conditions (Polle et al., 2003). Site-directed mutagenesis of enzyme phytoene desaturase has also been reported for *H. pluvialis* astaxanthin production (Steinbrenner and Sandmann, 2006).

Extensive studies have been conducted to transform the model microalgae, like *C. reinhardtii*, *N. gaditana* and *P. tricornutum* (diatom) (Jinkerson et al., 2013; Varela et al., 2015), while other strains are beginning to be understood, like *Nannochloropsis sp*. (Kilian et al., 2011), *S. obliquus* (Guo et al., 2013), or β-carotene-producing *D. salina* (Feng et al., 2009) and astaxanthin-producing *H. pluvialis* (Kathiresan et al., 2009). Ghosh et al. (2016) have summarized the strains for which genome project and transformation have been successfully done.

The key metabolic steps controlling carotenogenesis are discussed by Giuliano (2014) and the vector construction and gene selection strategies are reviewed by Qin et al. (2012). The genetic engineering of microalgae toward carotenoid production requires sufficient isoprenoid precursor supply, which represents one of the major approaches and may be realized by the overexpression of the important enzymes in the pathway or silencing/suppressing branch pathways via RNA interference (RNAi) (Varela et al., 2015). The enzymes to be highlighted are PSY, PDS, BKT in conventional genetic engineering strategies to increase carotenoid production. Extensive attempts by single or multi-gene overexpression of these proteins have been conducted and summarized by Gimpel et al. (2015). The coding gene is different for the main target PSY in different strains (Ye et al., 2008), by overexpression of the corresponding gene in *Chlamydomonas*, a 2-fold increase in carotenoid level was displayed (B F Cordero et al., 2011). By PDS gene mutation, the astaxanthin levels can be increased in *H. pluvialis* (Steinbrenner and Sandmann, 2006). Additionally, expression of BKT in *C. reinhardtii* can lead to the synthesizing of keto-carotenoids not present in the wild strain (León et al., 2007), and hydroxylase (CHYb) genes are also associated with astaxanthin overproduction as revealed in a study with *C. zofingiensis* while PDS gene is the dominant factor (Liu et al., 2014). For fucoxanthin from *P. tricornutum* (diatom), the DXS transformants reached 2.8 fold higher fucoxanthin content, while PSY transformants reached up to 1.8 fold than the wild type (Eilers et al., 2016). Additional carotenoid increase can be achieved when combined with the central carbon metabolism transformation (Heider et al., 2014). Since a nitrogen source is essential for protein synthesis and cell division, the deprivation of nitrogen source would enhance LCYb enzyme synthesis, hence increase the pigmentation rate (Cordero et al., 2010). The

approaches for sufficient supply of IPP and DMAPP were reviewed previously (Harada and Misawa 2009). Besides, balanced expression of the target genes and the creation of sufficient storage space for overproduced carotenoids are also necessary (Heider et al., 2014; Mulders et al., 2014; Varela et al., 2015). Addition of the transport route for the biosynthesized pigments out of the photosystem to cytoplasm may help increase the carotenoids productivity as well (Mulders et al., 2014).

The complex multi-enzyme pathways of carotenoid biosynthesis has impeded classical approaches' success on genetic improvement based on random mutagenesis or multiple transgenes overexpression (Daboussi et al., 2014). By the manipulation of the central regulatory carotenoid transcription factors (TF), ideally changing only one central regulator of a pathway to activate multiple components, the emerging transcriptional engineering (TE) may provide a better solution (Bajhaiya et al., 2016). Identification and characterization is vital for the success of TE; the possible methods include mapping target genes and determining *cis* elements, importing foreign TFs from other biologically relevant organisms, and generating synthetic TFs by *in silico* design. Emerging genome editing tools during the past decade include zinc-finger nucleases, meganucleases (MNs), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) (Bajhaiya et al., 2016; Daboussi et al., 2014; Scranton et al., 2015).

To date, the modification of the genome of microalgae is reported by only a few studies. The feasibility of TE is suggested by studies of *Dunaliella bardawil* (Lao et al., 2014) and *Chlamydomonas* (Gargouri et al., 2015). Baek et al. (2016) successfully used DNA-free CRISPR/Cas9 to knock out genes and enhanced zeaxanthin production. *P. tricornutum* (diatom) genome is stably modified by target editing tools of meganucleases and TALEN; while zinc-finger nuclease technology is used for *Nannochloropsis* and *C. reinhardtii* genome modification (Daboussi et al., 2014; Kilian et al., 2011). Therefore, genetic engineering including TE is a promising biotechnology for future carotenoids production, while much work is yet necessary to achieve high productivity and stability of the transformants (Bajhaiya et al., 2016; Varela et al., 2015).

2.8 Downstream processing for carotenoids

Kim (2013) stated that harvesting and extraction are the two most expensive steps in microalgal carotenoids production. Cost reduction in downstream processing hence needs to be emphasized (Park, 2015).

2.8.1 Harvesting

Harvesting of suspended microalgae is a major challenge, and the difficulty increases as the cell size decreases. The harvesting process accounts for 20-30% of the total cost to produce microalgae (Georgianna and Mayfield, 2012). Currently no suitable method is present for microalgae harvesting, especially for the carotenoid production process as the latter usually requires non-toxicity and minimal carotenoid degradation. Large cell size and auto-flocculation might be of interest, and the experiences from water treatment can be borrowed owing to comparable techniques (Uduman et al., 2010).

2.8.1.1 Physical methods

Centrifugation is a reliable, fast and efficient method, most widely applied for harvesting microalgae in both lab scale and small industrial applications especially for astaxanthin production. It is suitable for most algal strains. Over 80% of microalgae are reported to be recovered from a suspension within 2-5 minutes (Chen et al., 2011). However, the high capital cost and continuous energy investment during the operation have largely limited the further scaling-up. In addition, there is potential to damage the cell structure during high speed centrifugation. Grima et al. (2003) have reviewed the technical aspects of microalgae harvesting. Sedimentation is considered as an economical approach.

Filtration or screening is greatly dependent on the particle size. For small size microalgae, this process may be extremely time and energy consuming. Counter-current technologies or turbulent flow can be used to reduce the fouling or clogging of the filter or membrane.

Gravity sedimentation is an inexpensive method, but it requires a very long time for small uniformly suspended culture when no additional flocculants are present. Around 15% cell density can be achieved by this means, but this is very species specific; therefore, more

suitable for large, dense, and non-motile cells such as some diatoms. Currently, sedimentation is sometimes used as a first stage treatment to reduce the energy consumption when combined with other methods, most commonly centrifugation to get less moisture content.

2.8.1.2 Chemical method for cell harvesting

Compared with mechanical methods, the chemical harvesting method consumes much less energy, and requires lower capital investment. The major cost is for the use of flocculant chemicals. Although not as efficient as mechanical methods; and with a higher final water content in the slurry, flocculation has received much attention due to the possibility to treat large scale microalgae suspensions at a lower cost. This method is also widely applied in industry, in particular, for water and wastewater treatment (Gorin et al., 2015). Flocculation works by adding coagulants to neutralize the surface charge on suspended particles and/or increase the particle size to accelerate the sedimentation process. The type and dose of flocculent is species dependent; the required concentration of flocculent may range from 10-50 mg/L or more, while the types can vary from inorganic salts, mainly aluminum or ferric based, polymer based or nanoparticles and magnetic particles (Hu et al., 2013). Polymer based flocculation poses less of an environmental burden, and may be potentially non-toxic. Some literature however has reported decreased quality of the subsequent product and reduced quantity of carotenoids, mainly due to the covalent bonding through the coagulant to the polar functional groups of the pigments (Utomo et al., 2013). Meanwhile, the addition of chemicals also adds a complexity to the subsequent treatment (Hu et al., 2013), and the technology may not work well with marine micro-algae.

Flotation is another method in contrast to sedimentation/flocculation. The solids float to the surface of the liquid assisted by gas bubbles. In dissolved air flotation, the pressure of the gas pipeline is essential as it is associated with both bubble size and the prevention of back flow. Dispersed air flotation is slightly different, interaction of air bubbles with the negatively charged surfaces of algal cells is important for effective harvesting (Pragya et al., 2013). In general, flotation is species specific and involves high capital cost and

operational requirements, therefore it is not the best recommended method at present (Uduman et al., 2010).

More advanced harvesting methods usually involve electromagnetic techniques. No addition of chemicals make them more environment compatible. However, the fouling in the electro-cathodes may cause problems in the large-scale operation (Chen et al., 2011). Generally, development of an efficient and cheap harvesting method is of urgent need to produce micro-algal carotenoids.

2.8.2 Cell disruption

Cell disruption is often suggested as a necessary step to increase the carotenoids or lipid recovery yield by several fold. Therefore, although it introduces additional processing cost, the pre-treatment step is still considered necessary. It has been pointed out that the selection of a suitable cell disruption method is algae species specific (McMillan et al., 2013). Cold soaking with solvents is enough for some frustule absent species; for example, *C. reinhardtii* is a good carotenoid source without cell disruption, or diatoms are good candidates as well. Kim et al. (2012) reported that approximately 95% fucoxanthin in *Isochrysis galbana* can be released by a single solvent extraction. For many other algae, the thick rigid cell wall requires cell disruption to release the inner contents. Without cell disruption, the extraction results can be very inefficient (Chan et al., 2013; Gille et al., 2016). Michalak and Chojnacka (2014) summarized the advantages and disadvantages of some cell disruption techniques to extract biologically active compounds from algae without their degradation. However, most previous studies on cell disruption are dedicated for lipid recovery, rather than carotenoids, but the basic approaches are similar. The efficiency and advantages/disadvantages for different cell disruption methods as well as extraction methods are also compared in Table **2-2**.

Table 2-2 Summary of carotenoids extraction technologies from microalgae

Step	Methodology	Efficiency	Advantages disadvantages	References
Cell disruption	Grinding	++	Time-consuming	(Hu et al., 2013)
	Cryogenic grinding	+++	Expensive	(Grima et al., 2003; Zheng et al., 2011)
	Bead milling	+++	Most efficient in some studies; not as efficient in several studies; the inconsistent result may due to the treatment is strain specific; generates heat	(Chan et al., 2013; Halim et al., 2012a; J. Y. Lee et al., 2010; Prabakaran and Ravindran, 2011; Taucher et al., 2016)
	High pressure homogenizer	+++	Comparable with bead milling and ultrasound assisted extraction	(Grima et al., 2003; Halim et al., 2012b; Kim et al., 2015)
	Autoclave	-	Damage of carotenoids occurs	(Chan et al., 2013)

	Microwave	+++	Comparable efficient with bead milling; Low energy consumption; Simple method; Generates heat	(McMillan et al. 2013) Lee et al. (2012) Li et al. (2015)
	Ultrasonication	+++ / +	Most efficient in some studies; Not efficient in other studies	(Cravotto et al., 2008; Mercer and Armenta, 2011) (Halim et al., 2012b; McMillan et al., 2013; Pasquet et al., 2011)
	Enzymatic hydrolysis	+	Highly selective; mild condition; Expensive, Strict condition maintaince; Long treatment time	(Deenu et al., 2013; Kadam et al., 2013; Zheng et al., 2011)
	Pulsed electric field	++	Highly selective; Retain bioactivity of carotenoids	(Grimi et al., 2014; Lai et al., 2014; Sánchez-

			Short treatment time; Small solvent requirements	Moreno et al., 2005; Yu et al., 2015);
	Osmotic shock, Acid/ alkaline treatment,	+ -	Not efficient; Cause carotenoids to degrade	(Halim et al., 2012a) (Halim et al., 2012a)
	Ionic liquids/ Switchable solvent	-	High price; Toxicity; Cause carotenoids to degrade	(Park et al., 2015)
Solvent extraction	Conventional solvent extraction	++	Cheap and easy to scale up; Long extraction time; Multi-step operation; Use large amount of solvents	(Gil-Chávez et al., 2013; Reverchon and De Marco, 2006; Taucher et al., 2016)
	Super-/sub-critical solvent extraction	+++	Polarity of solvent is tunable; Fast; Safe;	(Du et al., 2015; Halim et al., 2012a; Hong-Wei Yen, Sheng-Chung Yang, Chi-Hui Chen, Jesisca,

	Easy separetion of carotenoids; Expensive	2015; Reverchon and De Marco, 2006)

The symbol "-" represents carotenoids degradation; "+", slightly efficient; "++", efficient; "+++",highly efficient.

2.8.2.1 Mechanical disruption methods

Grinding, bead-milling and high pressure homogenizers are the most commonly adopted mechanical cell disruption techniques for microalgae in both lab and pilot scale. They are easier to scale up than other novel methods (Taucher et al., 2016). However, the high energy requirement for these approaches is a significant limitation during bioprocess scale-up and careful control is needed to remove excessive heat generated to avoid carotenoid degradation.

2.8.2.1.1 Grinding

Manual grinding with wet biomass is reported to be able to efficiently extract pigments from microalgae (Hu et al., 2013). However, it is time-consuming and almost impossible to scale up (Utomo et al., 2013). Cryogenic grinding, or grinding with liquid nitrogen, is reported to be extremely efficient, but its high cost also made it un-realistic for industrial applications (Grima et al., 2003; Zheng et al., 2011). Review on extraction method, both traditionally and novel methods, was available by Kadam et al. (2013) for bioactive compounds including carotenoids.

2.8.2.1.2 Bead milling

Bead milling offers a better potential for larger scale operations as compared to grinding. The mechanical solid shear in bead mills offers a non-specific and highly effective cell disruption. Two types of bead mill are currently available, one type involves shaker vessels (bead beating) for laboratory use, and the other involves agitated beads for larger scale. In

both types, high speed spinning of beads leads to collision or friction of the cells. This has the advantage of generally high efficiency and minimized contamination risk from the environment, hence this method is often adopted for DNA extraction (Mercer and Armenta, 2011). One challenge with this approach is that the efficiency is low when treating the small micron size microalgae like *C. vulgaris* (J. Y. Lee et al., 2010). Extensive heat can as well be generated during the extraction, which would easily heat up the solvent, leading to degradation of functional compounds (Kim et al., 2015).

The container shape, shaking rate, bead amount, sample amount, bead size and bead type all have effects on the final cell disruption efficiency. Previous studies demonstrated that bead-beating is the most efficient for carotenoids extraction among various approaches such as autoclaving, bead-beating, microwaves, sonication, osmosis shock, French press, freeze and thaw, and lyophilization (Chan et al., 2013; Taucher et al., 2016). Wet milling of *S. dimorphus* or *C. prototheco*ides also provide improved results than other extraction methods (Mercer and Armenta, 2011). In another study for *Botryococcus sp*., bead beater and microwave assisted extraction (MAE) showed the best results (Pragya et al., 2013). However, the extraction may be strain specific, since for some cyanobacteria bead milling was reported to be not as efficient as some other treatments like ultrasound assisted extraction (UAE), MAE, or autoclaving (Prabakaran and Ravindran, 2011). The addition of beads also adds complexity to the system, which may require further separation of the beads. Continuous recycling bead milling may be potentially used to recycle beads as well as increase extraction efficiency (Ho et al., 2008).

2.8.2.1.3 High pressure homogenizer

The high pressure homogenizer (HPH) is a continuous system that can deal with slurry algal suspension and allows for easy scale-up. The cell suspension is forced through a narrow nozzle outlet by high pressure pumping. A rotor-stator homogenizer is also available, but only suitable for low viscosity liquid treatment. The working principle of high pressure homogenizer is not well understood, but it is generally believed to be caused by the high shear force and cavitation in the liquid coupled with the sudden pressure drop between the nozzle and the outer environment (Ho et al., 2008).

The applied pressure, cell size, and nozzle diameter are the main factors for high pressure homogenizer system control. Cooling is also essential to prevent carotenoid degradation (Lee et al., 2012). High pressure homogenizers showed superior results than traditional pre-treatment methods like osmotic shock or enzymatic hydrolysis (Grima et al., 2003). This was also reported to be particularly suitable for *Chlorococcum* cells compared with bead milling and ultrasound assisted extraction (Halim et al., 2012b; Kim et al., 2015) while the results were found to be contrary for *Botryococcus braunii*: the high pressure homogenizer was not as efficient (Pragya et al., 2013).

2.8.2.1.4 Autoclave

An autoclave involves high temperature steam and can efficiently break the microbial cell wall and extract lipids. This approach showed good results in a study by Lee et al. (2010) for lipid extraction from *C. vulgaris*. However, due to the temperature sensitive nature of carotenoids, such methods may not work for carotenoids extraction. This conclusion has been supported by the study of Chan et al. (2013) where lutein from autoclave treated algae was only 25% of that from bead milling.

2.8.2.2 Non-mechanical disruption methods

2.8.2.2.1 Microwave

Microwave assisted extraction (MAE) is a relatively new method (developed within the last 30 years) for cell disruption and currently employed in vegetable oil and animal oil extraction (Lee et al. 2010). The MAE generates high frequency waves with wavelength from 0.001 m to 1 m. The electromagenatic radiation is transmitted to the medium and can be absorbed to homogeneously heat up the mixture, and lyse the microalgae via rapid heat shock. The moisture content inside the cell is vaporized, producing a high pressure inside the cell towards the cell wall (Gil-Chávez et al., 2013). This allows better disruption of the cells and can be particularly effective for those algae with strong mechanical resistance (Barba et al., 2015). In addition to the operational factors of MAE (such as power, working volume, temperature), the dissipation factor, heat capacity of the solvent, and the polarity of solvent, are the other important factors affecting the extraction. Understanding the target compound, polarity is essential for the design of the pre-treatment process (Zheng et al.,

2011). High heat dissipation factors coupled with a high dielectric constant would in general facilitate the extraction process (Gil-Chávez et al., 2013).

Less energy is required for MAE than for the mechanical methods mentioned earlier. Lee et al. (2012) and McMillan et al. (2013) revealed that MAE have similar high efficiency as for bead milling, and MAE could break 94.92% *N. oculata* cells with 13 fold less energy consumption. It was reported that MAE can accelerate lipid extraction when combined with grinding (Soštarič et al., 2012). In addition, the advantage of no thermogradient in MAE eliminated the heat transfer requirements, which is a concern for other treatment methods especially ultrasonication. However, degradation of carotenoids usually starts from 60^{o}C (Pasquet et al., 2011), so additional temperature control for carotenoid extraction is needed. This may be the major obstacle for applying MAE for carotenoid extraction (Kadam et al., 2013).

Overall, MAE is a simple but efficient method for carotenoids extraction, since it requires less solvents and has demonstrated potential for further scale up, making it potentially more economical. The reaction mechanism needs to be further investigated, and MAE reactor design of large scale systems is still needed. Li et al. (2015) suggested that a microwave reflection tank may be applied, while more efficient microwave absorbents need to be developed. It is also very important to monitor the temperature change to minimize carotenoid loss.

2.8.2.2.2 Ultrasonication

Ultrasound assisted extraction (UAE) was widely studied for the extraction of proteins, sugars and lipids. Cavitation is considered the underlying mechanism for UAE, where micro-bubbles form and collapse near the cells, creating micro turbulence, high liquid shear and pressure shock. All of these factors help to break the cell wall. UAE has the benefits of higher efficiency, reduced extraction time, low to moderate cost, negligible toxicity, and simple handling. Frequency and working power play important roles in the performance of UAE efficiency (Wang et al., 2014). The sample volume too is particularly important for this method as the energy dissipates easily through transmittance (Zheng et al., 2011). UAE is a scalable process owing to the recent developments of installations by arranging

multiple devices with flowing fluid. OriginOil has applied this technology with electromagnetic pulses to disrupt cells (Mercer and Armenta, 2011).

When coupled with Soxhlet extraction, UAE is the most efficient in extracting lipids from *Crypthecodinium cohnii* (dinoflagellate) (Cravotto et al., 2008; Mercer and Armenta, 2011). For *Nostoc sp*. and *Chlorella sp*. cell disruption, UAE also showed the best results (Grima et al., 2003; Plaza et al., 2012). However, although reported to be efficient in some studies, some conflicting results have been reported. In one study, UAE is reported to be insufficient to break microalgae cell wall (Pasquet et al., 2011). McMillan et al. (2013) concluded that UAE was not as efficient as heating in a water bath, Halim et al. (2012b) showed that high pressure homogenization and bead milling gives much better results than UAE. However, since the comparisons were usually not conducted at the same energy output, the insufficiency of UAE may result from its low power input compared with other methods, (e.g. Ultrasonicator 40-130 W; bead beater 850 W; MAE 1000 W) (Halim et al., 2012b); The sample volumes vary with each other (350 mL for UAE versus 10 mL in MAE in McMillan et al.'s study (2013)), which indicates another possible reason for the inconsistent results (McMillan et al., 2013). Therefore, it is hard to conclude definitely whether UAE is sufficient enough to treat microalgae based on the reported studies.

2.8.2.2.3 Enzymatic hydrolysis

Enzymatic hydrolysis is an expensive process due to the high cost of enzymes. The major advantage of enzymatic hydrolysis is it decreases the activation energy of the chemical reaction, provided milder condition for the process. Moreover, the selectivity is higher, leading to less by-product formation; no corrosion issue is involved and higher yield may be achieved. These benefits made it appealing for carotenoids extraction (Deenu et al., 2013). However, the high price of the enzymes and the requirement to maintain a stable condition largely limit its applications, and the longer hydrolysis time offers less potential for processing microalgae for larger scale industrial applications (Kadam et al., 2013; Zheng et al., 2011). Chen et al. (2013) conducted the co-cultivation of bacteria with algae to lyse cell walls, which would be an interesting alternative to traditional enzymatic hydrolysis (Kim et al., 2015).

2.8.2.2.4 Pulsed electric field

The pulsed electric field approach for cell disruption was developed since the 1990s. This is a non-thermal treatment that requires much lower energy input. By applying high intensity intermittent electric field on the cells for periods of time in the order of micro-seconds, pulsed electric field assisted extraction improves the membrane permeability in the cell membranes by electroporation (Luengo et al., 2014). The pores formed can be controlled to be reversible or irreversible by adjusting the intensity of the electric field. In the last decade, pulsed electric field has been shown to be an efficient method to extract lipids and bioactive compounds from microalgae and plant tissues (Barba et al., 2015; Mercer and Armenta, 2011); this highly selective extraction method showed good ability in retaining the bioactivity of carotenoids (Grimi et al., 2014; Sánchez-Moreno et al., 2005). The short treatment time and smaller solvent requirements (Yu et al., 2015) makes it extremely attractive for carotenoids extraction. However, although this method can improve the extraction efficiency, it does not perform as well as HPH (Lai et al., 2014); and for the extraction of carotenoids by this method, organic solvent requirements remain essential (Luengo et al., 2014).

2.8.2.2.5 Osmotic shock, acid/ alkaline treatment, ionic liquids

The advantages of easy scale-up, low energy input, and modest capital cost make the physical or chemical treatment methods such as osmotic shock, acid/alkaline treatment methods attractive particularly in the operation aspects (Kim et al., 2015). However, osmotic shock is not capable of extracting pigments for cells with rigid cell wall; on the contrary, and although cell breakage was achieved at high efficiency by acid/alkaline treatment, the carotenoids were destroyed (Halim et al., 2012a).

Ionic liquids have superior solubility of biomass and recently have been studied extensively. Ionic liquids act as cell destabilizers in algae suspensions (Park et al., 2015), but the high price of ionic liquid based solvents, high energy requirement, and toxicity currently has prevented their industrial applications. In addition, the ionic liquids would cause carotenoids to degrade since they are not inert solvents. Therefore, ionic liquids are not suitable for carotenoids production.

2.8.3 Extraction

Extraction is a vital step for pigment production. In addition to the conventional solvent extraction approaches, the development of super-/sub-critical extraction offers a new concept of operation while the cost greatly limited the use.

2.8.3.1 Conventional solvent extraction

Since “like dissolves like”, a similar polarity is vital for efficient recovery of target compounds from a mixture. Chloroform/methanol, hexane/isopropanol, ethanol and other solvents were widely used for efficient extraction (Sicaire et al., 2014). The Bligh & Dyer method and Soxhlet are most commonly adopted in the small scale chemical engineering processing as mature protocols (Dejoye Tanzi et al., 2013; Hita Peña et al., 2015). The principles and operational considerations of solvent extraction of lipids or bioactive products from microalgae have been widely reviewed, but few focused specifically on the extraction of carotenoids (Gil-Chávez et al., 2013; Reverchon and De Marco, 2006). In Taucher et al.'s study (2016), dichloromethane is the optimal solvent among the six tested solvents. The best extraction temperature is at 60°C. Apart from single solvent extraction, binary extraction systems are also reported. Dichloromethane and methanol, chloroform and methanol, acetone and petroleum ether, and hexane and ethanol all presented better results than single solvent for carotenoids extraction (Soares et al., 2016).

The conventional solvent extraction is cheap and easy to scale up, the concern is these processes may take a long time, require further treatment like evaporation to concentrate the extract, and cost large amounts solvents, which brings an environmental burden, so other more advanced extraction methods are developed (Halim et al., 2012a).

2.8.3.2 Super-/sub-critical solvent extraction

Supercritical fluid extraction (SFE) has been well documented in the literature for valuable compound recovery from microalgae (Liau et al., 2010; Patil et al., 2013). Supercritical fluids have a similar density as fluid, but a similar viscosity as gas. The high pressure forces supercritical liquid into the cells, as the supercritical liquids have a diffusion rate similar to gas, the mass transfer is greatly enhanced, thus the extraction time can be much shorter

than for conventional solvent extraction. Mature processes have been developed to use SFE for decaffeination and essential oil extraction (Gil-Chávez et al., 2013). Also, as the polarity of solvent is tunable, SFE can be more selectively used to extract the target product than conventional solvent extraction (Guedes et al., 2013). Yen et al. (2015) have recently reviewed the advantages and challenges facing SFE extraction from microalgae biomass. Halim et al. (2012a) reported that eight minutes of SFE treatment would have a better extraction result than 5.5 hours conventional solvent extraction.

In SFE, chemical solvent usage is minimized or eliminated. CO_2, for its relatively cheap price, safety, non-toxicity, and chemical inertness, and suitable critical temperature, become the most popular solvent (Daintree et al., 2008; Wang et al., 2008). In this case, the extractant can be separated easily from solvent if no co-solvent is added since CO_2 is gaseous under normal conditions. The drawback of CO_2 as a supercritical solvent is its low polarity is not suitable for polar compound extraction. To compensate for this, polar co-solvents can be added. Ethanol is a good choice for carotenoids extraction due to its suitable polarity and non-toxic nature; moreover, it is miscible with CO_2 and is approved for nutraceutical and pharmaceutical use (Reverchon and De Marco, 2006). The addition of co-solvents may not be attractive in terms of extractant separation since ethanol is a liquid at room temperature. Meanwhile, the addition would alter the mixture's critical point, requiring harsher conditions (Reverchon and De Marco, 2006).

Sub-critical CO_2 extraction is more practical due to less strict environment control requirement than SFE. Sub-critical refers to a condition at which the temperature ranges from boiling point to critical point, and pressure sufficient to maintain the fluid state. Lutein, β-carotene, and astaxanthin extraction by sub-critical CO_2 have been extensively studied and numerous investigations have focussed on the optimization of extraction conditions (Mendes et al., 2003; Herrero et al., 2006). Pressure, temperature, fluid flow rate, and the addition of co-solvents are the critical factors that affect the extraction yield (Chen et al., 2012). Pressure up to 35 MPa, temperature from 40-45 ^{o}C, with 5% ethanol addition are the common conditions for sub-critical CO_2 extraction (Du et al., 2015). 1,1,1,2-tetrafluoroethane is also used for the extraction of carotenoids for it has more reasonable critical points (Lu et al., 2014).

Water is another environmental-friendly solvent for SFE use. The change of liquid parameters would turn water into a less polar solvent having similar dielectric constant as ethanol. However, it may not work as well with carotenoids, since high temperature may destroy the functional activity of the carotenoids (Gil-Chávez et al., 2013).

A two-stage operation of SFE is also suggested for more selective outcome, with the first stage using low density CO_2 (300 bar) for non-polar or volatile lipid compounds, and the second stage with high density CO_2 with co-solvent (500 bar) for more polar carotenoids and other products (Reverchon and De Marco, 2006). The pilot plant design is available in Soto's review (Rosello-Soto et al., 2016).

2.8.3.3 Other extraction methods

Other extraction processes have been considered previously. Switchable solvent extraction system refers to a solvent that can switch its polarity under different atmospheres, it has been proposed for the lipid extraction from algae. However, similar to ionic liquids discussed in section 2.8.2.2.5, it may not be suitable for carotenoids extraction (Boyd et al., 2012; Du et al., 2015). OriginOil has developed a single-step electromagnetic field process for dewatering, cell disruption, and lipid recovery. *In situ* extraction has recently gained more attention, it aims to obtain the target carotenoid without killing the cells. For review, refer to Kleinegris et al. (2011). However, to date, the solvents used for extraction are not capable to efficiently extract out the carotenoids.

2.8.3.4 Wet extraction

Similar to cell disruption, drying is another energy-consuming step. Attempts are focused on elimination of this step by using wet algae for direct extraction. Freeze drying is a more preferred drying method due to its mild conditions compared with spray or oven drying, which often lead to degradation of thermal-liable products and loss of volatile lipids coupled with the nonuniform particle size (Park et al., 2015). Wet extraction methods have been developed to overcome the bottleneck of high energy consumption (Park et al., 2015).

The effects of wet algae on extraction efficiency is still unclear. On the one side, the hypothesis is that the presence of residual water will adversely affect the extraction

efficiency as water forms a barrier that prohibits the solvent mass transfer from inside the cell to the outside. Although wet algae have been successfully used for carotenoid extraction by SFE in the presence of co-solvent (Chen et al., 2012), the presence of water may cause many problems, since super-/subcritical CO_2 may degrade carotenoids by its potential catalytic effects for the hydrolytic-based reactions in the presence of water. The presence of water may result in flow impedance and restrictor plugging and channeling, and formation of highly compacted bed within the vessel (Barbosa-cánovas, 2015).

An alternative hypothesis is that residual water in the biomass will improve the carotenoids extraction, as the presence of water swells the cell and facilitates the lysis of cell wall, allowing better chance of solvent to access the inner cell content and enhance mass transfer. Du et al. (2015) suggested that the existence of water may swell the center of the cell matrix and act as a polar co-solvent to facilitate extraction.

Soh and Zimmerman (2011) studied the effect of moisture content (up to 20%) on the extraction efficiency; no obvious change was observed in their study. Unlike SFE, mechanical cell disruption methods and solvent extraction are highly effective with wet extraction. Jiménez Callejón et al. (2014) harvested tripled amount of lipids than the conventional Bligh & Dyer method. The study by Halim et al. (2012b) showed reduced efficiency for lipid extraction using non-polar system, while in the polar system, the extraction efficiency was enhanced with moisture. Sarada et al. (1999) also concluded that the usage of fresh biomass may reduce up to 50% pigment loss during the drying process. Therefore, wet processing has more potential for future carotenoids production studies.

2.8.4 Purification

The current carotenoids purification method is developed based on the Willstatter method (Burdick, 1956). Organic solvents are used for separation of carotenoids after saponification for the solution containing crude carotenoids. The detailed method is described in Fig. **2-5**, after microalgae extraction, NaOH or KOH are usually added to the microalgae extract as saponification agents to release the carotenoids from their naturally occurring ester form. In this step, temperature is usually kept below 60^{o}C to prevent carotenoids degradation (Yuan and Chen, 2000). Saponification conditions such as time,

temperature, and alkali concentration, as factors affecting yield, have been investigated in many studies (Chan et al., 2013; Palumpitag et al., 2011). KOH concentration of 2.5-40% are most commonly used below 60^{o}C, reacting for hours to overnight.

Solvent extraction is then applied to obtain the non-water-soluble compounds from the saponified microalgae extract. In the past, hexane was often used, while EtOH-Water-CH_2Cl_2 solvent system was used to treat saponified solution with better efficiency (Li et al., 2006). Chlorophyll is removed by alkaline hydrolysis, and converted to salts that are soluble in water. The unsaponifiable compounds will appear in the organic phase in the following extraction step, including carotenes, xanthophylls, waxes, phospholipids, sterols and phytol split from chlorophylls (Burdick, 1956). Relative solubility and intermolecular attractions of different binary solvent systems were also studied for the better separation of carotenoids (Dineshkumar et al., 2015).

The solvents are then concentrated for subsequent purification, recrystallization or chromatography can be used to further purify the crude product. Due to the presence of residual water in the organic solvent, Na_2SO_4 can be added then filtered out as $Na_2SO_4 \cdot (H_2O)_2$ to remove trace water (Nobre et al., 2006).

The described multi-step process is time-consuming and requires large amount of solvents. More advanced purification methods are also reported such as selective absorption of lutein on solid phase (Shen et al., 2011); expanded bed coupled column chromatography method (Fernández-Sevilla et al., 2010); and reversed phase HPLC or high speed counter current chromatography for a small quantity of high purity carotenoids (Chen et al., 2005; Li et al., 2006; Li and Chen, 2001). Supercritical anti-solvent precipitation can generate solid carotenoids within a few minutes (Liau et al., 2010; Shen et al., 2011). However, the cost of these methods are higher than the conventional multi-step process. Therefore, a cost effective simple process is desirable for high purity carotenoids production.

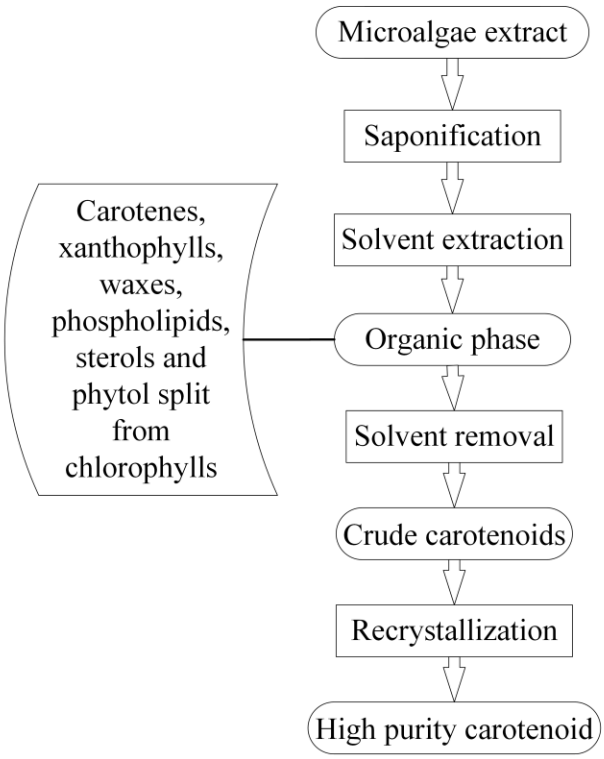

Figure 2-5 Schematic description of the general purification of carotenoids

2.9 Storage stability

Carotenoid degradation is catalyzed by oxygen and light and accelerated by heat (Shen & Quek, 2014). Carotenoids shown to be more stable than chlorophylls under short time (5 min) high temperature and high pressure treatments up to 117 ^{o}C and 625 MPa (Sánchez et al., 2014). Dias et al. (2014) studied carotenoids stability over a long term. An inert atmosphere, lower temperature (-20^{o}C), dark with the presence of anti-oxidant butylated hydroxytoluene (BHT) are suitable conditions for carotenoids storage up to 6 months (Chan et al., 2013). During degradation, the all *trans* form pigment would be transformed into *cis* forms. The rate is reported to fit a first order model (Tang and Chen, 2000). Crystalline lutein is easy to degrade (Fernández-Sevilla et al., 2010), so it is more commonly sold in ester form or suspended in vegetable oil. Sunflower oil is reported to be a better choice than olive oil since the tocopherols from it can act as an antioxidant (C. Y. Chen et al., 2016). Microencapsulation can better protect carotenoids as well (L. Chen et

al., 2016). Similiarly, the complex of astaxanthin with hydroxypropyl-beta-cyclodextrin may slightly increase the stability (Yuan et al., 2013). To prevent astaxanthin degradation, 180/110 °C is the best temperature to spray dry *H. pluvialis* biomass while -21 °C under nitrogen can preserve astaxanthin for nine weeks (Raposo et al., 2012). In addition, the lycopene stability is also higher when not extrcted out from tomato, indicating free form of carotenoids may alleviate the degradation (Sharma and Le Maguer, 1996). More studies would benefit the understanding of storage conditions of carotenoids.

2.10 Market and economics

Due to the vibrant color and anti-oxidant related health boost properties, strong demands on carotenoids was raised in recent years. The overall carotenoid market was estimated to be $1.5 billion, with β-carotene, lutein, and astaxanthin occupied over 60% market share, and the global market was estimated to reach $1.8 billion by 2019 (Business Communications Company, 2015). Among the ten carotenoids best marketed, β-carotene, lutein, astaxanthin, and canthaxanthin could be efficiently produced by microalgae (Zaghdoudi et al., 2015). Their applications include food supplements, food colorant, feed additives, cosmetics, and drugs (Borowitzka and Mohemani, 2012).

To date, two successful stories of commercialized microalgal carotenoids production are astaxanthin from *H. pluvialis* and β-carotene from *D. salina*. The cost of synthetic astaxanthin is over $1000/kg, while the sales price is over double of that (Li et al., 2011). Li et al. estmated $718/kg cost to produce astaxanthin from microalgae, while it may be further decreased by lower microalgae cost according to Kleinegris et al. (2011), see also in section 2.7.1. Apart from raising the production rate and pigment content, a few attempts have been conducted to reduce the cost. Tran et al. (2014) tried to reduce the cost by recycling medium. Currently, the bottleneck to further reduce production cost is in the harvesting and extraction steps (Barba et al., 2015).

Lutein is another major market sharer. Currently produced by marigold flowers with a price of $500/kg, the natural product has a cost even lower than synthetic products. Growth rate, nutrient requirements, pigment content, tolerance to the environmental fluctuations are all significant for the strain selection and economic considerations. Additionally, consumer

acceptance of new functional food may as well affect the microalgal carotenoids market development (Freitas et al., 2012).

Apart from the carotenoids, microalgae can potentially be used for valuable by-products producing, like biodiesel, EPA, DHA, vitamins, proteins, and enzymes. Therefore, considering other compounds like biofuels as a by-product in the microalgal carotenoids production might be a new potential. Dineshkumar et al. (2015) extracted lutein and biodiesel using the same algae in one process, and got satisfactory yield for both products (6 mg/g lutein and 94 mg/g FAME). A few works have been conducted in this area for integrated one-step biodiesel and lutein production (Araya et al., 2014; Dineshkumar et al., 2015; Hodgson et al., 2016). Fucoxanthin and lipids from hexane-ethanol system was also established (Kim et al., 2012). Other products have not been reported to be produced simultaneously with carotenoids, but since microalgae is known to produce sterols, protein, sugars, vitamins, and moreover, the biomass itself is an edible product that is common in Korea and Japan. Hence the potential to obtain multiple products from microalgae exists, given better separation technology is developed.

2.11 Conclusions

In this review, the entire process starting from carotenoid synthesis, cultivation of microalgae, harvesting, extraction, till purification and storage is discussed. The chemical total synthesis is a well established process, contributing to the majority of the global market, but its safety to human direct consumption is questionable (Ye et al., 2008). Natural carotenoids from biosynthesis are gaining market preference due to the health effect of carotenoids are better understood nowadays. Microalgae are excellent hosts for the mass production of carotenoids since these uni-cellular microorganisms have high carotenoid content, fast growth and many other advantages. The biotechnologies including high efficiency photobioreactors and optimized growth conditions are applied in the cultivation of carotenoid rich microalgae. However, cheaper and more scalable cultivation strategy is still under investigation. Great challenges remain in the downstream processing especially the harvesting and cell disruption. While centrifugation is one of the most popular harvesting methods, appropriate energy reduction approaches are necessary. Flocculation

can be an alternative, but the efficiency remains to be improved. For the carotenoids extraction, although the pretreatment step is generally considered necessary these years, the efficiency still needs to be improved especially for the small cells with a rigid cell wall. The recent developments of extraction process enabled the scale-up of some cell disruption methods, and allowed some non-conventional extraction and purification; among the various methods, microwave assisted extraction and pulsed electric field are the most promising methods considering efficiency and cost.

Further research should emphasize on the productivity improvement and cost reduction. Advanced metabolic engineering tools such as CRISPR/Cas9 can be applied for high through-put strain development. For the production of high quality carotenoids, the concern of degradation during the production and storage should not be ignored. The linkage between physiological mechanisms of carotenogenesis should be considered to develop the species-specific growth strategy and the cell wall disruption method. The novel methods like *in situ* extraction may be interesting directions for further investigation, while switchable solvents extraction may have good potential if non-toxic solvent can be screened out and the bioactivity can be retained. To save the drying cost, wet extraction methods present another future direction. In addition, as microalgae generate a variety of valuable products, research attempts should be given to the simultaneously production of multiple products to develop a more economically attractive and sustainable microalgae industry. Microalgal carotenoids production have good potential with a lot of challenges to overcome, especially in the cost reduction in downstream processing. In summary, carotenoid production from microalgae is an attractive and potentially growing market. There is still a need for better engineering design and innovation to make the processes more cost competitive.

Chapter 3

3 *Chlorella vulgaris* UTEX 265 cultivation under light and low temperature stressed conditions for lutein production in the flask and the coiled tree photo-bioreactor (CTPBR)

The information presented in this Chapter is based on the paper of the same title, submitted to Applied Biochemistry and Biotechnology. The sections in this chapter present the results towards the completion of objectives 1 and 2 of the thesis (see section 1.2.2).

3.1 Abstract

Lutein has an increasing share in the pharmaceutical and nutraceutical market due to its benefits to eye health. Microalgae may be a potential source for lutein production while the expense limits the commercialization. In this study, a coiled tubular tree photobioreactor (CTPBR) design was investigated for cultivating the cold tolerant microalgae *Chlorella vulgaris* UTEX 265 under various conditions for lutein production. The influence and interaction of light irradiance strength, lighting cycle and temperature on microalgae and lutein production efficiency at low temperature range were also studied in flasks via response surface method (RSM). The results demonstrated that moderate light, shorter light cycle, and higher temperature were favorable to the growth and lutein production of *C. vulgaris* at experimental ranges. *Chlorella vulgaris* UTEX 265 showed good potential to produce lutein at cold weather, and the optimum lutein production was contrary to the specific lutein content but corresponds to the trend of optimum growth. Additionally, fast growth and good lutein recovery in CTPBR were also achieved at the low irradiance stress condition and the low temperature photo-inhibition condition compared to stirred vessels or flasks.

3.2 Introduction

Belonging to the diverse group of carotenoids, lutein is a dietary xanthophyll that has been clinically proven to protect people against age-related macular degeneration and cataract formation.(Bone and Landrum, 2003) For this reason, lutein had the second largest share (US $233 million) in the $1.2 billion global carotenoid market in 2010, and should have an

increasing share in this market in next few years as the overall carotenoid market increased to $1.5 billion in 2014 (Gong and Bassi, 2016). Traditionally, lutein is produced from marigold flowers (*Tagete erecta* and *Tagete patula*); however, due to the biological and operational benefits of microalgae, including higher lutein content, flexible cultivation conditions, fast growth, and the less labor required for operation, microalgae have become an interesting alternative for lutein production (Gong and Bassi, 2016). The bottle-neck preventing it from commercialization is the lack of a cost-effective method for the large-scale production in non-tropical weather areas.

Microalgae have been investigated extensively for the production of biodiesel and other bioactive compounds. To produce microalgae, the closed photo-bioreactor (PBR) systems are considered more efficient than open pond systems. Tubular PBRs offer higher light capture efficiency through higher surface to volume ratios, and are easier to scale-up (Briassoulis et al., 2010). In this experiment, a conically shaped helical tubular reactor was selected addressing the concerns of both the land-use footprint in regular tubular reactors and light shading in helical tubular reactors. Few studies have reported on conical, helical tubular photo-bioreactors with respect to photo-efficiency and performance except Morita *et al.* (Morita et al., 2002). They revealed enhanced photosynthetic productivity in a reverse conical shaped helical tubular photo-bioreactor.

Two types of stress conditions concerning light and temperature are identified in this study. Firstly, since 250 to 500 µmol photon m^{-2} s^{-1} is typically the favorable light range at around 20^{o}C for microalgae cultivation (Shriwastav and Bose, 2015), few studies have reported on bioreactor performance below 100 µmol photon m^{-2} s^{-1}. However, indoor lighting, a common approach for closed PBR operations, often fall in this low light range (Zigman and Review, 2008). Among the previous studies under limited irradiance, the phototrophic microalgae growth was found to be poor, *i.e.* Javanmardian and Palsson (Javanmardian and Palsson, 1991) found the growth rate below 0.1 d^{-1} under 76 µmol photon m^{-2} s^{-1}. Therefore, a coiled tree photo-bioreactor (CTPBR) was developed for rapid microalgae growth at light limited conditions, e.g. at light intensity below 85 µmol photon m^{-2} s^{-1}. The growth kinetics of *C. vulgaris* and lutein production in CTPBR under light limited conditions were systematically investigated and is discussed in this paper.

The other stress condition lays on the outdoor cultivations. Large scale PBRs operating year round will have to rely on natural light to minimize energy costs and hence must address the significant challenges of the cold weather (Canadian) environment. For the Canadian context and environment with long winters, low temperatures are common (average annual temperatures of 0-20^{o}C) (Environment Canada (climate.weather.gc.ca)). However, most microalgae, apart from some psychrophilic algae species, prefer high or moderate temperatures to grow (Mayo, 1997). Cold temperatures are often associated with the energy imbalance with light, or low-temperature introduced photo-inhibition due to the lowered metabolic enzyme activities (Davison, 1991; Gray G.R., 1998). Thus it is important to investigate suitable PBR configurations for such conditions. Sánchez et al. (J. F. Sánchez et al., 2008) have studied the effect of light and temperature on lutein productivity from *Scenedesmus almeriensis* at high temperature conditions up to 48°C. However, cultivation at low temperatures and different irradiance has not been previously investigated, not to mention for lutein production.

In this current study, growth kinetics of *C. vulgaris* under the two types of stress conditions are compared for a conical configuration in a coiled tree photo-bioreactor (CTPBR). The feasibility to cultivate microalgae in this CTPBR under varied temperatures ranging from 4^{o}C to 22^{o}C was first systematically investigated for biomass and lutein accumulation. The influence and interaction of light irradiance strength, lighting cycle and temperature on microalgae and lutein production efficiency at low temperature range were also investigated via response surface method (RSM) to capture a holistic picture of the capabilities of microalgae *C. vulgaris* for lutein production.

3.3 Materials and methods

3.3.1 Algal strain and culture medium

Chlorella vulgaris UTEX 265 (University of Texas, Austin, TX) was maintained in Bold's Basal Medium (BBM), containing (per L): 0.25 g $NaNO_3$, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.075 g K_2HPO_4 0.175 g KH_2PO_4, 0.025 g NaCl, 0.005 g EDTA (anhydrous), 3.1 mg KOH, 0.05 mg $FeSO_4 \cdot 7H2O$, 0.11 mg H_3BO_3, 0.088 mg $ZnSO4 \cdot 7H_2O$, 0.014 mg $MnCl_2 \cdot 4H_2O$, 0.007 mg, 0.016 mg $CuSO_4 \cdot 5H_2O$, 0.005 mg

$Co(NO_3)_2 \cdot 6H_2O$. All the chemicals were dissolved in double-distilled water. The initial pH of culture was adjusted to 6.8 ± 0.2.

3.3.2 CTPBR design

The coiled tubular tree photobioreactor (CTPBR) was made of borosilicate glass with an effective volume of 0.34 L (Figure **3-1**). The largest circular diameter of the reactor loop was at the bottom and was 150 mm; the height of the reactor was 500 mm, with inner and outer coil reactor tube diameters of 9 and 12 mm. As shown in the scheme in Fig. **3-1**, the tubes were coiled and spaced in the shape of a conical helix (inclined 6.5° to the vertical axis).

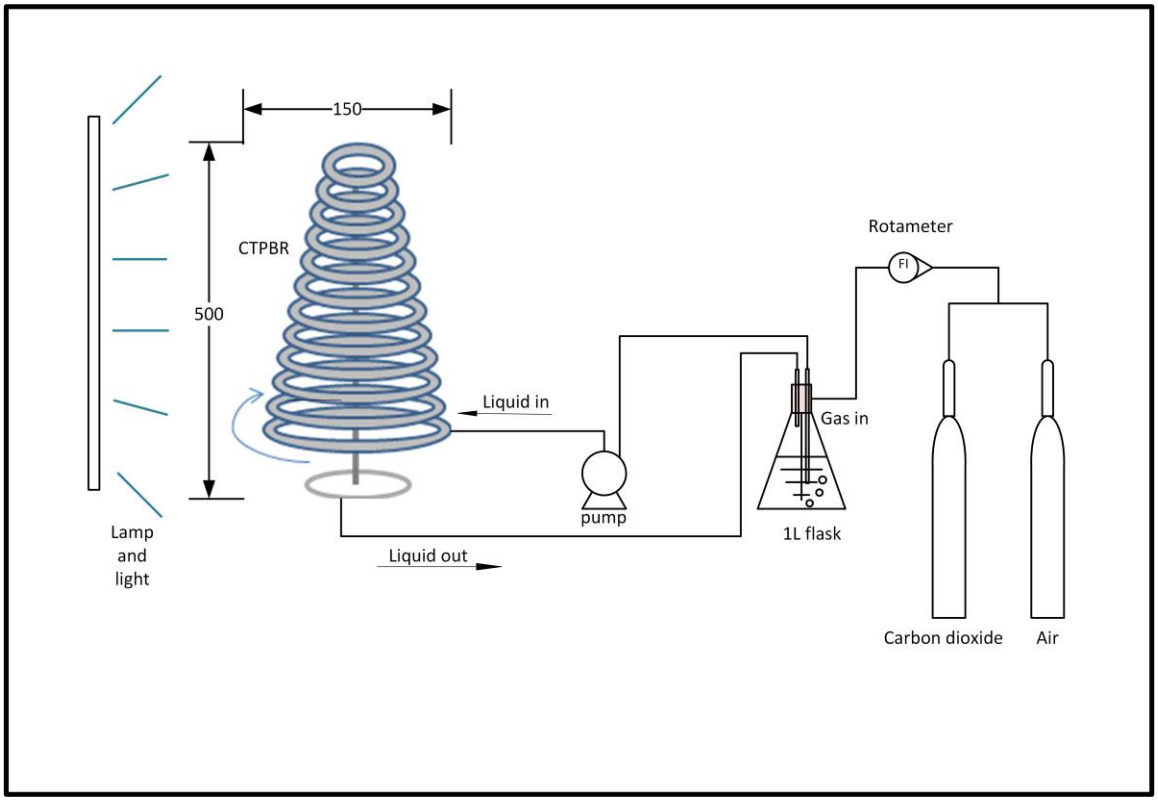

Figure 3-1 Illustration of the coiled tubular tree photobioreactor (CTPBR) and its operation system. The arrows show the direction of inoculum flow with the rotation of the pump. A peristaltic pump was used for liquid circulation at a rate of 8 mL s^{-1}, culture enters the reactor from bottom and exits though the top before arrive the 1 L Erlenmeyer flask which functioned as a mixer, carbonator, and degasser. Air was humidified and

filter sterilized through a 0.2 µm pore size filter before being introduced into the flask containing growth medium through a spherical stone sparger (diameter of 2 cm) at flow rate of 0.20 vvm (volume per volume per minute).

3.3.3 Culture system and operation conditions

The entire setup of the CTPBR and associated tubing was autoclaved to avoid contamination and the nutrient medium was autoclaved separately and added later. The bioreactor system illustrated in Figure **3-1** had a total working volume of 1.2 L, including flask, tubing and the CTPBR.

Standard 1 L Erlenmeyer flasks were used in contrast to CTPBR. The bottom diameter of the flask is 12 cm, diameter of the flask neck is 5 cm, and the total height of the flask is 20 cm. For each experiment in flask and the CTPBR cultivation, the microalgae culture inside the flask was controlled to be 825±25 mL. The working liquid height is 9 cm in flask. For each experiment, 9±1% (v/v) *C. vulgaris* inoculum was added to make the starting concentration ~0.04 g/L, the concentration was determined by optical density measurements at 687 nm (OD_{687}). The inoculum was pre-prepared by culturing in sterilized BBM. The CTPBR and the Erlenmeyer flasks were illuminated by 18 inch GE cool-white fluorescent lamps or by the lighting fixtures in the environmental growth chambers in Biotron Experimental Climate Change Research Centre at Western University, London Ontario. The light intensity, specified as the photo-synthetic activated radiation (PAR), was measured by an Apogee MQ-200 quantum meter (Apogee Instruments Inc., USA). The external irradiance of the flasks was measured in the center of the flask filled with cell-free BBM. Light falling on the top, middle and bottom of the CTPBR was measured by attaching the light probe outside the reactor wall toward the light source, and the average PAR was calculated to be the external irradiance of the CTPBR. The light intensity was adjusted by controlling the number of the fluorescent lamps used in the lab. Specifically, due to the physical arrangement limitations in space, one lamp corresponds to 25 µmol photon m^{-2} s^{-1}, two lamps 54 µmol photon m^{-2} s^{-1}, and 4 lamps 85 µmol photon m^{-2} s^{-1}. In the environmental growth chambers, the light intensity was adjusted by controlling the

distance of the culturing flask or reactor to the light source. Temperature control was achieved by adjusting the settings of the environmental growth chambers. Air was bubbled in as the sole carbon source (CO_2). Each experimental run was triplicated and as well triplicate samples were taken for each cell density measurement.

3.3.4 Determination of cell dry mass

The dry mass of microalgae was measured using the method described by Aguirre and Bassi (Aguirre and Bassi, 2013). Three 1.5 mL samples were taken on a daily basis from each inoculum. The OD_{687} readings were taken by a Cary Bio 50 UV/Visible Spectrophotometer (Varian, Inc., USA) and the mean of three samples was taken. For cell dry weight measurements, the diluted solution was vacuum filtered through a 0.45 µm pore-size cellulose nitrate membrane, then the microalgae with the membrane were dried at 60^{o}C for 24 hours or till the weight became constant to get the cell dry mass. The biomass was collected at each growth condition at the end of exponential phase to develop the standard curve for dry cell weight versus OD_{687} developed. For instance, for cultivation at 22 ± 1^{o}C, 16 h/8 h (light/dark) photoperiod cycle, at 54 µmol photon m^{-2} s^{-1} gave a standard curve as: Cell dry mass (g L^{-1}) = 0.18×OD_{687}, R^2 = 0.998.

3.3.5 Determination of specific growth rate

The specific growth rate (µ) was measured as per equation (3-1) below:

$$\mu = \frac{1}{t_m} \ln \frac{X_m}{X_0} \qquad (3\text{-}1)$$

where X_m is the maximum biomass concentration and X_0 is the initial biomass concentration; t_m is the time at which X_m was obtained.

3.3.6 Nitrate level measurement

The nitrite concentration was measured by chromotropic acid method using Hach DR 2800 (0.2 to 30.0 mg/L NO_3–N) Test ‘N Tube™ Vials (Hach Canada Ltd., London, ON, Canada).

3.3.7 Lutein measurements

The lutein analysis was carried out by HPLC (method adapted from Maxwell *et al.* (1994) with minor changes). An Acclaim C30 column (Thermo Scientific, USA) was used in an Agilent LC 1100 series (Agilent Technologies, Canada) system. The sample was eluted at 1 ml/min (acetonitrile/methanol (9/1, v/v)) for 5 minutes then in a 4-minute gradient change to 100% ethyl acetate. Re-equilibration between sample injections was carried out for 6 minutes. The total analytical run time was 13 minutes. Lutein standard was purchased from Sigma-Aldrich (NJ, USA). Standard curves were obtained by injecting five serial dilution samples ranging from 5 to 100 mg mL^{-1}. Each point was triplicated and the R^2 was above 0.990.

The retention time and peak area were used to determine the pigment contents in analytical samples. The dry weight of sample was determined by taking 50 mL of cell culture in a centrifuge tube, centrifuged and then washed with distilled water, and the cell pellet was weighed after dried at 60°C for 24 hours, three samples were taken, and the mean was used as the accurate cell dry weight. Equal amount of cell culture was centrifuged, washed and transferred to a 2-mL sample vial with 0.4 g of zirconia/silica beads (0. 1 mm diameter). Ethanol was added to fill the vial and get rid of air. The wet cells were bead-beaten in a Mini-Beadbeater (Biospec, USA) for one minute. The treated cells were cooled in an ice bath for 1 minute, then centrifuged for 3 minutes at 3500 rpm. The supernatant was then collected and fresh solvent added. The same process was repeated till the extract was colorless. The supernatant samples were combined and fresh solvent were added to make a 10 mL volume and filtered through a 0.22 um PTFE filter for HPLC analysis.

3.3.8 Experimental design and statistics

Response surface methodology was employed to study the irradiance strength, temperature and light/dark cycles. The Central Composite Design (CCD) was used with three factors and five levels. The codified CCD design sheet is presented in Table **3-1** with actual factor values, in columns 2, 3 and 4. The experiments were conducted in randomized sequence. The response variables were modeled by experimental parameters of Temperature (X), external irradiance (Y), and hours of light per day (Z). The interaction coefficients and

quadratic terms were also included to develop a second order equation. All the experiments were carried out in 15 days, and the lutein measurements were carried out on the last day. The analysis of variance (ANOVA) was employed to measure the significance of regression coefficients with a confidence level of 95%, and the model accuracy was evaluated by the regression coefficients of R^2 and adjusted R^2 (adj-R^2). The software Minitab 17.0 (Minitab Inc., USA) and Matlab 2016b (Mathworks, USA) were used to help carry out the statistical analysis.

Table 3-1 The low temperature study of specific cell growth rate (μ), lutein content and specific lutein productivity of *C. vulgaris* in 1 L flasks by response surface method (RSM) with three factors: Temperature, irradiance, and light/dark cycle. The design sheet of actual experimental ranges is reported with normalized values in the bracket. The response values are recorded with the model fitted values.

R-un N-o.	Temperat-ure	Irradiance strength	light hours per day	specific growth rate	fitted value	Lutein content	fitted value	specific lutein productivity	fitted value
	°C	(μmol m^{-2} s^{-1})	h d^{-1}	d^{-1}	d^{-1}	mg g^{-1}	mg g^{-1}	mg $g^{-1}d^{-1}$	mg $g^{-1}d^{-1}$
5	1 (-2)	242.5 (0)	17 (0)	--	--	3.21	--	--	--
21	4 (-1)	125 (-1)	14 (-1)	0.18	0.16	7.80	7.98	1.40	1.35
10	4 (-1)	125 (-1)	14 (-1)	0.19	0.16	8.20	7.98	1.56	1.35
16	4 (-1)	125 (-1)	20 (1)	0.07	0.09	5.92	6.30	0.41	0.56
20	4 (-1)	125 (-1)	20 (1)	0.08	0.09	6.75	6.30	0.54	0.56
4	4 (-1)	360 (1)	14 (-1)	0.17	0.19	5.46	5.38	0.94	1.04
11	4 (-1)	360 (1)	14 (-1)	0.18	0.19	5.18	5.38	0.93	1.04
23	4 (-1)	360 (1)	20 (1)	0.07	0.07	4.68	4.63	0.33	0.31
8	4 (-1)	360 (1)	20 (1)	0.08	0.07	5.01	4.63	0.39	0.31

14	7 (0)	7.5 (-2)	17 (0)	0.12	0.13	6.40	6.50	0.77	0.86
9	7 (0)	242.5 (0)	11 (-2)	0.29	0.31	5.70	5.40	1.68	1.73
2	7 (0)	242.5 (0)	17 (0)	0.16	0.16	4.86	4.71	0.78	0.73
7	7 (0)	242.5 (0)	17 (0)	0.14	0.16	4.68	4.71	0.66	0.73
18	7 (0)	242.5 (0)	17 (0)	0.17	0.16	4.39	4.71	0.75	0.73
20	7 (0)	242.5 (0)	17 (0)	0.16	0.16	4.43	4.71	0.71	0.73
13	7 (0)	242.5 (0)	17 (0)	0.18	0.16	4.32	4.71	0.78	0.73
3	7 (0)	242.5 (0)	23 (2)	0.18	0.15	3.77	4.03	0.68	0.62
22	7 (0)	477.5 (2)	17 (0)	0.06	0.03	3.90	4.21	0.23	0.14
15	10 (1)	125 (-1)	14 (-1)	0.28	0.29	4.23	4.59	1.18	1.34
26	10 (1)	125 (-1)	14 (-1)	0.28	0.29	4.94	4.59	1.38	1.34
17	10 (1)	125 (-1)	20 (1)	0.26	0.25	4.14	3.96	1.08	0.96
25	10 (1)	125 (-1)	20 (1)	0.24	0.25	4.31	3.96	1.03	0.96
1	10 (1)	360 (1)	14 (-1)	0.24	0.22	4.33	3.98	1.04	0.88
12	10 (1)	360 (1)	20 (1)	0.10	0.13	4.32	4.28	0.43	0.56
19	10 (1)	360 (1)	20 (1)	0.09	0.13	4.61	4.28	0.41	0.56
6	13 (2)	242.5 (0)	17 (0)	0.35	0.33	3.48	3.90	1.22	1.21

3.4 Results and discussion

The unicellular micro-algae *Chlorella vulgaris* UTEX 265 was selected for its high growth rate, easy handling, and good temperature adaptability. Since the focus was specifically on growth kinetics, the volume and size of the coiled tubular tree photobioreactor (CTPBR) was deliberately kept small to avoid mass transfer, heat transfer, and light limitations due to the low light conditions in the study.

3.4.1 Effectiveness of CTPBR at low irradiance stress condition

The light irradiance strength plays a major role in the microalgae cell growth and product profile, and is a common limiting factor of autotrophic growth (Chu et al., 2013). The coiled tubular tree photobioreactor (CTPBR) was designed to enhance light utilization at low light intensities. This configuration demonstrated advantages over standard stirred systems in both growth rate and the biomass density, as presented in Fig. **3-2** (a). The biomass concentration in both CTPBR and the flask exhibited linearly incremental trend under 25 µmol photon m^{-2} s^{-1}, while the slope was smaller in the flask. The final biomass concentration obtained was 0.56 g L^{-1} in the CTPBR and 0.34 g L^{-1} in the conical flask. The initial µ in CTPBR was 0.59 d^{-1}, which was 64% higher than the value of the flask (0.36 d^{-1}).

Figure **3-2** (b) describes the experiments in the CTPBR under two different low light conditions, *i. e.* 54 µmol photon m^{-2} s^{-1} and 85 µmol photon m^{-2} s^{-1}, and also in the 1 L Erlenmeyer flask under 54 µmol photon m^{-2} s^{-1}. An exponential trend was observed for the growth profiles of CTPBR under 54 and 85 µmol photon m^{-2} s^{-1}. In contrast, the cells in the flasks showed a linear growth behavior, which is typically seen in algal cultures and indicates light limitations. The initial specific growth rate, µ, achieved for 54, 85 µmol photon m^{-2} s^{-1} in CTPBR and 54 µmol photon m^{-2} s^{-1} in flask in first three days' growth were 0.93, 1.50, and 0.63 d^{-1}, respectively. The average µ for *C. vulgaris* in flask under 54 µmol photon m^{-2} s^{-1} during 7 days' growth (0.23 d^{-1}) was 57% lower than that in CTPBR (0.54 d^{-1}), while the initial growth rate from the first three days was 32% slower. Under both irradiance strength the final biomass concentrations obtained in CTPBR were quite similar, the numbers were in the range of 1.62 ± 0.09 g L^{-1}, 3 times higher than that of the flasks, and 2 times higher than CTPBR under 25 µmol photon m^{-2} s^{-1}. Due to the microalgae self-shading in autotrophic conditions, the cell density usually stay below 1.0 g L^{-1} (Feng et al., 2011). Therefore, CTPBR is shown to be superior in both growth rate and biomass density.

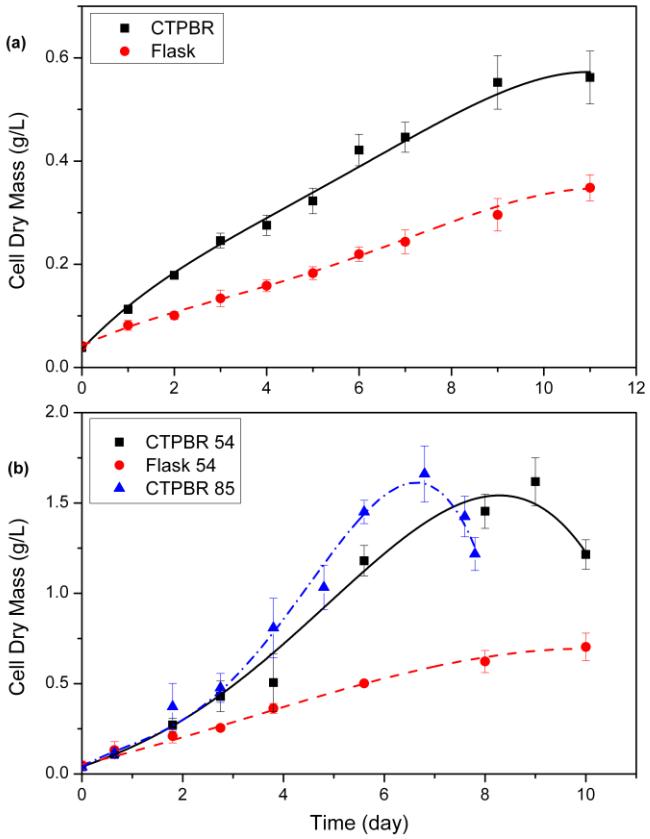

Figure 3-2 The growth curves of *C.vulgaris* in coiled tubular tree photobioreactor (CTPBR) and a 1 L Erlenmeyer flask at 22 ± 1°C, with atmospheric CO_2 at (a) 25 µmol photon m^{-2} s^{-1}, 12 h/12 h (Light/Dark) photoperiod cycle; (b) 54 µmol photon m^{-2} s^{-1} in CTPBR and a 1 L Erlenmeyer flask and 85 µmol photon m^{-2} s^{-1} in CTPBR under 16/8 (L/D) photoperiod cycle. Results shown are the mean of triplicated experiments ± STDEV.

The advantage was far greater at low light of 25 µmol photon m^{-2} s^{-1} because the CTPBR configuration has a larger surface/volume (S/V) ratio (400 m^{-1}) compared to the Erlenmeyer flasks (37 m^{-1}), allowing more photons falling on the microalgae suspension for their photosynthesis. The focus effect of light in the tubular glass tube also offered better light distribution for cell growth.

Only a few studies have been previously conducted at such low irradiance to study the indoor photobioreactor (PBR) productivity, despite the fact that indoor lighting is a

common approach for PBR operations (Zigman and Review, 2008). The previous studies of *C. vulgaris* growth under low light are summarized in Table **3-2**. Compared with other configurations under low light conditions reported, the best result was achieved in a flat plate PBR: the cell density was 2.3 g L^{-1} and the μ was 0.94 d^{-1} under 120 μmol photon m^{-2} s^{-1} with CO_2 addition (Chang et al., 2016). Growth of *C. vulgaris* under similar conditions in a vertical tubular reactor has a cell density of 1.31 g L^{-1} and μ value of 0.31 d^{-1}, less than half of that in our coiled tree configuration of the CTPBR. Aguirre and Bassi (Aguirre and Bassi, 2013) reported a similar μ value of 0.38 d^{-1} at even higher light intensity, 120 μmol photon m^{-2} s^{-1}. The results in bubble column and 0.5 L flask were even worse (Khoo et al., 2016; C. Li et al., 2013). Therefore, the CTPBR has proven its effectiveness, most likely attributing to the larger surface area. Although it is difficult to compare the PBR configurations due to the varied growth conditions, a generally increasing trend could be observed as the specific irradiance (μmol photon s^{-1} L^{-1}) increases and CTPBR shown advantages in both growth rates and biomass density. Also, the experiments under 25 μmol photon m^{-2} s^{-1}, 12 h/12 h (Light/Dark) in CTPBR has a growth rate of 0.59 ± 0.08 d^{-1}, while the μ fast elevated to 1.50 ± 0.09 d^{-1} as the irradiance level increased to 85 μmol photon m^{-2} s^{-1}, 16 h/8 h (L/D). Meanwhile, the biomass density was increased from 1.56 g L^{-1} to 2.93 g L^{-1} when temperature was increased from 23ºC to 27ºC. The fact that 250 - 500 μmol photon m^{-2} s^{-1} is the favorable light range at around 20ºC (Shriwastav and Bose, 2015) encouraged the confidence that CTPBR would have an even better performance at corresponding light range.

Table 3-2 Biomass density and cell growth rate (μ) of *C. vulgaris* reported in the literature for some photobioreactors under low light conditions with comparison to the

coiled tubular tree photobioreactor (CTPBR). The light availability was calculated as light intensity* surface area/volume.

Bioreactor configuration	Light (µmol m^{-2} s^{-1})	Biomass density (g L^{-1})	μ_{max} (d^{-1})	Light availability	CO_2 (%)	Temperature (ºC)	Volume (L)	Reference
4 L flask	150.00	1.07	0.38	4.35	4	23.00	3.50	(Aguirre and Bassi, 2013)
flat-plate PBR	120.00	2.30	0.94	6.96	1	27.00	1.60	(Chang et al., 2016)
Vertical tubular	70.00	1.31	0.31	5.74	6	30.00	1.80	(De Morais and Costa, 2007)
Bubble column	60-70	0.94	--	0.18	air	30.00	56.00	(Khoo et al., 2016)
0.5 L flask	40-60	0.98	0.45	4.60	air	25.00	0.15	(C. Li et al., 2013)
1L flask	54.00	0.70	0.63	2.59	air	21.00	0.80	This study
CTPBR	54.00	1.61	0.93	7.83	air	21.00	1.14	This study
CTPBR	85.00	1.56	1.50	12.33	air	21.00	1.14	This study
CTPBR	70.00	2.93	1.47	10.15	air	27.00	1.14	This study

Further studies on large scale configurations require taking into account hydrodynamic gradients and mass transfer and light limitations, but a previous reversed conical helical tubular reactor was reported by Morita *et al.* (2002). The 14 L reactor offered 1.5 d^{-1} growth rate in outdoor conditions, around 30ºC, 580 µmol photon m^{-2} s^{-1}. Khoeyi et al. (2011) reported that the µ for microalgae *C. vulgaris* was 0.8 d^{-1} at 25ºC. Based on these reported results, it can be concluded that the CTPBR offers comparable growth rates under light limiting conditions with a smaller footprint. Although the influence of scaling up CTPBR

is unknown yet, the performance of CTPBR is promising as suggested by the above-mentioned studies.

The lutein productivities in both flasks and CTPBR are summarized in Table **3-3**. It can be noticed that the lutein content is similar in both configurations at comparable conditions, while higher light intensity in the experimental range is associated with significant decrease of lutein content. It can be concluded that the lutein content is higher at conditions not favorable for the growth. The highest lutein content obtained was in the range of 8-10 mg/g, and highest lutein productivity 11.98±1.42 mg $g^{-1}d^{-1}$. These values were higher than reported values, around 3-7 mg/g (Cordero et al., 2011; Shi et al., 2000), due to the differing growth conditions of previous studies. A plausible reason for the increased lutein content at lower irradiance should reason back to lutein's function in photosynthesis system II (PS II), which, under low irradiance, is to widen the light absorbing spectrum and transmit the energy to the chloroplast (Gong and Bassi, 2016). In terms of specific lutein productivity, the growth rate plays a more dominant role than the specific lutein content, agrees with lutein's definition of being a primary pigment. As the cultivation temperature increased toward the optimal, consistent increase trends were shown in both flasks (0.34 – 0.99 mg g^{-1} d^{-1}) and CTPBR (0.96 – 1.20 mg g^{-1} d^{-1}), indicating that the temperature is the most significant factor for enhancing the lutein productivity. The highest value was obtained at 85 µmol photons m^{-2} s^{-1}, where it was 11.98 mg g^{-1} d^{-1}, three times that in the flasks. The previous study in bubble columns showed similar increase trend of lutein accumulation as the growth condition moved toward the optimal, their maximum lutein production rate was calculated to be 2.12 mg L^{-1} d^{-1} (J. F. F. Sánchez et al., 2008). The CTPBR exhibited great potential for highly value-added lutein production. Therefore, it would be interesting to optimize lutein productivity by manipulating temperature and irradiance strength.

Table 3-3 Comparison of specific growth rates, lutein content, and lutein productivity between CTPBR and 1 L Erlenmeyer flasks at 22.0±1.0 °C under various light intensity as specified. All the experiments were started at 0.04 g L^{-1}, at least triplicated and the STDEV were shown as error. The lutein contents were expressed as mg free lutein per gram dry cell.

	Light Intensity	Initial specific growth rate	Lutein content	Lutein productivity
	μmol $m^{-2}s^{-1}$	d^{-1}	mg g^{-1}	mg $g^{-1}d^{-1}$
Flask	25	0.44±0.11	9.13±0.57	4.02±1.31
	54	0.63±0.09	8.15±0.06	3.91±0.93
CTPBR	25	0.76±0.11	9.82±0.12	7.46±1.19
	54	0.93±0.14	8.55±0.27	7.95±1.49
	85	1.50±0.11	7.99±0.33	11.98±1.42

3.4.2 Low temperature RSM study for growth

Twenty-six batch cultivations in 1 L Erlenmeyer flasks at low temperatures were conducted in random order by modified CCD design, the centre point was repeated for 5 times, and the factorial points were duplicated. The complete design matrix with three parameters: temperature (X), irradiance strength (Y), and light hours per day (Z); experimental responses are shown in Table **3-1**. The parameters and their ranges were determined from previous literature information and the knowledge gap of *C. vulgaris* growth at low temperatures (Maxwell et al., 1994). The data are fitted into the quantitative surface models using a second-order polynomial regression equation (Gai et al., 2014). The details of models, including the estimated coefficients of corresponding response variables, test of significance of terms and models, and the goodness of fit (R^2 and adj-R^2), are shown in Table **3-4**. The normality test is also conducted, and the residuals are evenly distributed.

This information can be found in the supplemental materials (Appendix I). The model of growth is established as below in equation (3-2):

$$\mu\ (d^{-1}) = 0.728 - 0.0101\ X + 0.001564\ Y - 0.0750\ Z + 0.002230\ X^2 - 0.000001\ Y^2 + 0.001921\ Z^2 - 0.000069\ XY + 0.000639\ XZ - 0.000035\ YZ \quad (3\text{-}2)$$

Three terms were not of statistical significance, namely X, XY and YZ. the coefficients of Y, X^2, Z^2, XZ are positive, showing a positive effect of these parameters. While the other terms are negative, meaning not beneficial effects on the growth rate. Figure **3-4** is plotted to visualize the effects of temperature and irradiance on growth, at different day light cycles. The optimal growth was at 13°C, around 200 µmol photons m^{-2} s^{-1}, and 14 hours of light per day. As expected, the microalgae grew faster at higher temperatures. The µ at 10ºC in flask was around 0.24 d^{-1}, and was almost the same as that reported by Mayo (Mayo, 1997). The values are significantly higher than that achieved from 4ºC, typically below 0.10 d^{-1}. The best growth for 10ºC was observed under conditions of 125 µmol photons m^{-2} s^{-1}. A clear photo-inhibition can be observed from the decreasing trend of growth rate towards the higher light intensity. Moreover, the prolonged daylight also triggered worse growth. As the daylight hours increased, the response surface sank lower, indicating slower growth rates, proving the existence of photo-inhibition. Noticeably, the lower the temperatures were, the longer the daylight existed, at the lower light intensity the inhibition effect occurs. The reason for that is that at low temperatures, the enzyme activities are slower, so the PS can process less photons than at higher temperatures. Further, the cell density throughout the growth was below 0.6 g L^{-1}, so the shading within cells were minimum, which alleviated the light inhibition effect.

Table 3-4 Analysis of Variance, model summary and test of significance of the coefficient terms

	Growth		Lutein content		Specific lutein productivity	
	Adj SS	P-Value	Adj SS	P-Value	Adj SS	P-Value
Regression	0.145	0.000	32.050	0.000	3.553	0.000
t	0.000	0.547	5.332	0.000	0.177	0.004
light	0.011	0.001	4.540	0.000	0.001	0.783
ir	0.008	0.002	0.882	0.019	0.534	0.000
t*t	0.006	0.007	1.093	0.010	0.115	0.017
light*light	0.009	0.001	0.785	0.025	0.081	0.038
ir*ir	0.007	0.003	0.145	0.303	0.290	0.001
t*light	0.009	0.002	3.619	0.000	0.020	0.282
t*ir	0.000	0.377	0.992	0.014	0.152	0.007
light*ir	0.002	0.071	0.786	0.025	0.003	0.654
Error	0.009		1.909		0.237	
Lack of Fit	0.007	0.000	0.881	0.118	0.183	0.001
Pure error	0.001		1.028		0.053	
Total	0.154		33.959		3.789	
R^2	94.35%		94.38%		93.76%	
Adj. R^2	90.96%		91.01%		90.01%	

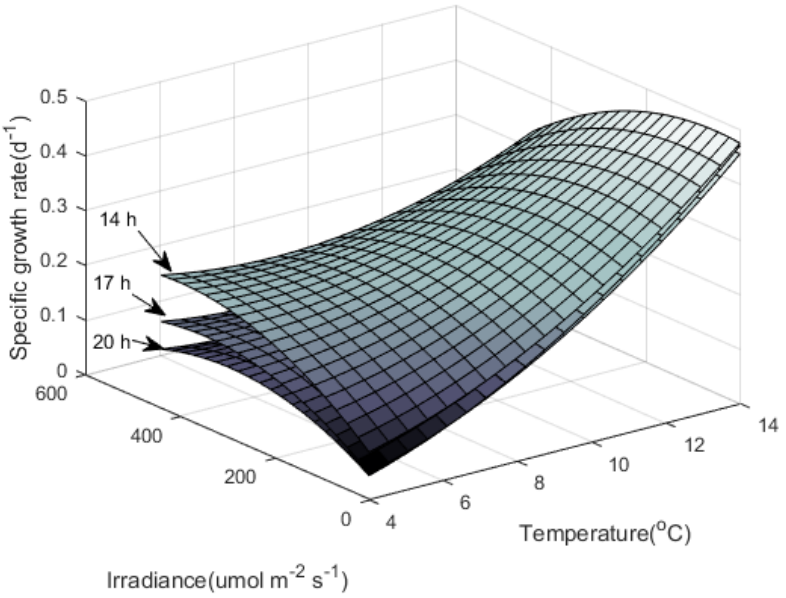

Figure 3-3 The fitted RSM model for microalgae specific growth rates in the designed temperature, irradiance strength, and light hours per day.

3.4.3 Lutein content and lutein production

Beside the effects of cell growth, the lutein productivity was also investigated by RSM. The experimental results ranged from 3.21 to 8.20 mg g^{-1}, and agreed with reported in literature (Shi et al., 2000). Employing the same method but changing the response variable to specific lutein content, the empirical model can be written as equation (3-3). The 1°C run was excluded due to the abnormal growth (no growth observed).

$$\text{Specific lutein content (mg } g^{-1}) = 23.27 - 1.577\ X - 0.03233\ Y - 0.773\ Z + 0.0309\ X^2 + 0.000013\ Y^2 + 0.00869\ Z^2 + 0.001413\ XY + 0.0290\ XZ + 0.000658\ YZ \quad (3\text{-}3)$$

The evaluation of the model and ANOVA results are shown in Table **3-3**. According to the ANOVA analysis, the quadratic terms and interaction terms are of greater significance than linear terms to the specific lutein content at given conditions. Figure **3-4** is plotted to show the response surfaces of lutein content at specified day light hours, temperature and irradiance. At lower temperatures, the lutein content decreased as the irradiance got stronger, or as the light hours got longer. At low irradiance level given any light/dark cycle, the lutein content decreased as the temperature increased. The trends are caused by the complex physiological changes in the cells. This should relate to the other role of lutein in PS II that responsible for the increase of lutein content at high light conditions, which is to dissipate excess energy light energy and protect the chlorophylls from being photo-oxidized (Niyogi et al., 1997). However, although the specific lutein content increased, the photosynthesis pool size is decreased at inhibiting light level. Meanwhile, the cells became heavier at low temperature due to the increased cell wall thickness, which is also a critical reason for the decreased lutein content. Therefore, the trends are different at higher temperatures since the increased temperature lowered the level of inhibition, enlarged the PS pool, and consequently increased the total pigment content. Further, the cells' specific gravity decreased (Yap et al., 2016), causing an increase in specific lutein content mathematically. At higher irradiances, increased light inhibition increased the demand of lutein, and may be responsible for the different lutein accumulation trend in the corresponding region. Some increase of lutein content is observed at high temperature, high irradiance, which should dominantly attribute to the change in the specific gravity of cells.

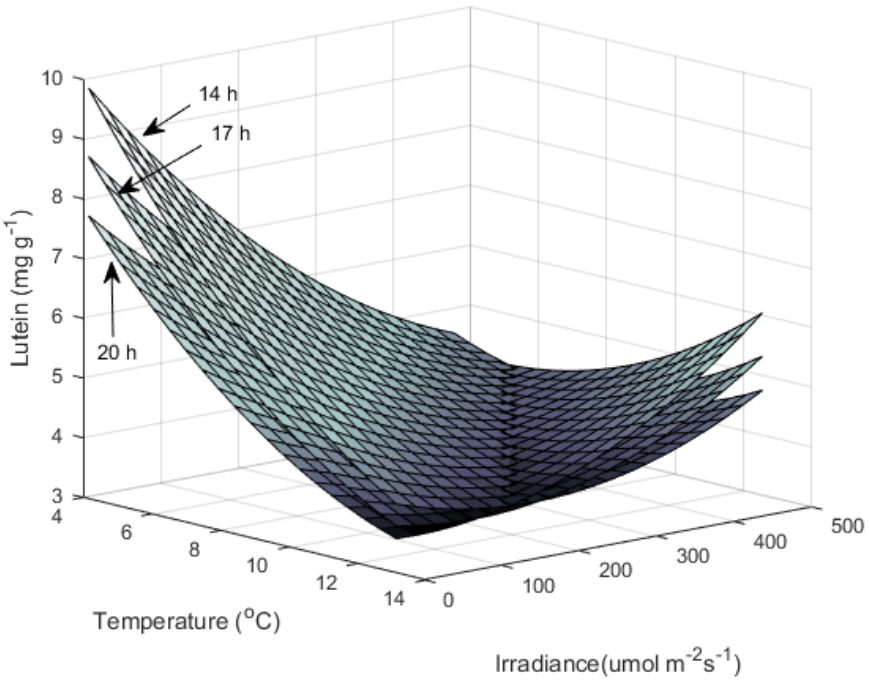

Figure 3-4 The fitted RSM model for microalgae lutein content in the designed temperature, irradiance strength, and light hours per day.

By considering both the growth rate and lutein content, the model for the specific lutein productivity is listed below as equation (3-4):

Specific lutein productivity (mg $g^{-1}d^{-1}$) = 7.69 - 0.2877 X + 0.00053 Y - 0.601 Z + 0.0100 X^2 - 0.000004 Y^2 + 0.01230 Z^2 - 0.000104 XY + 0.01134 XZ + 0.000043 YZ (3-4)

Overall, the coefficients of determination (R^2, adj-R^2) for the three models (3-2)-(3-4) were all above 90%, indicating a good fit under the specified conditions. The plot of the model (3-4) in Figure **3-5** indicates that at any given day-light cycles, increasing temperature always benefits the lutein productivity. The response surfaces showed close resemblance to Fig. **3-3**, besides the values are higher at low irradiance levels due to the effect of lutein content. The optimum productivity is achieved at 14 h daily light hours, 120 µmol photons m^{-2} s^{-1} and 10°C in the plotted area. However, if further reduction of light hours, or increase temperatures, higher lutein productivities can be observed, while the optimal irradiance should become slightly stronger. The higher productivities of the axis test points at 13°C

or 11 h daily light proved this, and the highest value was 1.73 mg $g^{-1}d^{-1}$. Not enough comparable results are found to our knowledge, while the interaction of temperature and light may require further investigation.

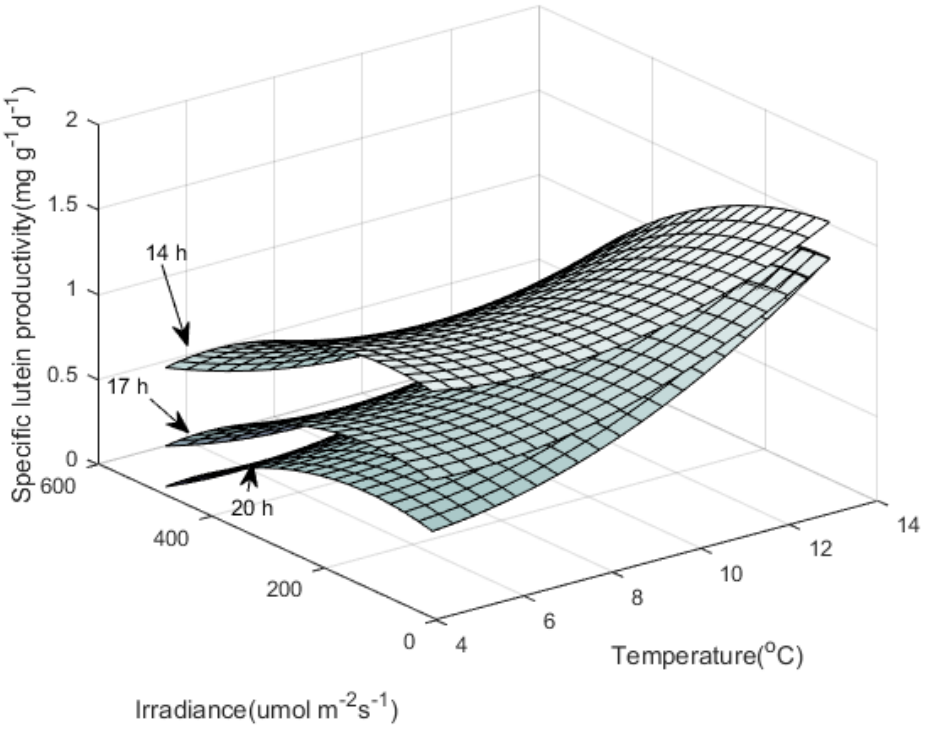

Figure 3-5 The fitted RSM model for microalgae specific lutein productivity in the designed temperature, irradiance strength, and light hours per day.

3.4.4 Growth kinetics of *C. vulgaris* under low temperature cultivation in CTPBR

To evaluate the growth kinetics of *C. vulgaris* at low temperatures, a series of experiments were conducted under temperatures of 4, 7 and 10°C in the CTPBR, and the results are summarized in Table **3-5**. The light intensity was fixed at 470 µmol photons m^{-2} s^{-1} due to the growth chamber configuration restrictions. Previously, Maxwell *et al*. (1994) reported that the µ in a tubular reactor at 5°C under 150 µmol photons m^{-2} s^{-1} was 0.34 d^{-1}. The lower values in our study may be caused by the higher light intensity (Maxwell et al., 1995). Experiments were also carried out at 1 °C, but no obvious growth were observed (data not shown) although the cells survived after two weeks' incubation. The cells in the CTPBR

at low temperature range grew faster than those in the flasks, probably due to the more even light distribution achieved through the focus effect of glass tubes.

Table 3-5 Comparison of specific growth rates, lutein content, and lutein productivity in CTPBR under various light intensity 470 μmol $m^{-2}·s^{-1}$ at various temperatures as specified. All the experiments were started at 0.04 g L^{-1}, at least triplicated and the STDEV were shown as error. The lutein contents were expressed as mg free lutein per gram dry cell.

Temperature	Initial specific growth rate	Lutein content	Lutein productivity
°C	d^{-1}	mg g^{-1}	mg $g^{-1}·d^{-1}$
4	0.16±0.03	5.97±0.20	0.96±0.21
7	0.22±0.01	4.87±0.18	1.07±0.09
10	0.31±0.06	3.86±0.36	1.20±0.36

The specific growth rate in the temperature range of 4 to 10°C using the specific growth rates were fitted to the Arrhenius equation:

$$\mu_{max} = A \exp(- Ea / R T) \quad (3\text{-}5)$$

where A is the constant related with the formation of growth related enzymes, Ea is the corresponding activation energy, T expresses temperature in Kelvin. The parameters obtained are $A=5.69\times10^{12}$ d^{-1}, $Ea = 1.04\times10^{3}$ J mol^{-1}, $R^2 = 0.999$. The values are lower than reported at higher temperatures, such as 3.75×10^{4} J mol^{-1} for *S. almeriensis*, 6.8×10^{4} J mol^{-1} for *E. coli*, indicating the *C. vulgaris* have a lower sensitivity to temperature in the tested stress conditions (J. F. Sánchez et al., 2008). Hence the CTPBR configuration a suitable system for *C. vulgaris* cultivation at temperatures down upon 4°C in experimental conditions. A similar trend of lutein accumulation and productivity was found in cells cultured in the CTPBR as those in the flasks, the highest productivity was 1.20±0.36 mg g^{-1}

$^{1}d^{-1}$. The lutein content of the cells cultured in CTPBR was agreed well with reported in literature (4.58 mg g^{-1}) (Shi et al., 2000).

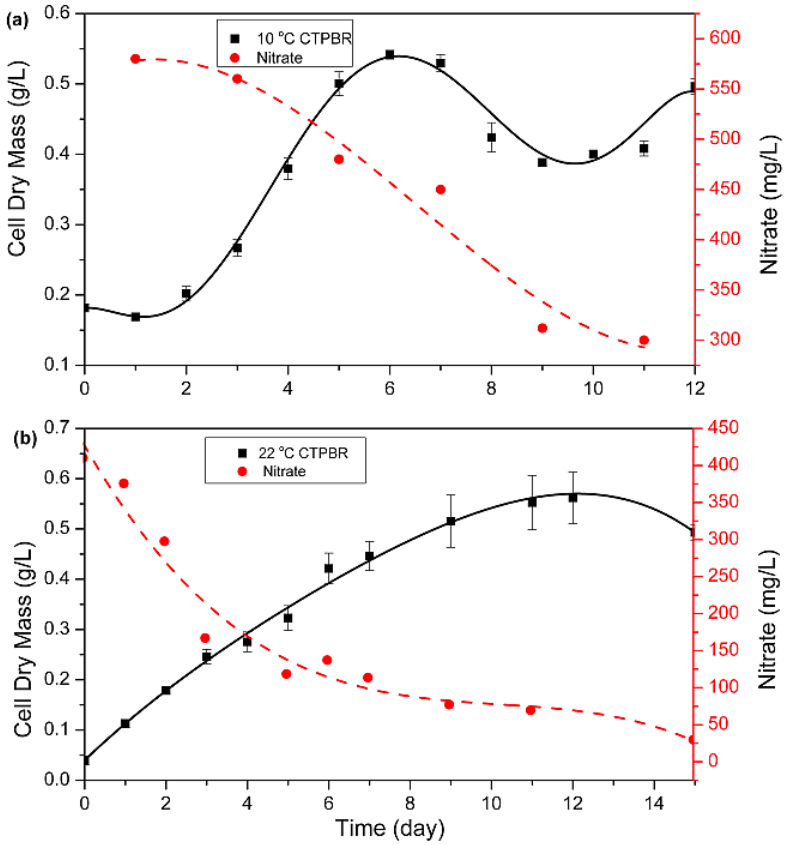

Figure 3-6 Comparison of nitrate consumption by C. vulgaris cultivated under (a) 470 µmol photons m^{-2} s^{-1} at 10±0.5ºC, and (b) 54 µmol photons m^{-2} s^{-1} 22±0.5ºC CTPBR with atmospheric CO_2.

The growth curve and nitrate consumption rates were compared for the two stress conditions [(low temperature/high light) and (high temperature/low light)]. From Fig. **3-6** (a), at 10±0.5ºC, 470 µmol photons m^{-2} s^{-1} in CTPBR, the concentration of nitrate decreased consistently from 600 to 300 mg L^{-1} in 11 days, and the corresponding biomass concentration increased from 0.2 to 0.5 g L^{-1}. The highest biomass was achieved at day 6 (0.55 g L^{-1}). Cultivation studies were also carried out at 22±0.5ºC, 54 µmol photons m^{-2} s^{-1} in the CTPBR, and the results were presented in Fig. **3-6** (b). Nitrate was almost depleted after 2 weeks' cultivation at 20ºC. The nitrate consumption rate was comparable to those

reported by Yang *et al.*(Yang et al., 2013) In their study, the nitrate concentration dropped from 190 to 130 mg L^{-1} within 11 days under conditions of 100 rpm, 70 μmol photons m^{-2} s^{-1} in 125 mL shake flasks at 10^{o}C (Yang et al., 2013). It should also be noticed that the microalgae cells tend to settle, as mentioned before, under low temperatures due to their increased specific gravity. No dropping was observed at room temperature growth, but a drop in biomass concentration at day 8 can be noticed, at 10±0.5^{o}C, where the actual biomass concentration inside the PBR should not have decreased, according to the constant decreasing rate of nitrate. Therefore, that number is due to measurement limitation. One more proof of this is on the last day of growth, a through mixing was given to wash out all the biomass inside the PBR, and the biomass concentration increased again. In our study of the CTPBR, higher nitrate reduction efficiency is achieved when compared with other studies (George et al., 2014). Thus the CTPBR proved to be an effective configuration under different environmental conditions.

3.5 Conclusions

This study emphasizes the importance of lutein production under stressed conditions especially at low temperatures (outdoor) or lower light intensities (indoor). The proposed models are accurate in predicting microalgae growth rate, lutein content and productivity under specified conditions, which is 4-10°C, 125-360 μmol photons m^{-2} s^{-1}, and 14-20 h daylight per day. Moreover, the models would be useful to investigate the effect and interactions of the environmental conditions for future lutein production from *C. vulgaris*. The coiled tubular tree photobioreactor (CTPBR) configuration was demonstrated to be an efficient photo-bioreactor for cultivating microalgae at low light. The *C. vulgaris* specific growth rate and biomass concentration in CTPBR were all more than 2 times higher than that in Erlenmeyer flasks under comparable conditions. It is possible to produce lutein in cold weather by microalgae, and in general the conditions that are favor for microalgae growth would be more beneficial for the lutein production due to cellular lutein content decrease slower than the growth rate, and indoor conditions are more beneficial for lutein productivity, while outdoor conditions have lower cost. More research shall be done to further optimize the lutein productivity and to scale up the unit in a cost-effective manner.

Chapter 4

4 Investigation of simultaneous lutein and lipid extraction from wet microalgae using Nile Red as a solvatochromic shift probe

The information presented in this Chapter is based on the paper of same title submitted to Algal Research. The sections in Chapter 4 present the results towards the completion of objectives 3 and 4 of the thesis.

4.1 Abstract

Microalgae have been proposed as an alternative lutein source due to their high productivity, reliability and versatility. In this study, the lutein and lipid extraction from wet microalgae *Chlorella vulgaris* UTEX 265 was investigated. The lutein production was monitored throughout the micro-algal growth phase, and several extraction parameters such as the biomass to solvent ratio, drying method, cell disruption method were investigated. The performance of solvents on lutein extraction was compared using Nile Red as a solvatochromic polarity probe. The simultaneous lipid and lutein extraction was also studied for different polarities using an ethanol-hexane binary solvent at the optimal solvent compositions suitable for lutein extraction. Among the solvents investigated in this research, 80% (v/v) ethanol in hexane was recognized as the optimal solvent for lutein and lipid co-extraction, which contributed to a 13.03 mg/g lutein and 7% (w/w) lipid yield. Based on our results, wet extraction approach exhibits good potential, while the bead-beater is the most suitable technique for cell disruption and lutein extraction.

4.2 Introduction

Microalgae are photosynthetic microorganisms; they have long been studied for the production of biodiesel, proteins and other functional ingredients such as pigments and vitamins. Lutein is a commercial carotenoid approved by the European Union and FDA as a food colorant and in food additives (Taylor et al., 2012). Its application can be extended as a nutritional supplement since it has shown the potential for reducing the risk of cataract and macular degeneration (Manayi et al., 2015)., Microalgae exhibit certain advantages as

alternative lutein sources compared to the conventional source, i.e. marigold flowers. The microalgae can be cultivated in a shorter time frame, have richer lutein content, and can survive in harsh growth conditions. In addition, microalgal production of lutein may be less labor-intensive and uses less arable land. In spite of all these advantages, the major challenge for lutein production from microalgae is the high capital and operational costs especially in the downstream processing (Kim et al., 2013). Thus, more studies are needed to evaluate improved approaches and strategies for lutein recovery.

Hexane has been commonly applied to extract products from microalgae. However, according to Craft and Soares (1992), the lutein solubility of hexane is poor, due to the existence of the two hydroxyl groups. Other solvents with higher polarity such as ethanol, THF, and ethyl ether are theoretically better choices. Ethanol, due to its low toxicity and higher lutein selectivity is favored for lutein recovery (Balasubramanian et al., 2011; Zhengyun et al., 2007). Binary solvent mixtures consisting of non-polar and polar solvent can be applied in an integrated process for both lutein and lipid production. The polarity difference between lutein and neutral lipids may be exploited for this purpose (Araya et al., 2014; Dineshkumar et al., 2015; Hodgson et al., 2016). Dineshkumar et al. (Dineshkumar et al., 2015) have previously reported on such an approach and achieved satisfactory yield for both products (6 mg/g lutein and 94 mg/g FAME). The solvatochromic behavior of Nile Red can be applied as an approach to indicate the solvent polarity (Deye et al., 1990; Jessop, 2005). To the best of our knowledge, a quantitative study of lutein extraction using Nile red for the effects of mixture ratio and solvent polarity has not been previously attempted.

The simultaneous extraction of lutein and lipid extraction using Nile Red as solvatochromic polarity probe was investigated in this study. The strategies used include (i) targeting the proper growth phase to determine the optimal harvesting time for microalgae cells; (ii) optimization of the extraction parameters such as biomass to solvent ratio, drying method, cell disruption method and solvent polarity; (iii) and comparing the polarity effect on integrated lutein and lipid extraction.

4.3 Methods

4.3.1 Microalgae type and cultivation

The microalgae type and cultivation methods were previously discussed in section 3.3.1, *Chlorella vulgaris* UTEX 265 was used. The microalgae were harvested at the end of growth period by centrifugation at 3,500 rpm for 15 min at 4 °C in a Sorvall R40 centrifuge (ThermoScientific, USA). The wet microalgae pellet was washed three times with deionized water and used fresh, frozen or dried for further analysis. Frozen cells were frozen and storage at −20°C or −86 °C. Dried cells were either freeze dried (described below) or oven dried at 60°C till constant weight. To freeze dry, the wet biomass pellets were first frozen at −86 °C then vacuum dried using a 4.5-L freeze-drier (Labconco, Kansas City, MO, USA).

4.3.2 Extraction method

Five cell disruption methods were examined in this study and a non-treatment control was also included. The approaches and treatment time are described as in Table **4-1**. For the bead-beater, 0.4 g of 0.1 mm diameter Zirconia/Silica beads were added to each vial. For ultra-sonication, two different shapes: 20 mL Scintillation vial and 10 mL test tube were used as sample containers. All other procedures are the same for all cell disruption methods as followed: For each experiment, 20 mg dry weight equivalent biomass were used for a five-milliliter solvent extraction, then the samples were centrifuged at 3,500 rpm for 5 min, the supernatant was collected and the cells were re-suspended in the solvent and the procedure was repeated another two times. All the extract was combined and filtered through a 0.22 µm PTFE filter for HPLC analysis. The procedure is depicted in Figure **4-1a**.

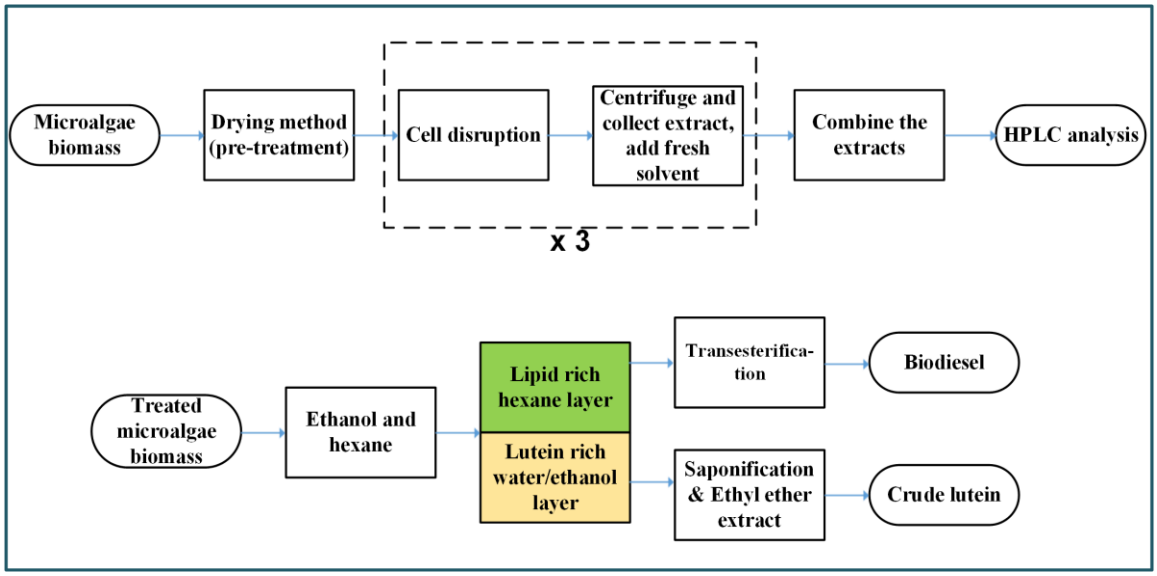

Figure 4-1 Schematic block diagrams for (a) lutein extraction (b) simultaneous lutein and lipid extraction processes

Table 4-1 Conditions for different cell disruption methods used with corresponding treatment time and energy consumed if applicable

Treatment method	Mini bead-beater (biospec, USA)	Ultra-sonication (Hielscher, Germany)	Microwave oven (Sunbeam, Canada)	Pestle and mortar	Solvent soaking (ethanol, −20 °C)	No treatment
Rated power	70 W	50 W	700 W	--	--	--
Treatment time	200s	280s	20s	300s	24 h	60s
Energy (J)	14000	14000	14000			

4.3.3 Analysis methods

4.3.3.1 Cell concentration and dry weight

The cell density of microalgae was measured spectroscopically as below using the method previously described by Orr and Rehmann (2015) with minor modifications. To achieve a better accuracy, the cells were first bleached before measuring optical density (OD_{680}) to

avoid the error from different cellular chlorophyll contents (Orr and Rehmann, 2015). A Cary Bio 50 UV/Visible Spectrophotometer (Varian, USA) was used for OD measurement. Twenty milliliter series diluted microalgae solution was vacuum filtered through a pre-dried and weighted 0.45 µm pore-size cellulose nitrate membrane, then the microalgae with the membrane were dried at 60°C for 24 hours or till constant weight to get the cell dry mass. The cell dry mass was related with the corresponding spectroscopic reading via a calibration curve, Cell dry mass (g/L) = $0.22*OD_{680}$, $R^2 = 0.998$. All the readings were triplicated.

4.3.3.2 Solvents polarity measurements

The Nile Red dye (Sigma-Aldrich, Oakville) was used to determine the polarity of the liquids. A known quantity of dye was dissolved in the solvent for λ_{max} scan from 800 nm to 200 nm. The λ_{max} is then related with the polarity index, for instance, the correlation of ten popular solvents are shown in Figure **4-2**.

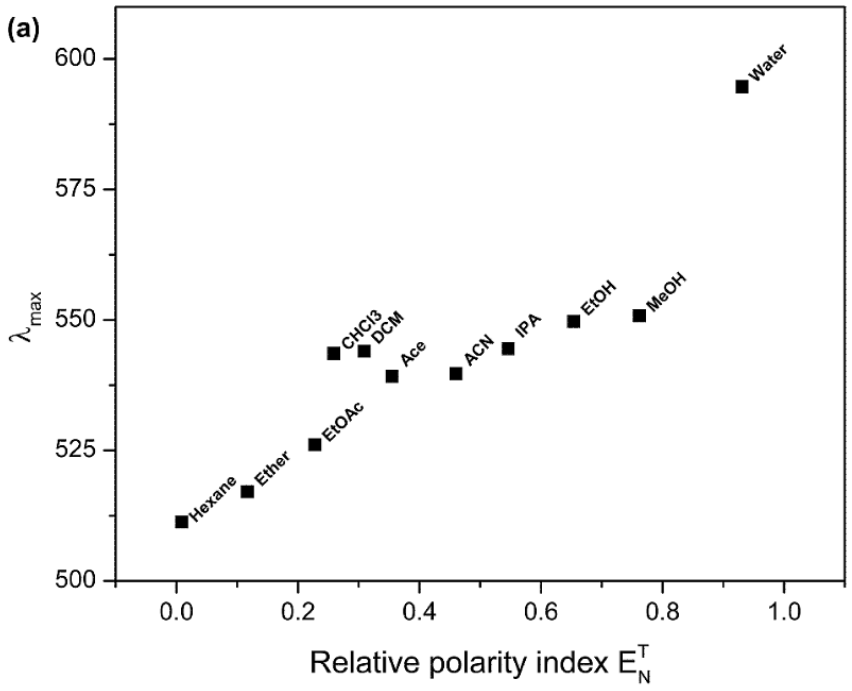

Figure 4-2 Solvent polarity vs λ_{max} of Nile Red solution. Polarity was measured using Nile Red as solvatochromic shift probe and represented as relative polarity index E^T_N.

4.3.3.3 Pigments quantification

The pigment analysis was carried out by HPLC (see section 3.3.7). Then the analytic sample retention time and peak area were used to determine the pigment contents in analytical samples.

4.3.3.4 Lipid content

The total lipid content was determined according to the method described by Bligh and Dyer (Bligh EG and Dyer W J, 1959). In the simultaneously lutein and lipid extraction, the solvent hexane/ethanol was used instead of chloroform/methanol.

4.3.4 Statistics

All the experiments were at least triplicated unless otherwise stated. The mean of the samples was reported and the STDEV was shown as error. ANOVA test using a confidence level of 95% was used to establish the statistical significance.

4.4 Results and discussion

4.4.1 Effect of harvesting time on lutein content in microalgae

First the growth kinetics of *Chlorella vulgaris* were investigated to locate the optimal harvest time for lutein production from batch cultivation. The pigment contents (lutein, chlorophyll *a* and chlorophyll *b*) and the growth curve were shown in figure **4-3**a. The highest total pigment was observed at day 11, which was also consistent with the trend of biomass density. However, according to figure **4-3**b, the pigment content per cell dry weight decreased with the increased cell density, which was most likely due to the lack of nitrogen source at that point (Xie et al., 2013). The chlorophyll *a/b* ratio also decreased since the self-shading increased as the cell density increased. The value as well as the trend agreed with Maxwell et al. (1994).

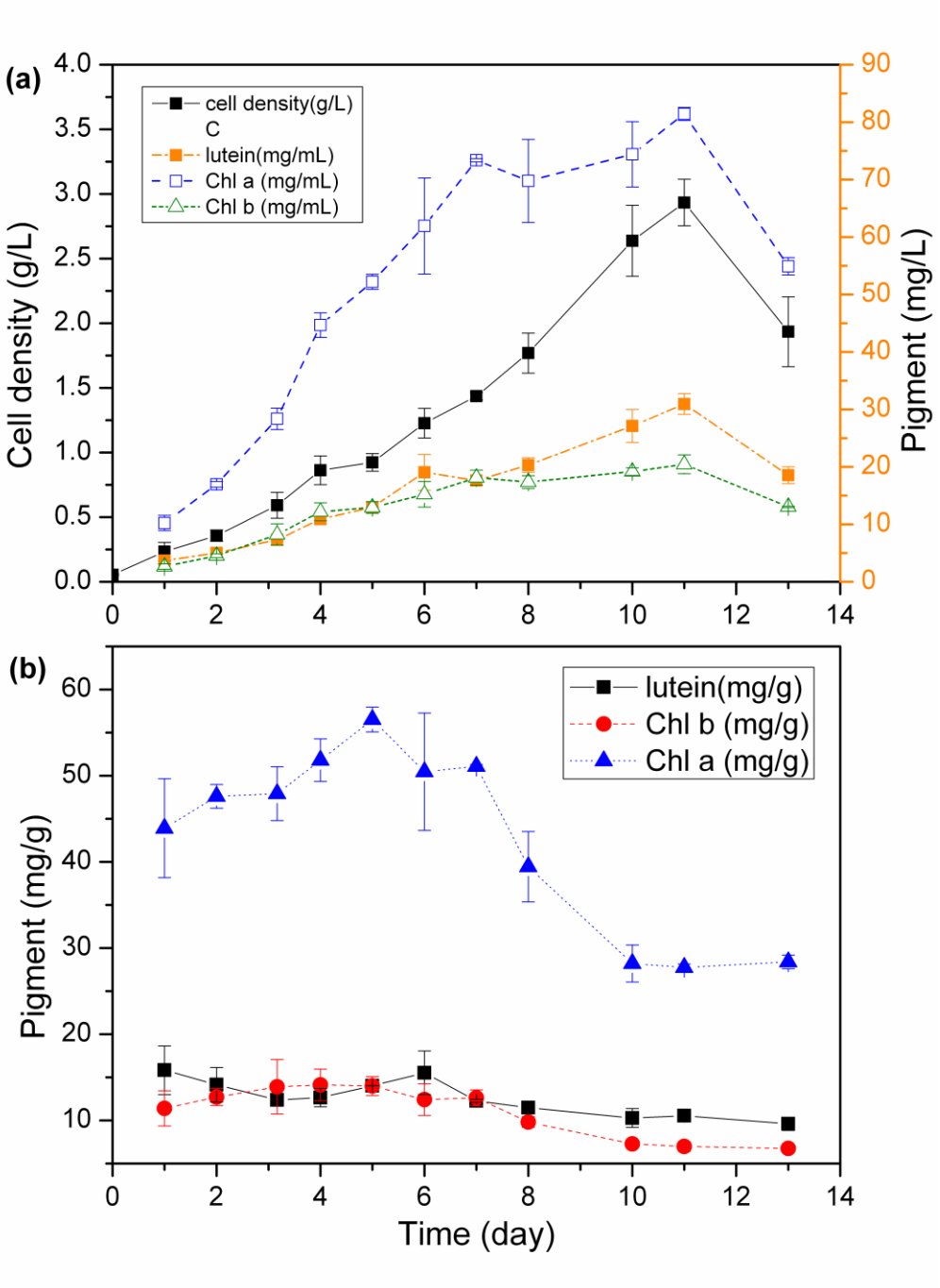

Figure 4-3 (a) Growth kinetics of *Chlorella vulgaris* and its volumetric lutein, chlorophyll *a*, chlorophyll *b* content as a function of time. On day 0 the pigment concentration was below detection limit so was not displayed in the data. **(b) The pigment content per dry weight as a function of time**

Lutein leveled at around 15 mg/g for the first 6 days, although a slight but statistically significant drop was observed in the later growth phase after day 6, it remained relatively more stable compared with chlorophyll *a*. The effect of biomass to solvent ratios was studied to exclude the interference from different cell densities. As discussed later in the following section, the variance in biomass to solvent ratios resulted in insignificant difference. Therefore, the pigment accumulation was mostly dependent on the cell growth stages. To the best of our current knowledge, this is the first study on growth phase vs. pigment content, but more comprehensive and continuous studies may be necessary to relate the nutrient availability and pigment content for continuous growth.

The lutein content decreased 33.81% from 15.53 to 10.28 mg/g. The highest volumetric lutein content was 30.94 mg/mL, where the cell density was also at its highest value, 2.93 g/L. Unlike the reported secondary carotenoids (Shah et al., 2016), lutein is a primary carotenoid and the content change is not regulated with photosynthesis. The other pigments also changed during the growth: chl *a* decreased from 51.79 to 28.21 mg/g, chl *b* from 13.91 to 6.96 mg/g, and chl *a/b* ratio was kept around 4.0. The reduced chl content indicated a decrease in photosynthesis pool size. Therefore, it is also important to retain the PS pool size when targeting primary xanthophylls and chlorophylls (Porcar-Castell et al., 2014). A conflict hence exists for lipid and pigments, since unfavorable growth condition is beneficial for lipids but not for pigments. The best harvesting time in our study was day 11 for highest lutein production per volume, while day 6 during the exponential phase is more appropriate for a higher content and higher productivity if harvesting is not a major concern.

The lutein content is significantly higher than reported in other batch studies in literature (Dineshkumar et al., 2015) The biomass to solvent ratio is small which enables more efficient extraction, and the wet extraction used enabled 40.78% higher extraction efficiency (see section 4.4.2.2); 2. The harvesting was carried out at before the onset of stationary phase of algae. The growth conditions also influence the lutein content. The algal cells grown on agar plates have a significant lower content, 2.37±0.45 mg/g.

4.4.2 Cell disruption

The lutein content can vary depending on the pre-treatment applied. Therefore, these effects were first examined to adopt a uniform approach for further studies.

4.4.2.1 Effect of microalgae biomass to solvent ratio

Since the best time for cell harvesting was first determined, the effect of microalgae biomass to solvent ratio was studied for any potential effects on the extraction. biomass to solvent ratios ranging from 0.2 to 30 mg/L was prepared in the same solvent volume. Interestingly, the results showed that the concentration of microalgae had negligible influence on lutein, chl *a*, and chl *b* yield when it was below 1 mg/L (fig.**4**-**4**.a) but there was an effect at larger biomass to solvent ratios. The less polar β-carotene exhibited a slightly different trend compared to other pigments since its solubility in ethanol was very limited (10 mg/L). Consequently, the extraction result was poor at higher biomass to solvent ratios. The lutein yield decreased significantly when the biomass to solvent ratios exceeded 10 mg/L. This trend at a larger scale is shown in Figure **4**-**4**b. The reverse trend was possibly due to decreased mass transfer efficiency as viscosity increased. As biomass to solvent ratios below 10 mg/L does not interfere with the extraction efficiency, therefore 4 mg/L was selected for further experiments.

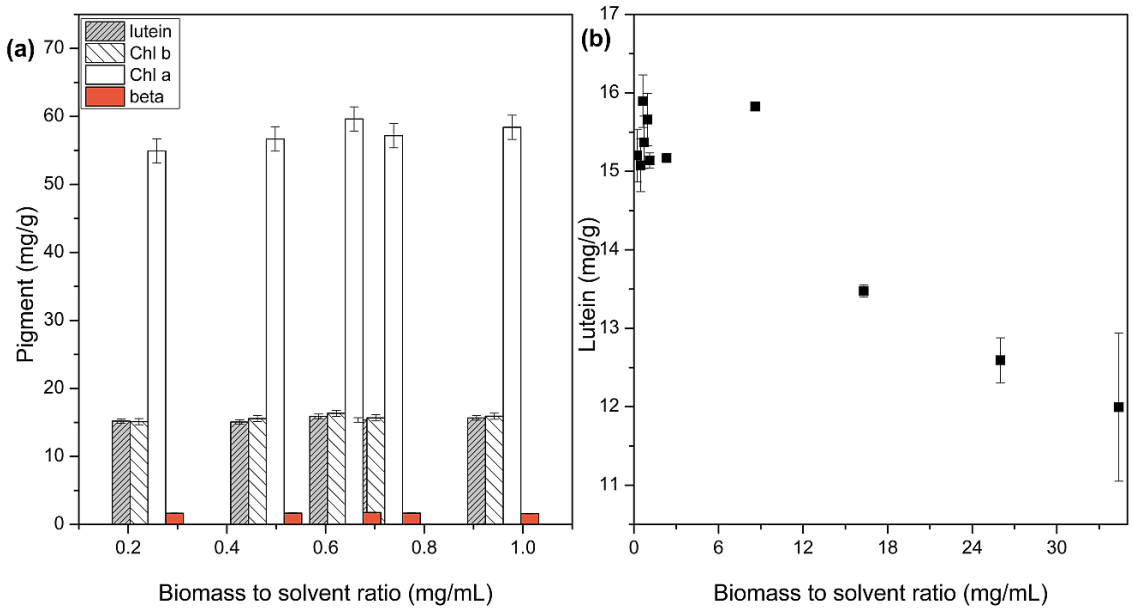

Figure 4-4 (a) The interference from varied biomass to solvent ratios in the range of 1-5 mg was negligible in terms of lutein, chlorophyll *a* and *b* extraction, but it was not the case for beta-carotene. **(b) Expanded biomass to solvent ratios affected lutein extraction at a larger scale**

4.4.2.2 Effect of the drying method

Few studies have previously compared the efficacy of different drying methods for microalgae. In this research, different methods including fresh wet cells, oven dried at 60°C, freeze dried, -85°C frozen cells, and -20°C frozen cells were compared for lutein yields. The results are shown in figure **4-5**a. The highest yield (15.5 mg/g) was obtained from wet cells regardless of fresh or frozen. No statistical difference was observed from the three methods, though frozen cells yielded slightly higher lutein, probably was due to the cell lysis during the phase change (cell wall damage). The dry processes gave much lower yield, and as expected, freeze dried cells have higher content (9.18 mg/g) than oven dried cells (5.20 mg/g), indicating that temperature would lead to pigments degradation. The lower yield after drying process could be explained by (i) the additional drying step lead to lutein degradation; (ii) it was more difficult for the extracting solvent to reach the central matrix of the dried cell where lutein is located thus impeded interaction between lutein and solvent (Amaro et al., 2015). Therefore, wet extraction should be preferred as it

not only has higher extraction yield, but can reduce the processing cost from elimination of a drying step.

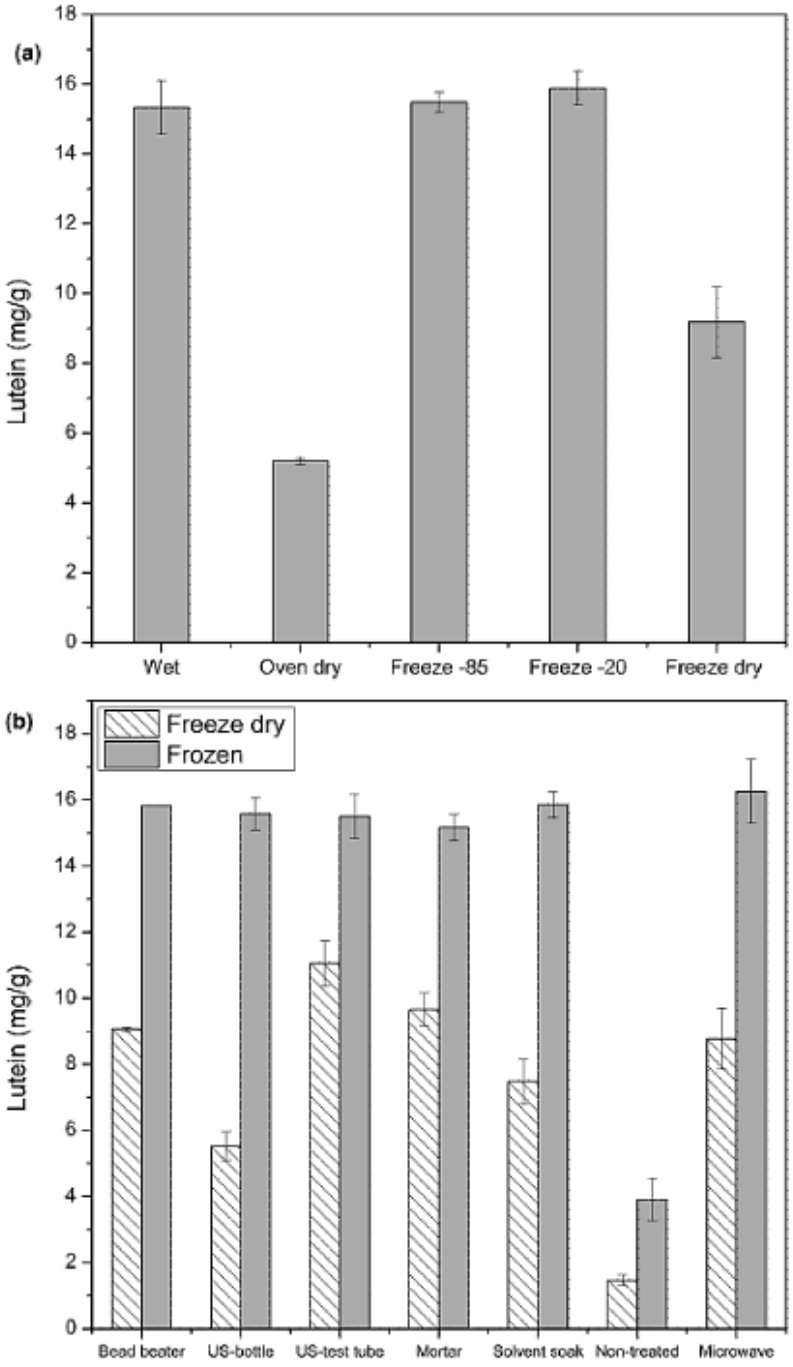

Figure 4-5 Lutein yield from (a) different pre-treatment/drying methods, and (b) different cell disruption methods for wet frozen cells and freeze dried cells

The effect of using wet algae on higher extraction efficiency are unclear since two popular but opposite viewpoints are mentioned in literature. One is that the existence of water would impede solvent extraction via formation of a water barrier; the second one is wet biomass would improve the extraction by swelling the cell, which facilliated the lysis of cell wall and allows solvent to access the inner cell content (Barbosa-cánovas, 2015; Du et al., 2015). Previously, approximately 95% recovery was achieved by the optimized hexane extraction method for dried *S. almeriensis* (Cerón et al., 2008), but in our study wet extraction resulted in negligible lutein yield from hexane. Meanwhile, for water misible solvents, over 50% increase in extraction efficiency was observed (Sarada et al., 1999). Similarily, the lipid extraction yield is reduced with presence of moisture in non-polar system, while in the polar system, the extraction efficiency is enhanced using wet biomass Halim et al. (2012b). The drawback for wet extraction may be the increased cost in handling and storage as the wet algae perish in a week under ambient temperature. So, it is suggested to either use the biomass immediately or store at below -20°C. Overall, wet fresh or frozen cells are the best choices for lutein extraction.

4.4.2.3 Effect of cell disruption

Different cell treatment methods were also studied for both freeze dried and -20°C frozen wet cells, including ultra-sonication in flat bottom bottles or test tubes, microwave, solvent soaking, bead beater, pestle and mortar, and no treatment. The results shown (Figure **4-5**b) are quite different for the two pre-treatment types, for the frozen wet cells, lutein yield is maintained 15.5 mg/g regardless of disruption methods, except that for the no treatment gave a lower result, 3.90 mg/g.

However, the freeze-dried cells were sensitive to the disruption method. Among all the treatments, ultra-sonication gave one of the best results, 11.05 mg/g, while bead beater had the most stable and reproducible result. Again, since the cells were freeze dried, the extraction efficiency was lower than that from wet cells. Moreover, ultra-sonication shape had pronounced effect on the extraction efficiency, similar to the study by Kulkarni and Rathod (2014). The explanation is the energy diffusion is radiated spherically, hereby a

more concentrated shape is more beneficial for cavitation to take place, therefore a more centralized shape of liquid is more favorable for the extraction.

The application of bead beater, microwave, and pestle and mortar gave similar results (approximately 9.1 mg/g), the efficiency was in between the two ultra-sonication methods (Figure **4-5**b). The microwave treatment yielded similar results as for the bead beater. These results are agreeble with the results reported by Lee et al. (2012) and McMillan et al. (2013). However, our results differ from Chan et al. (2013) who used a varying energy input. The bead beater, although not the most efficient method among all the treatments, was selected and applied in further studies due to its stable performance (small STDEV of lutein yield) and low energy operation.

4.4.3 Effect of solvent polarity

Nile Red was used as an indicator of polarity as its functional groups absorb different wavelengths in different environments (Figure **4-2**). Generally speaking, the more polar a liquid is, the more the color shifts toward blue. Clear color change can be observed and a standard curve can be established. The deviations from the curve may arise because of hydrogen bonding and ion-dipole interactions (Deye et al., 1990; Katritzky et al., 2004).

Eight typical different solvents were used to extract lutein from the *C. vulgaris* microalgae; the results are shown in Figure **4-6**c. Among the solvents investigated, ethanol showed the best results for the extraction, followed by isopropanol, acetone, and ethyl acetate. The result agreed with the hypothesis of Orr and Rehmann (2015), the solvents gave better extraction yield had similar polarities as ethanol. On the contrary, hexane gave inferior result than otherwise reported (Cerón et al., 2008), because in this study the wet biomass was used. In general, a quadratic polynomial relationship can describe the lutein yield and polarity and the relation agrees with Hansen's equation (Kislik, 2012). However, since the other effects such as hydrogen bonding and dispersion also existed but the trend was not very clear. Also, solvents that are miscible with water generally have better results than those form two distinct phases due to the dispersion effect, this prediction was validated by the results which diethyl ether had a high lutein solubility but could not compare to

isopropanol or acetone (Craft and Soares, 1992). Therefore, it may be more reasonable to compare the results in similar solvent systems than different types of solvents.

Ethanol as a solvent was reported previously (Gil-Chávez et al., 2013) and our study also confirmed it to be a good solvent for lutein extraction, while hexane can be used for lipids extraction. The mixture of these two solvents was studied for simultaneous lutein and lipid extraction, also considering the water in wet algae, a three-solvent system was formed.

Some experiments were done to determine extractability of lutein vs. polarity in different polarities among the mixture of the three solvents. The polarity of the solvent mixtures was again measured by Nile Red and was shown in Fig. **4-6**.b and Fig **4-6**.c. A strong correlation can be observed for the polarity trend. Comparing the lutein extraction results from the mixed solvents as shown in Figure **4-6**.c with that of the pure solvents, the data trend fitted more linearly, indirectly indicated that the hydrogen bonding and other factors have influence on the extract yield. The highest value was obtained from 25% (v/v) hexane in ethanol, giving 16.91±0.51 mg/g. The best yields were obtained from the polarity region of 0.45 to 0.65. The conclusion agreed with Ryckebosch *et al.* (Ryckebosch et al., 2014) where the best extraction was from a mixture of dichloromethane and ethanol. The relatively similar polarity (25% hexane in ethanol) facilitated the dissolution of lutein adhered to cell membrane, meanwhile, enabled solvent diffusion into the plastids, where xanthophyll accumulated (Amaro et al., 2015). This obeys the theory of similarity and intermiscibility. The medium polar solvents form hydrogen bonds and weaken van der Waals attraction between pigment-fatty acid esters associations and cell membrane, thus enables higher complex dissolution. The addition of another polar solvent to adjust the polarity may improve the extraction yield. However, considering the convenience and low toxicity, pure ethanol is recommended for lutein production than other mixed ones.

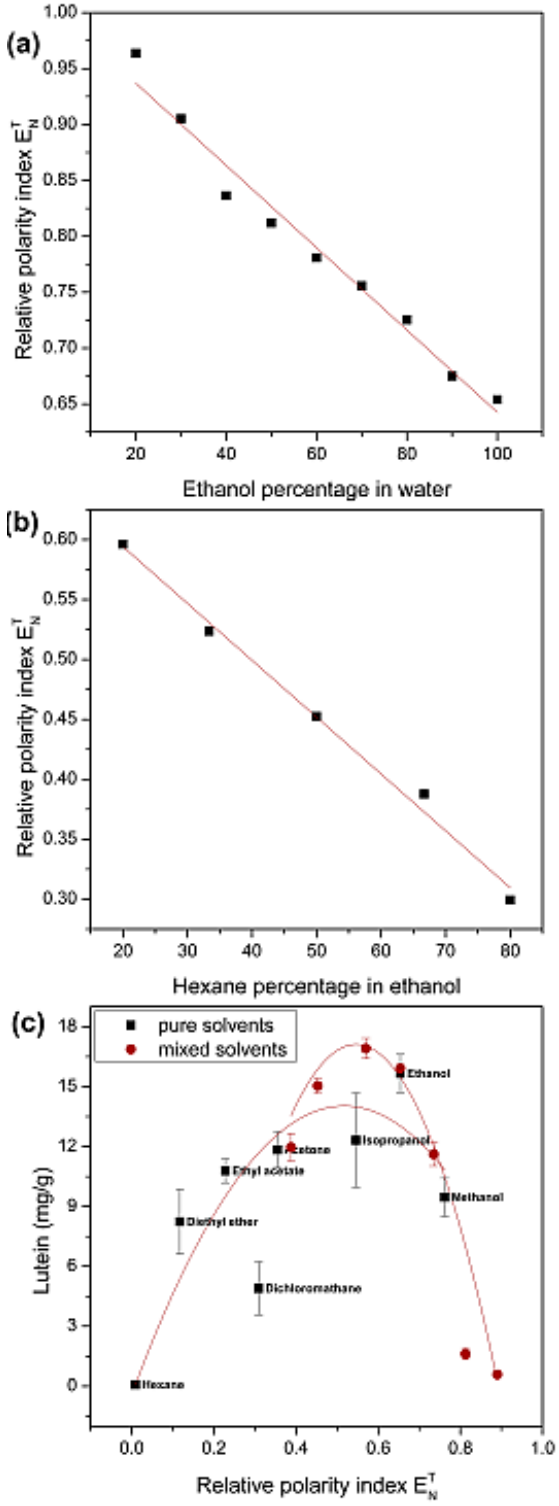

Figure 4-6 Lutein extraction yield from different solvent mixtures (a) ethanol-water, (b) hexane-ethanol vs. polarity index E_N^T. The polarity was obtained from λ_{max} of Nile Red solution. (c) the lutein extraction yields from different pure or mixed solvents

4.4.4 Simultaneous lipid and lutein extraction

Further studies were done towards the simultaneous lipid and lutein extraction from microalgae. The process diagram was shown in fig. **4-1**.b. The process was proven to successfully extract lutein and lipids at the same time. For a 10-mL sample, a minimum amount of water, usually 1 mL, was added to achieve phase separation.

Similar as in section 4.4.2.1, variance of biomass to solvent ratios in current experiment (below 10 mg/mL) had little effect on extracted yield (data not shown). And as expected, the solvent polarity significantly affects the lutein and lipids yield. The results are shown in Fig. **4-7**. The more ethanol, the higher the polarity, the more lutein and less lipids could be recovered.

Comparing with pure ethanol method for lutein extraction, the integrated process extracted slightly lower amount of lutein, around 13.03 mg/g, approximately 85.0% lutein recovery. The trend can be observed as in section 4.4.3, in general, the higher the polarity, the more lutein can be recovered. On the contrary, lipid extraction exhibited a relatively lower efficiency. Around 58.8% compared with B&D method lipid recovery was obtained at 25% ethanol condition.

By mixing hexane and ethanol together, the polarity of the solvent can be switched easily, thus better selectivity for the target product (lutein here). However, the reason speculated as to why it did not improve lutein productivity as shown in Fig.**4-6**.c is that part of lutein was dissolved or bound with lipids in hexane, and unlike before the solvent was evaporated then analyzed, so the lutein retained in ethanol layer decreased. The content analysis of the hexane layer and ethanol layer, and later ether layer gave direct evidence for this, as shown in Fig.**4-7**. Since the hexane and ethanol layers were analyzed directly after phase separation, if the extraction method was improved, e.g. careful wash of both phases with the opposite solvent, it is possible to further increase both lipid and lutein productivity. The process of simultaneous lipid and lutein extraction can be easily adapted for the extraction of other products as well, and is very promising for scaling up and continuous operation (Hodgson et al., 2016).

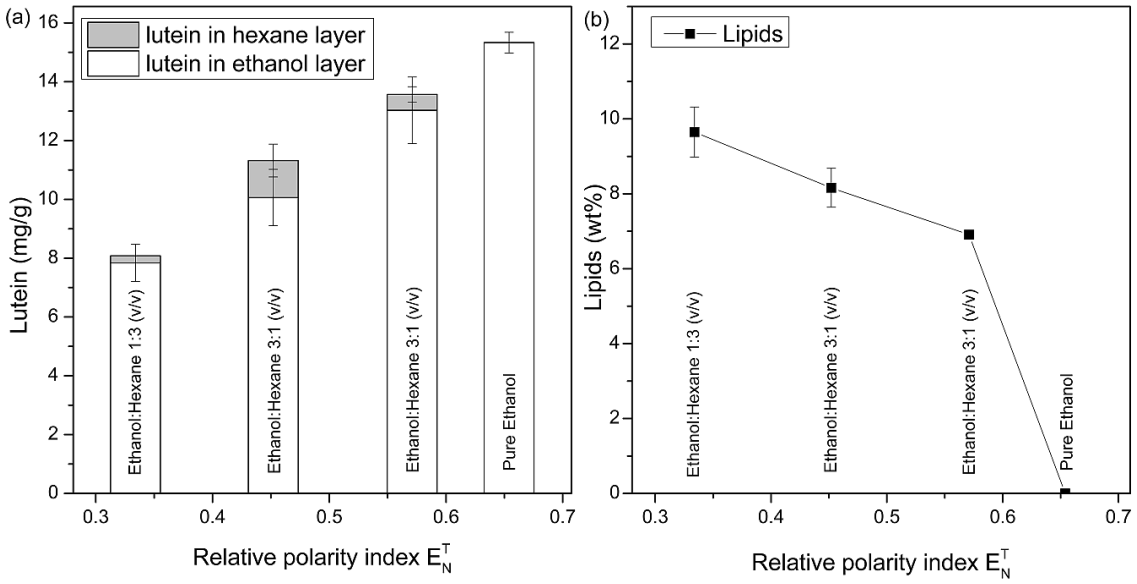

Figure 4-7 Effect of ethanol-hexane binary solvent polarity on (a) lutein and (b) lipid extraction. A small fraction of lutein was extracted to the hexane layer and the lutein distribution is shown for the hexane and ethanol layers. For E_N^T value 0.654, which is pure ethanol, there was no hexane layer so the entire lutein is present in ethanol layer and the lipid recovery from hexane layer is zero.

4.5 Conclusions

Different extraction approaches were compared in both dry and wet extraction. Bead-beater and ultrasonication showed good result for dry extraction, while solvent soaking for wet biomass may be a more energy saving extraction method. Biomass to solvent ratios did not affect the extraction in our experiment, but its effect cannot be ignored to a larger scale. To optimize the lutein yield, harvesting in the late exponential phase achieved highest productivity, and may be suitable for continuous production. Nile Red has been used as a solvatochromic polarity probe to relate the extraction efficiency with solvent polarity, good linearity was revealed in the ethanol-hexane-water mixture when compared with different pure solvents. The wet extraction of lutein and lipids from microalgae is a very promising method, and a proper proportioned ethanol-hexane binary solvent enhanced the extraction yield. The major drawback for the integrated extraction is the low yield of lipids, due to the non-optimizable growth condition and the lipid loss during the extraction.

Chapter 5

5 Development and Modelling of a Single-step Simultaneous Extraction, Saponification and Primary Purification Process for Free Lutein Production from Wet Microalgae

The information presented in this Chapter is based on the paper of same title submitted to Biotechnology and Bioengineering. The sections in Chapter 5 present the results towards the completion of objectives 5 and 6 of the thesis.

5.1 Abstract

Lutein is a commercial yellow to orange carotenoid with potential health benefits. Microalgae are alternative sources to conventional approaches using marigold flowers for the production of lutein. In this study, a single-step simultaneous extraction, saponification and primary purification process for free lutein production from wet microalgae biomass was investigated. The feasibility of binary solvent mixtures for wet biomass extraction was successfully demonstrated, and the extraction kinetics of lutein from chloroplast were evaluated. The effects of type of organic solvent, solvent polarity, method of cell disruption, alkali and solvent usage on lutein yields were examined. The apparent mass transfer rate of lutein extraction was found to be controlled by the intra-particle diffusion. A mathematical model based on Fick's second law of diffusion was applied to model the experimental data. The best conditions for extraction efficiency were found to be pre-treatment with ultrasonication at 0.5s working cycle per second, react 0.5-1 hour in 160 mL final solvent volume of 1:3 ether/ethanol (v/v) with 2 mL 10% (w/v) ethanolic KOH. In addition, the extraction rate was found more significantly related with alkali ratio to solvent than to biomass.

5.2 Introduction

Lutein is a commercially available high value-added product. It belongs to the group of the carotenoids, and is responsible for the natural yellow to orange color (Taylor et al., 2012). Therefore, lutein is an important food colorant; moreover, lutein is clinically proven to

improve human eye health, especially for reducing the risk of cataract and age-related macular degeneration (Manayi et al., 2015). Traditionally, marigold flowers are used as natural lutein sources; however, microalgae have attracted increasing attention as an alternative source for lutein production due to faster growth rate, higher lutein content, less labor requirements, and reduced land requirement (Gong and Bassi, 2016; Kim et al., 2013). The major challenge for commercial production of lutein from microalgae lies in downstream extraction and separation, which account for 50 – 80% of the total production cost (Amaro et al., 2015). This is because of the existence of a rigid cell wall in many algal species limited the yield and rate of pigment extraction (Chan et al., 2013; McMillan et al., 2013). Thus, research is needed for extraction procedures that are fast, simple, selective, and cost-effective.

Conventionally, the natural lutein esters are first extracted from the dried source biomass, then saponification is used to produce lutein in free form (Wang et al., 2016), followed by further extraction for improved purification. Solvent extraction is widely employed to separate and purify lutein on an industrial scale. In general, during the extraction, the internal diffusion of the solute occurs from inside the particle to the surface, then the solute transfers to the stagnant solvent film around the particle, finally to the bulk solvent (Hojnik et al., 2008). The diffusion step in the particle is most commonly assumed as the rate-limiting step in this mass transfer mechanism, and can be predicted by the simplified unsteady state second order Fick's equation (Hojnik et al., 2008; Shi et al., 2003).

The development of a new single-step method that skips drying, and combines extraction, saponification and purification approach may save both time and solvent. Previously, Wang et al. (2016) have developed a procedure for a combined procedure of lutein extraction from marigold flowers, but similar studies for more microalgal lutein extraction and purification are rare. In this study, a single-step extraction, saponification and purification method is investigated for extraction of lutein from the wet biomass of the microalgae *Chlorella vulgaris* UTEX 265. The feasibility of a binary solvent for biomass extraction were investigated, and the extraction kinetics of microalgal lutein extraction were monitored under different conditions for a better understanding and optimization of the process. The experimental data was also fitted using mathematical modelling and the

diffusion coefficients were determined to represent the apparent mass transfer rate at different conditions.

5.3 Methods

5.3.1 Microalgae type and cultivation

Chlorella vulgaris UTEX 265 was maintained in Bold's Basal Medium (BBM) at 23°C, as described in section 3.3.1. Microalgae were harvested at the end of the growth period by centrifugation at 3,500 rpm for 15 min at 4 °C in a Sorvall R40 centrifuge (ThermoScientific, USA). The wet microalgae pellet was washed three times with deionized water and frozen at −20°C for further analysis.

5.3.2 Extraction method

Simultaneous extraction and saponification experiments were conducted in a batch extraction mode for five hours in a 500-mL flat bottom glass bottle with screw cap. For each experiment, 0.60 g dry weight equivalent frozen microalgae pellets were re-suspended in 10 mL ethanol, then subjected to ultrasonic treatment using a 50W Ultra-sonication probe (Hielscher, Germany) at 0.5 s per second for a specified time (0 to 30 min). The biomass suspension was then added to a specified known volume of extraction solvent mixture. In addition, ethanolic potassium hydroxide was added and this suspension was then well mixed using a magnetic stirrer. The binary solvents used were as follows (v/v): 1/3 ethanol/ether ($C_2H_5OH/C_2H_5OC_2H_5$), 1/1 ethanol/ether, 3/1 ethanol/ether, 1/1 ethanol/hexane (C_2H_5OH/C_6H_{14}), 3/1 ethanol/hexane. The final solvent volume was 80, 160 or 320 mL. Various ratios were investigated, i.e., 10% (w/v) alkali in ethanol added to the ratio of dried algae biomass (R_a) were 1, 2, 4, and 8 L/kg, consequently, the concentration of alkali in extraction solvent (C_a) was 0.313, 0.625, 1.25, or 2.5 g KOH/L solvent.

The samples were collected (1% total initial volume) at specified time intervals, centrifuged at 3,500 rpm for 5 min, then the supernatants were combined. Water was added to achieve phase separate lutein from other contents for purification purposes. The ether or

hexane phase was collected and filtered through a 0.22 µm PTFE filter for UV/Vis and HPLC analysis.

5.3.3 Analysis methods

5.3.3.1 Solvents polarity measurements

The Nile Red dye was used to determine the polarity of the liquids as described in section 4.3.3.2.

5.3.3.2 Pigments quantification

The pigment analysis was carried out by HPLC as in section 3.3.7. A Cary Bio 50 UV/Visible Spectrophotometer (Varian, USA) was used for simplified lutein measurement. The method was similar to described by Hojnik et al. (2008). Absorbance at 445 nm were measured and calibrated with HPLC to obtain the calibration curve. Each solution was measured five times and the average was taken. Once the concentration of lutein was known, it was multiplied by the total volume of solvent to get the mass of lutein, then divided by the mass of microalgae dry weight to get the lutein yield (mg lutein / g cell dry weigt).

5.3.4 Statistics

All of experiments were at least triplicated unless otherwise stated. The mean of the samples was reported and the STDEV was shown as error. ANOVA test using a confidence level of 95% was used to establish the statistical significance. The performance of model was evaluated by the average absolute relative deviation (AARD).

5.4 Theory

The method used by Hojnik et al. (2008) was used to model the free lutein recovery process. Lutein is located inside the chloroplast in microalgae (Camejo et al., 2006), once the cell wall was disrupted, the chloroplast would be released. The entire process happened during the free lutein extraction can be described as (1) the solvent forms a thin layer around the solid matrix and dissolution or desorption happens, (2) the diffusion of solute/solvent mixture from the inside to the surface of the solid particle happens, (3) the solute moves

across the stagnant film to the bulk solvent (Crank, 1975), and in our process an additional step need to be considered, which is (4) the hydrolysis of the extracted lutein esters into free lutein. Since usually the second step is the rate-limiting step (Chan et al., 2014), the dynamic behavior of the extraction and saponification of lutein can be modelled (Hojnik et al., 2008). However, due to the existence of saponification reaction, so instead of diffusion rates, the apparent mass transfer rates were actually measured. But this rate is controlled by diffusion rate, so we could apply the same approach as used for diffusion rates to model the apparent mass transfer rate of free lutein.

Therefore, the assumptions of symmetrical and porous solid sphere were made to model the lutein release from the chloroplast into the well-stirred bulk liquid. Besides, uniform concentration of lutein in chloroplast, constant extraction rate in each extraction stage, and 6 μm solid diameter (size for chloroplast) was assumed. The external mass transfer resistance was minimized by using very low biomass to solvent ratio.

Fick's law of diffusion was widely employed for the modeling of extraction process. Assuming uniform concentration of lutein in the chloroplast particle, homogeneous solid-liquid mixing and no interaction between the diffusion of solute and other compounds, the extraction of solute in solid particles depends on time, t, and radius, r. The equation can be written as:

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial r^2} \tag{5.1}$$

Here the term on the left hand side of Equation 5.1 represents the extraction rate. The respective initial and boundary conditions can be written as:

$$t = 0, \qquad C = C_0 \qquad 0 \leq r \leq R \tag{5.2}$$

$$t > 0, \qquad C = Ci = 0 \qquad r = R \tag{5.3}$$

$$t > 0, \qquad \frac{\partial C}{\partial r} = 0 \qquad r = 0 \tag{5.4}$$

where C_0 is the initial lutein concentration in chloroplast, Ci is the lutein concentration at the solid-liquid interface. Assuming negligible mass transfer resistance of lutein in the very diluted microalgae extracts, the general solution of equation (5.1) for spherical samples can be written as:

$$\frac{C-C_0}{C_i-C_0} = 1 + \left[\frac{2R}{\pi r}\sum_{n=1}^{\infty}\frac{(-1)^n}{n}\sin\frac{\pi n r}{R}\exp\left\{-\frac{Dn^2\pi^2 t}{R^2}\right\}\right] \quad (5.5)$$

The mass of solute, M, here calculated as free lutein, transferred from the sample particle sphere at any time t, can be calculated by solving equation (5.5):

$$\frac{M}{M_\infty} = 1 - \frac{6}{\pi^2}\sum_{n=1}^{\infty}\frac{1}{n^2}\exp\left\{-\frac{Dn^2\pi^2 t}{R^2}\right\} \quad (5.6)$$

As only the first term of eq. (5.6) remain significant after a short time, the equation can be expressed in the simplified form:

$$\ln\left(\frac{c_\infty}{c_\infty - c}\right) = 0.498 + \frac{9.87Dt}{R^2} \quad (5.7)$$

Where c is the lutein concentration in the solution at time t, c_∞ is the concentration in the solution after infinite time. Here the solid-liquid extraction can be divided into two stages. The first stage is the fast stage, which represents the period of fast extraction at a constant extraction rate limited by the film resistance. The internal diffusion is limiting the extraction rate in the second stage, where the extraction rate is much slower and keeps decreasing. By plotting equation (5.7) against time using experimental data, two intersecting straight lines can be drawn based on the points, representing the fast stage and the slow stage of the extraction, and the intersection of the two lines is the transition point of the two stages. As suggested by Osburn and Katz (1944), the two parallel diffusion processes should both be considered to achieve better modelling results, the eq. (5.7) can be rewritten as:

$$\frac{c_\infty}{c_\infty - c} = \frac{6}{\pi^2}\left[f_1\exp\left\{-\frac{\pi^2 D_{fast}t}{R^2}\right\} + f_2\exp\left\{-\frac{\pi^2 D_{slow}t}{R^2}\right\}\right] \quad (5.8)$$

where f_1 and f_2 are the fractions of the solute, in corresponding to the apparent mass transfer (dominated by diffusion) coefficients D_{fast} and D_{slow}.

5.5 Results and discussion

As illustrated in Figure **5-1**, the extraction, saponification, and primary purification steps for lutein recovery from the microalgal biomass were combined into a single step operation, and the overall extraction kinetics were monitored. A binary solvent system was used in this study to simplify the extraction process of lutein from microalgae biomass. As the alkali was added in the single step, the saponification of lutein esters into free lutein happened simultaneously with the solid-liquid extraction. The separation and purification was achieved in the separator by using the polar solvent (ethanol) and the non-polar solvent (diethyl ether or hexane) and subsequent addition of water. As water was added to the binary solvent system, the more polar components, i.e., KOH, chlorophylls, proteins, sugars, and saponified lipids, remained in the bottom water-ethanol layer, while the non-polar solvent was in the upper layer and contained lutein and other carotenoids, mainly β-carotene.

The use of binary solvent enabled easier lutein extraction from wet biomass. This is because the polar solvent can easily penetrate into the wet biomass containing water, and the non-polar solvent has stronger affinity for lutein extraction (Ryckebosch et al., 2014). The non-polar solvent served two roles in the process: extracting solvent of leaching as well as the liquid-liquid extraction solvent. This single-step approach potentially can save both overall extracting time and lead to reduced solvent usage. The choice of inter-miscible polar and non-polar solvent systems can also reduce the mass transfer resistance by avoiding additional liquid-liquid extraction steps. Ethanol was reported to be the best solvent for lutein extraction from wet microalgae, and hexane is the most conventional solvent applied for lutein extraction from marigold flowers on industrial scale (Soares et al., 2016). In addition, diethyl ether (ether), is reported as a good solvent due to the high solubility of lutein and its low boiling point (Chan et al., 2013). Therefore, ethanol/hexane and ethanol/ether solvent systems were chosen for further investigation in this study.

The overall lutein mass transfer extraction rate was a combination of saponification and the free lutein extraction rate. The effects of solvent type, polarity, volume, usage of alkali, and the presence of cell disruption pre-treatment on free lutein extraction kinetics were studied. The results were fitted into the model based on two term Fick's second law of diffusion to determine the apparent mass transfer rates during the free lutein extraction as described in the Theory section. The determination of a "fast-stage" and a "slow-stage" extraction is shown graphically in Figure **5-2** based on Equation (5-8). The slope and the intercept of a first-order fit were used to determine the apparent mass transfer coefficients as per Equation 5-8. Since the slow extraction stage is the only process that is significant at later stages, the parameters of slow stage fraction f_2 and apparent mass transfer coefficient D_{slow} can be first determined from the slope and intercept of the second stage by a plot of $\ln\left(\frac{c_\infty}{c_\infty - c}\right)$ vs. time. Then fast-stage fraction f_1 and apparent mass transfer coefficient D_{fast} of the early fast-stage extraction can be determined as the second term is close to unity in Equation 8. The average absolute relative deviation (AARD) was employed to estimate the model performance.

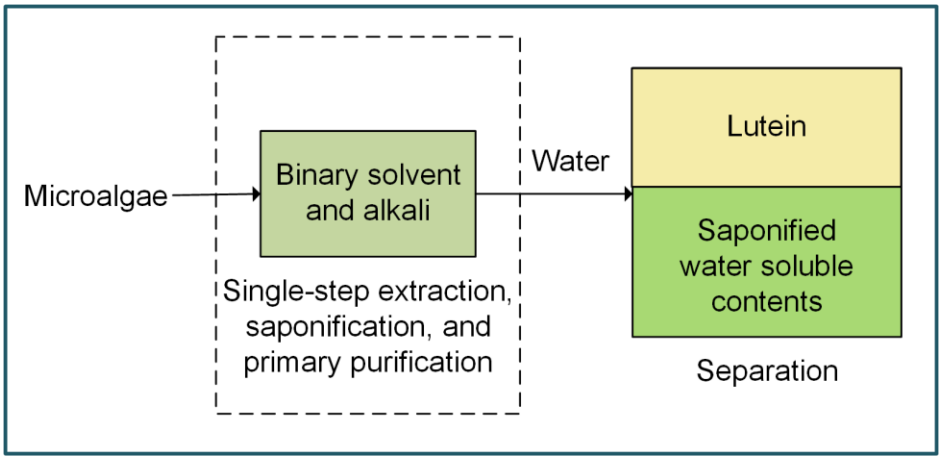

Figure 5-1 Schematic diagram of single step lutein extraction, saponification and primary purification, and subsequent separation process to acquire free lutein

As described in the methods, the determination of washing stage and slow stage are conducted graphically, as represented in figure **5-2**. The slope and the intercept of the first-order fitting plot was used to determine the apparent mass transfer coefficients.

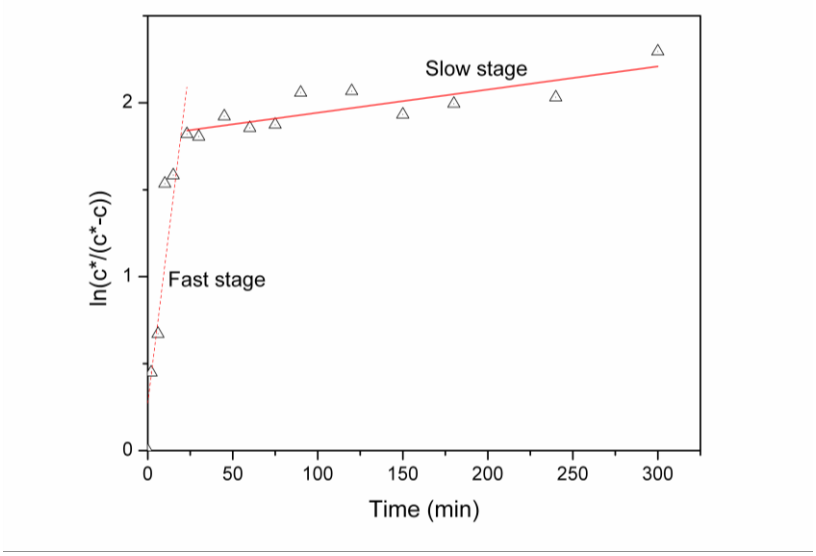

Figure 5-2 Representative first-order plot for the washing stage (solid line) and slow stage (dashed line) of lutein extraction. The points represent a typical extraction result with 160 ml 1:3 ether/ethanol (v/v) at 23 °C, 2 ml KOH, ultrasound pre-treatment of 10 minutes at 0.5s/s working cycle.

5.5.1 Effect of solvent type

The choice of solvent is known to affect the lutein extraction yield (Chan et al., 2013). Since polarity is also a significant factor for the lutein extraction yield, in addition to the different solubilities of lutein of various solvents, first the binary solvent mixtures of identical polarities were compared for best lutein extraction yield and extraction rate. This was achieved by adjusting the polarities of binary solvent systems to the same value using Nile Red dye as a polarity indicator. According to the maximum absorbance wavelength of the solvent (λ_{max}), 1:1 hexane/ethanol (v/v) has similar polarity with 3:2 ether/ethanol (v/v), with a polarity index E_N^T of 0.452. therefore, these two solvent mixtures were compared in the kinetics study. The extraction curves were plotted in Figure **5-3**. The lutein concentration in the bulk solvent, presented by the calculated lutein extraction yield, was plotted against extraction time.

In general, the extraction curves are similar to the conventional solid-liquid extraction curves of bioactive compounds (Tao et al., 2014). A fast stage occurred with a steep slope,

followed by the exponential shape. In the fast stage, the extractable components located on the exposed particle surfaces are washed into the bulk solvent rapidly; while in the slow stage, the diffusion of dissolved solute inside the solid particles controls the extraction rate (Tao et al., 2014). The extracted lutein increased rapidly during the first 15 minutes, then incremented slowly after the initial extraction stage passed. However, the ether/ethanol binary solvent had a much higher lutein extraction yield than the hexane system, despite the two solvent systems have similar polarity. The final yield in ether system was 6.5 mg/g, which was about 2.5 mg/g higher than that in hexane system after the five-hour extraction. The reason for the variation mainly contribute to the difference in lutein solubility, which is 20 mg/L for hexane, and 2000 mg/L in ether (Craft and Soares, 1992).

The apparent mass transfer coefficients D_{fast} and D_{slow} are good indicators of the rate of free lutein recovery. The extraction kinetics curves were fitted into equation (5-8) to obtain D_{fast} and D_{slow} and the fractions of fast stage f_1 and slow stage f_2. The kinetics parameters and model statistics were calculated and exhibited in Table **5-1**. The models fitted the curve quite well, judging from the small deviation. From figure **5-3**, the fraction of lutein released was much higher in the fast stage than in the slower stage. However, the calculated values of f_1 in Table 5-1 were low, below 0.50, which is due to the reason that a uniform c_0 8.5 mg/g was assumed to better compare the apparent mass transfer coefficients D_{fast} and D_{slow}. Although f_1 does not represent the true fraction of fast stage, the general trend still can be inferred from the values. The rate constants of apparent mass transfer (D_{fast} of $1.8\text{-}6.2\times10^{-11}$ cm^2/s) were much smaller to previous reported studies with extraction from marigold flowers (Hojnik et al., 2008). It is speculated that the complex chloroplast structure may be more difficult to extract inner contents out. Both D_{fast} and D_{slow} was higher in ether system than the hexane system, indicating more efficient mass transfer in ether system, so ether was chosen for the subsequent experiments.

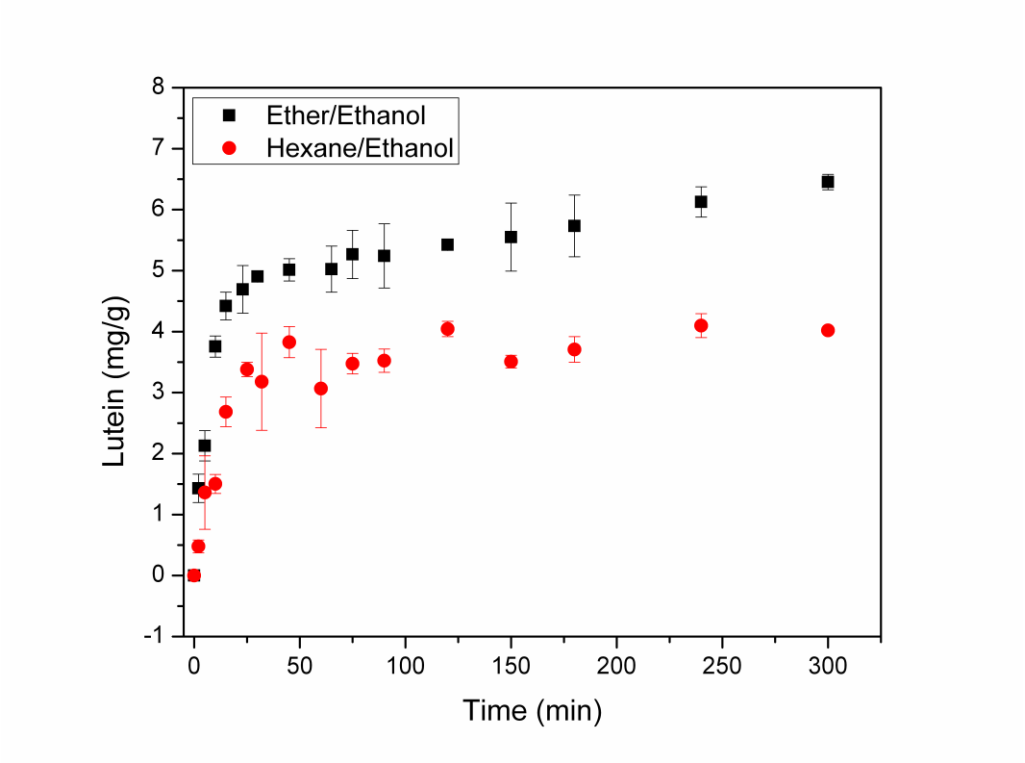

Figure 5-3 lutein extraction kinetics for two different solvent mixture types: 1:1 hexane/ethanol (v/v) and 3:2 ether/ethanol (v/v) with 10-minute ultrasound pre-treatment. The extraction was in 160 mL final solvent volume with 1 mL 10% (w/v) ethanolic KOH. The lutein concentration in the bulk solvent is presented by calculated lutein extraction yield ± STDEV, n=3.

The extraction kinetics curves were modeled by the two term Fick's second law of diffusion equation (5-8). The kinetics parameters and model statistics were calculated and exhibited in Table **5-1**. The models fitted the curve quite well, judging from the small deviation. From the figure, the fraction of lutein released was much higher in the washing stage than in the slower stage. However, the calculation of f_1 in table **5-1** did not agree, due to the uniform c_0 8.5 mg/g was used for better compare of the apparent mass transfer coefficients D_{fast} and D_{slow}. Although f1 does not represent the true fraction of washing stage, the general trend still can be told from the values. The values of lutein concentration derived from OD and HPLC were different (Fig. S4 in appendix II), due to the optical density could not differentiate lutein esters from free lutein at 445 nm, so the values

calculated from HPLC readings are reported. So, it can be determined that the extraction rate was faster than that of saponification. The rate constants of apparent mass transfer were close to reported (Hojnik et al., 2008). Both D_{fast} and D_{slow} were higher in ether system than the hexane system, indicating more efficient mass transfer in ether system, so ether was chosen for the subsequent experiments.

Table 5-1 Apparent mass transfer coefficients and model constants calculated for two different solvent systems. The values are the mean of three experiments.

Non-polar solvent	$D_{fast}\times10^{11}$ (cm^2/s)	f_1	$D_{slow}\times10^{11}$ (cm^2/s)	f_2	AARD
Ether	1.73	0.4514	0.033	0.5486	0.0746
Hexane	0.51	0.1529	0.018	0.8471	0.0254

5.5.2 Effect of polarity

Beside the solvent type, the polarity of the solvent as well plays an significant role in the lutein extraction. The polarity was adjusted by changing the ethanol to ether ratio at three levels: 1:3, 1:1, or 3:1 ether/ethanol (v/v). Fig. **5-4** shows the amount of lutein extracted vs. time. The calculated apparent mass transfer coefficients are summarized in Table **5-2**. The higher polarity improved the extraction yield, but had a negative effect on the extraction rate. The trend agreed with that of Ryckebosch et al. (2014). The rapid initial mass transfer of solute toward the bulk solvent is represented by the high values of D_{fast} of the less polar solvent mixtures, $3.61\text{-}4.74\times10^{-11}$ cm^2/s. These mixtures extract the surface content faster due to their stronger solvent power, but did not extract as much lutein in the slower stage, corresponded to poor mass transfer efficiencies, which is explicitly described by the decrease trend of D_{slow} from 0.042 to 0.023×10^{-11} cm^2/s as the ethanol ratio decreased. The potential reason is that ethanol could form hydrogen bonds, and can weaken the van der Waals force between lutein-lipid associations and the cell membrane. The larger ratio of polar solvent ethanol facilitated the dissolution of lutein bond to algae cell membrane, and enabled solvent diffusion into the chloroplast inside the center of the cell

matrix. The fractions of fast stage and slow stage of the three ratios of solvent mixture did not make significant difference.

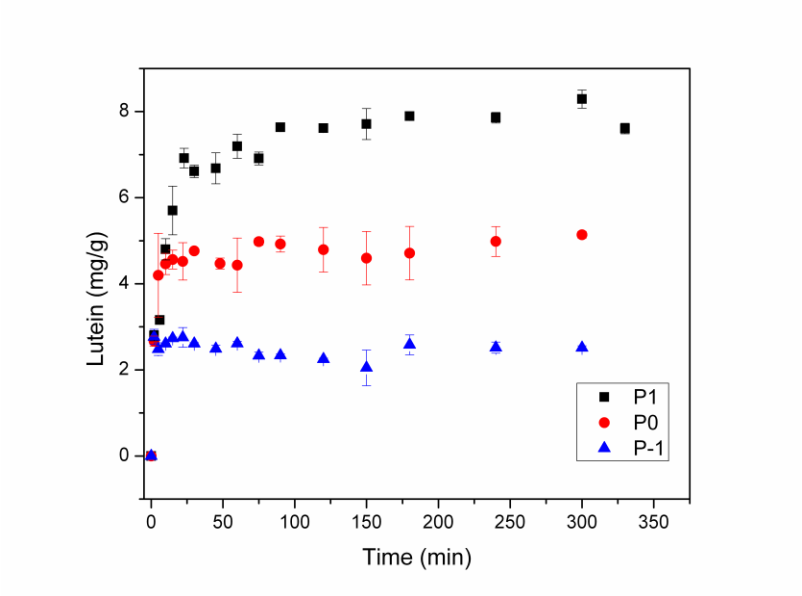

Figure 5-4 Lutein extraction kinetics for three different solvent mixture types: P1: 1/3, P0: 1/1, P-1: 3/1 ether/ethanol (v/v). The extraction was pre-treated with 10 minutes ultrasonication at 0.5s working cycle per second. The extraction was in 160 mL final solvent volume with 2 mL 10% (w/v) ethanolic KOH. The lutein concentration in the bulk solvent is presented by calculated lutein extraction yield ± STDEV, n=3.

Table 5-2 Apparent mass transfer coefficients and model constants calculated for three different solvent mixture types. The values are the mean of three experiments.

Ether/ethanol (v/v)	$D_{fast}\times10^{11}$ (cm^2/s)	f_1	$D_{slow}\times10^{11}$ (cm^2/s)	f_2	AARD
1/3	4.89	1.36	0.4868	0.042	0.5132
1/1	12.98	3.61	0.4623	0.036	0.5377
3/1	17.08	4.74	0.4728	0.023	0.5272

5.5.3 Effect of solvent volume

In this study, three solvent volume was tested for extraction efficiency: 320 mL, 160 mL, and 80 mL. For a fixed 0.5 g dry-weight equivalent wet biomass. The results are presented in Figure **5-5**. Generally, the concentration of active compounds in the solvent phase increases until the equilibrium is reached (Hojnik et al., 2008). The amount of solvent used was more than saturation to minimize the diffusion resistance from the intact film to bulk solvent. The three curves showed similar final lutein yield after five hours. However, slight variance can be observed in the slope of fast stage. As shown in Table **5-3**, the larger the amount of the solvent, the faster the mass transfer in fast stage. The D_{fast} of smallest amount of solvent volume, 1.08×10^{-11} cm^2/s, was approximately half that of the 320-mL trial. This is in good agreement with theory of the driving force of diffusion being the concentration gradient. As the final lutein concentration in solvent was way lower than the saturation, similar extraction yield of free lutein was eventually achieved at a yield around 7.6-8.0 mg/g.

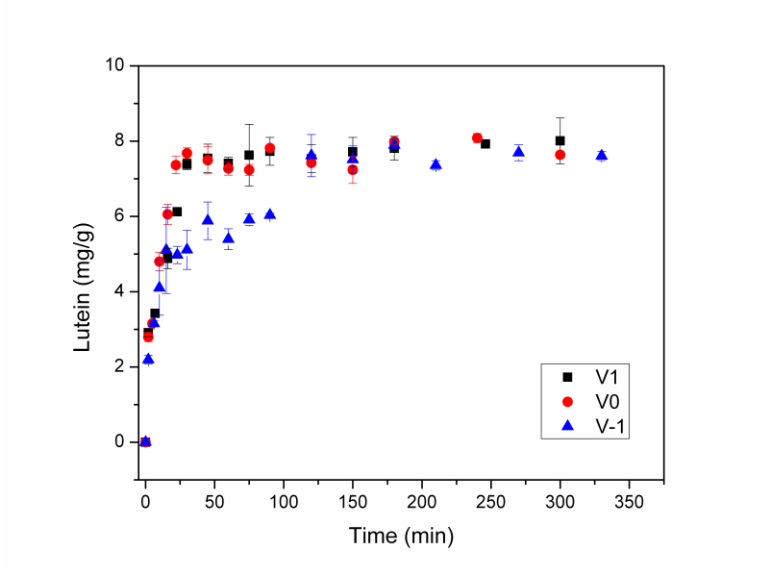

Figure 5-5 Lutein extraction kinetics for three different volume: V1: 320 mL, V0: 160 mL, V-1: 80 mL. The extraction was pre-treated with 10 minutes ultrasonication at 0.5s working cycle per second, and was conducted in 1:3 ether/ethanol (v/v) with 2 mL 10% (w/v) ethanolic KOH.

Table 5-3 Apparent mass transfer coefficients and model constants calculated for three different solvent volumes. The values are the mean of three experiments.

Solvent volume (mL)	$D_{fast}\times10^{11}$ (cm^2/s)	f_1	$D_{slow}\times10^{11}$ (cm^2/s)	f_2	AARD
320	2.05	0.5525	0.030	0.4475	0.0407
160	1.23	0.4864	0.042	0.5136	0.0892
80	1.08	0.4468	0.078	0.5532	0.1092

5.5.4 Effect of alkali usage

Since simultaneous saponification and extraction of lutein was conducted in a single step for simpler process, it was important to also study the effect of usage of alkali on final yield in addition to the traditional mass transfer operators. The alkali used in this experiment was KOH dissolved in ethanol in a volume percentage of 10% (w/v). Different levels of the 10% alkali solution were added, specifically 1, 2, 4 and 8 mL, which has a concentration of alkali in extraction solvent (C_a) of 0.313, 0.625, 1.25, and 2.5 g KOH/L, or alkali to dried algae biomass ratio (R_a) of 1, 2, 4, and 8 L/kg, respectively. The corresponding extraction curves are shown in Fig. **5-6**. Most noticeably, the smallest amount of alkali used, denoted as A-1, had a very low lutein yield. It was about half of the value of the other trials. This can be explained by the insufficient OH^- to cleave lutein esters into free form. No significant difference was observed for the 2 or 4-mL trials, both yielded around 8.0 mg/g lutein during the experiment, typical to most of the other experiments in this study. The lutein content was higher than other studies due to the improved operation procedure reduced the operation units and minimized the lutein loss during drying and transferring (Chiu et al., 2016; Dineshkumar et al., 2015). The 8-mL trial had a similar trend with the 2 or 4-mL one, but degradation was observed after the initial phase; therefore, the lutein yield was not as high. Its lutein content topped at 30 min, 7.1 mg/g, but decreased to 5.9 mg/g at the end. The rates of apparent mass transfer can be better monitored from table **5-**

4. The highest concentration of alkali had the fastest extraction rate, with a apparent mass transfer coefficient of 4.30×10^{-11} cm^2/s. As the molecular size of lutein esters are larger than the cell membrane pore size, in the traditional process, it was necessary to lyse the intact cell for bioactive compound extraction (Azencott et al., 2007). However, OH- and free lutein is small enough to travel through the cell wall pores, which may also benefit the extraction rate. The D_{fast} of the 2 or 4 mL experiments were $1.77\text{-}2.05\times10^{-11}$ cm^2/s. The values of D_{slow} showed an opposite trend as D_{fast}, decreased as the alkali concentration went higher, probably due to the reduced concentration difference, as more lutein were extracted to the bulk solvent in the fast stage. The lowest concentration of alkali performed poorly in every aspect. So, the alkali amount cannot go too low for full release of free lutein, nor too high to avoid degradation.

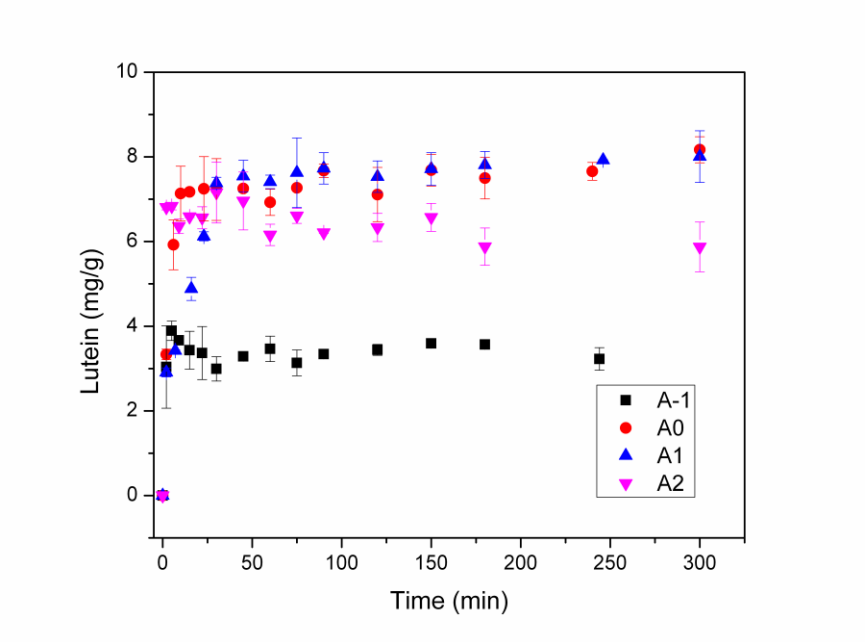

Figure 5-6 Lutein extraction kinetics for four alkali usage levels: A2: 8 ml 10% (w/v) ethanolic KOH, A1: 4 mL, A0: 2 mL, A-1: 1 mL. The extraction was pre-treated with 10 minutes ultrasonication at 0.5s working cycle per second, and was conducted in 320 mL 1:3 ether/ethanol (v/v) with 2 mL 10% (w/v) ethanolic KOH.

Table 5-4 Apparent mass transfer coefficients and model constants calculated for four different 10% (w/v) ethanolic KOH usage. The values are mean of three experiments

Alkali concentration (C_a) (g/L)	$D_{fast}\times10^{11}$ (cm^2/s)	f_1	$D_{slow}\times10^{11}$ (cm^2/s)	f_2	AARD
2.5	4.30	0.5063	0.027	0.4937	0.3278
1.25	2.05	0.5525	0.030	0.4475	0.0407
0.625	1.77	0.6291	0.065	0.3709	0.3217
0.313	1.32	0.5155	0.025	0.4845	0.3017

It is also important to understand whether the alkali concentration or the absolute alkali amount in the solvent is more significant for extraction and hydrolysis rate. This knowledge would offer a more precise guideline for the usage of solvent and alkali amount for optimal lutein extraction yield and efficiency. A 2-factor-3-level full factorial design was used for the interaction study solvent volume and alkali usage, as table **5-5** indicates. The ranges were determined from previous studied results (Hojnik et al., 2008). An ANOVA analysis was carried out which showed that the lutein extraction rate, as indicated D_{fast}, was more significantly related with the concentration of KOH in solvent, than with the ratio of KOH to microalgae dry mass ($p > 0.05$). Thus, it can be concluded that the lutein yield was limited by the amount of alkali added, though the extraction and hydrolysis rate is co-related to the concentration. The apparent mass transfer coefficients were fitted into the second-order polynomial model, and the fitted model for D_{fast} is listed below:

$$D_{fast} = 1.55 + 0.793\ V + 0.762\ A - 0.2\ V^2 + 0.552\ AV + 0.845\ A^2 \quad (R^2=0.959) \quad (5.9)$$

where A is the coded alkali concentration, V is the coded solvent volume.

Table 5-5 Experimental conditions for interaction study solvent volume and alkali usage and the corresponding apparent mass transfer coefficients and model constants. The values are mean of three experiments.

Solvent volume (mL)	Alkali volume (mL)	Ca	Coded solvent volume	Coded alkali volume	$D_{fast}\times10^8$ (cm^2/s)	f_1	$D_{slow}\times10^8$ (cm^2/s)	AARD
320	8	2.5	1	1	15.47	0.51	0.096	0.1736
320	4	1.25	1	0	7.386	0.55	0.108	0.2686
320	2	0.625	1	-1	6.354	0.63	0.234	0.3208
160	4	2.5	0	1	12.27	0.44	0.096	0.4007
160	2	1.25	0	0	4.476	0.54	0.150	0.1114
160	1	0.625	0	-1	6.100	0.52	0.216	0.4303
80	2	2.5	-1	1	4.894	0.36	0.174	0.3592
80	1	1.25	-1	0	3.470	0.47	0.180	0.143
80	0.5	0.625	-1	-1	3.734	0.46	0.396	0.3217

Since the factor parameters were codified, it can be easily seen that the alkali concentration has a more significant effect on lutein extraction rate than the solvent volume. The interaction term has a weight of 0.552, so it could not be ignored. Almost all the term coefficients are positive, indicated that the increase of both factors and their interaction within the tested range would benefit the extraction yield. The highest D_{fast} was obtained at 320 mL volume, 4 ml alkali. Figure **5-7** (a) showed the fitted model plot in the experimental range.

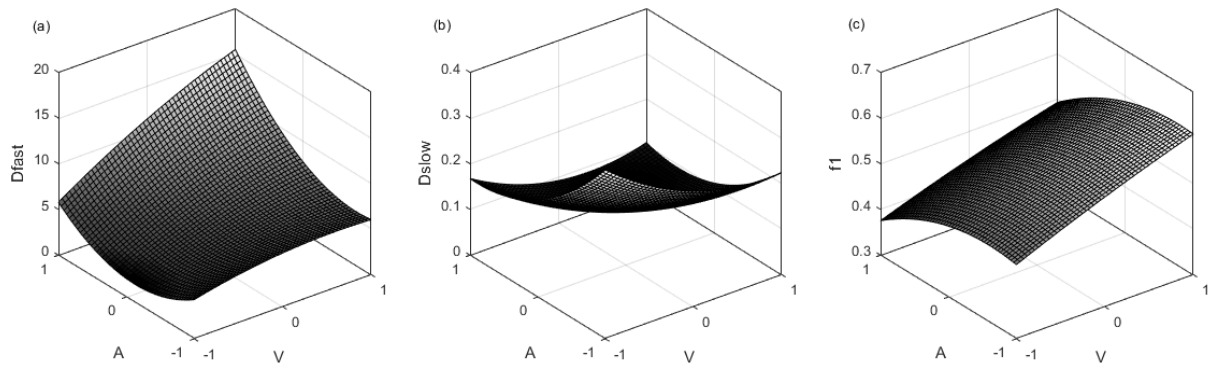

Figure 5-7 Lutein extraction kinetics fitted models for D_{fast} (a), D_{slow} (b), and fraction of washing stage (c)

The change in D_{slow} does not make a significant difference in the practice due to the low efficiency in the slow stage. The model, however, is still given and plotted in figure **5-7** (b).

$$D_{slow} = 0.0327 - 0.0143V - 0.0222A + 0.012V^2 + 0.006\,AV + 0.0155\,A^2 \quad (R^2=0.936) \quad (5.10)$$

Contrary to D_{fast}, the slow stage extraction rate was negatively related with the alkali concentration and solvent volume. All the quadratic terms have positive effect on extraction rate in the slow phase. The reason for the opposite trend should be the same as previously discussed in this section.

In addition to the apparent mass transfer coefficients, the fraction of fast stage within the entire extraction process was studied. Maximizing this portion would greatly enhance the extraction efficiency. The model is given below and plotted in figure **5-7** (c).

$$f_1 = 0.526 + 0.0658V - 0.0502A - 0.00517V^2 - 0.00675AV - 0.0362A^2 \quad (R^2=0.940) \quad (5.11)$$

The optimal point was at 320 mL solvent, 2 mL KOH. The terms beside solvent volume are all negative, showing that the parameters beside solvent volume have negative effects on the fast stage fraction. The reason for the lower values of f_1 compared with literature

(Tao, 2014) should attribute to the following three reasons: 1. Lutein is located inside the chloroplast in the center of the cell matrix, so the diffusion of lutein to particle surface takes time. 2. Saponification was conducted in parallel with extraction, which should take a long time than the one step extraction. 3. To acquire better compares of D_{fast} and D_{slow}, uniform c_0 value of 8.5 mg/g was used instead of the infinite lutein concentration in each extraction in the apparent mass transfer, or diffusion, model fitting.

5.5.5 Effect of biomass pre-treatment

The presence of biomass pre-treatment is widely recognized as an effective method to enhance lutein yield (Guedes et al., 2011). However, its effect on lutein extraction kinetics was seldom studied. Therefore, lutein extraction yield in presence of cell disruption was compared with non-treated biomass. Considering the scale of operation and cell disruption efficiency, ultrasonication was chosen, since it can be adapted to larger scale continuous extraction and was reported to maximize carotenoid yield from freeze dried thraustochytrids biomass (Singh et al., 2015). Additional cell disruption pre-treatment breaks up the cell wall, increases the contact surface area of biomass to solvent, and facilitates the mass transfer of intracellular solute. The results to determine the effect of pre-treatment for lutein extraction from wet microalgae biomass are presented in Figure **5-8**.

As seen in Figure **5-8**, The initial mass transfer rate together with lutein esters hydrolysis (saponification) rate was fast, obtained from the steep slopes of the fast stage in ultrasound treated 30 minutes, 10 minutes and no treatment, in corresponding to the D_{fast} values of 1.58, 1.36, and 1.03×10^{-11} cm^2/s in table **5-6**, respectively. The increasing trend of D_{fast} as the ultrasonication time increased indicated that the treatment of cell disruption benefited the washing effect. However, the fast stage fraction was highest at 10-minute level, rather than the 30-minute. This is probably due to the degradation of heat and oxygen sensitive lutein during the ultra-sonication process. The degradation was also observed in the final lutein yield: the 10-minute had a value of 8.3 mg/g while that of 30-minute was only 6.2 mg/g. The non-treated cells were much more difficult to release lutein, and the yield was less than half of those with cell disruption treatments. Therefore, the increased yield and

extraction efficiency of the pre-treatment of algal biomass make it worthwhile to include an additional operation step. Meanwhile, the temperature dissipated during the cell disruption should be closely monitored to avoid lutein degradation. Moreover, although the temperature rising can accelerate the mass transfer rate, its negative effects on lutein stability, temperature-control cost and solvent losses should be considered especially when ether was used, which has a boiling point of 34.6°C (Tao et al., 2014). Thus, the experiments were conducted at 23°C and the effect of temperature on extraction was not studied.

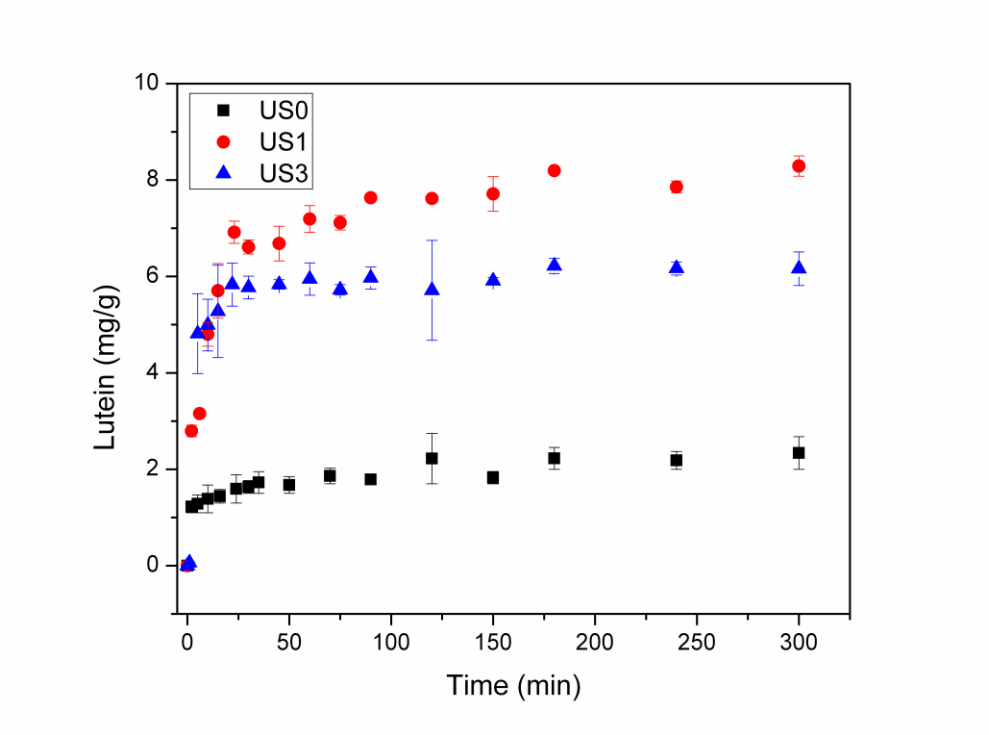

Figure 5-8 Lutein extraction kinetics for three different pre-treatment methods: US1: ultrasonication for 10 minutes at 0.5s working cycle per second, US3: ultrasonication for 30 minutes, US0: no pre-treatment. The extraction was in 160 mL 1:3 ether/ethanol (v/v) with 2 mL 10% (w/v) ethanolic KOH. The lutein concentration in the bulk solvent is presented by calculated lutein extraction yield ± STDEV, n=3.

Table 5-6 Apparent mass transfer coefficients and model constants calculated for three different pre-treatment methods. The values are mean of three experiments.

Pre-treatment time (min)	$D_{fast}\times10^8$ (cm^2/s)	f_1	$D_{slow}\times10^8$ (cm^2/s)	f_2	AARD
30	5.69	0.384	0.066	0.616	0.0518
10	4.89	0.4868	0.152	0.5132	0.1192
0	3.71	0.1194	0.126	0.8806	0.1087

5.6 Conclusions

This is the first study for lutein extraction kinetics from chloroplast in microalgae. The feasibility of binary solvent for wet biomass extraction was investigated. Mixing a polar and a non-polar solvent together is energy and time saving as polar solvent better extracts lutein from the wet cell matrix while the non-polar solvent has higher solubility of lutein and could be easily separated away by subsequent water addition. Therefore, a single-step simultaneous extraction, saponification and primary purification process for free lutein production from wet microalgae biomass was developed and optimized. The interaction of alkali usage and solvent volume was studied. Two parallel diffusion processes, fast and slow extraction processes were considered to model the kinetics of lutein extraction and hydrolysis.

In this study, the factors affecting lutein extraction was studied. The best conditions for extraction efficiency was with 10 minutes' pre-treatment of ultrasonication at 0.5s working cycle per second, react 0.5-1 hour in 160 mL final solvent volume of 1:3 ether/ethanol (v/v) with 2 mL 10% (w/v) ethanolic KOH. The use of alkali should correspond to the solvent volume, since the lutein extraction efficiency was more significantly related with the KOH concentration in solvent, rather than the ratio to biomass. Although more solvent volume can further increase the efficiency, considering the solvent cost, additional alkali needed and similar lutein yield, less solvent options are recommended. In this study, a higher lutein

yield was achieved by the single-step extraction than previously reported. This is due to the fewer operating steps and the direct use of wet microalgae. Overall, the results obtained in this study can lead to new and improved techniques for the design and optimization of the lutein extraction process, and scale up.

Chapter 6

6 Conclusions and Recommendations

In this Chapter the main conclusions of this study are presented as well as some future recommendations.

6.1 Conclusions

Current challenges in lutein production from microalgae were identified through the integrated process analysis. Two methodologies can be applied to enhance the lutein production: 1. increase the microalgal lutein productivity and 2. improve the lutein extraction yield. Therefore both aspects were investigated in this study toward the overall goal of production and recovery from microalgae using phototrophic cultivation.

Firstly, the coiled tubular tree photobioreactor (CTPBR) configuration was demonstrated to be an efficient photo-bioreactor for cultivating microalgae and for lutein production in chapter 3. More than 2 times higher biomass growth rate and concentration was achieved in the CTPBR than in Erlenmeyer flasks.

The lutein production under stressed conditions adapted for Canadian context were optimized in chapter 3. The microalgae strain *Chlorella vulgaris* was selected due to its excellent adaptability to low temperature conditions. The empirical models obtained by applying the experimental design of response surface method had good accuracy in predicting microalgae growth rate, lutein content and productivity under specified conditions, which is 4-10°C, 125-360 μmol photons m^{-2} s^{-1}, and 14-20 h day-light per day. The results indicated that the conditions that are favor for microalgae growth was also more beneficial for the lutein production. From chapter 4, it was determined that harvesting microalgae in the late exponential phase can optimize the lutein productivity. These knowledges can be applied for further scale up.

Secondly, different cell-pre-treatment and lutein extraction approaches were compared in chapter 4. The wet extraction of lutein from microalgae represent an energy saving and high yield operation. Bead-beater and ultrasonication showed good result for dry

extraction, while solvent soaking for wet biomass may be a more energy saving extraction method. The wet extraction of lutein and lipids from microalgae is a very promising method but require further optimization due to growth condition conflict in lutein and lipids for higher yields.

Solvatochromic polarity probe (Nile Red) was successfully used to study the effect of solvent polarity on the lutein extraction yield. This method was applied in chapter 4 and 5. The feasibility of binary solvent for wet biomass extraction was investigated, and the proper proportioned ethanol-hexane binary solvent enhanced the extraction yield.

Finally, a single-step simultaneous extraction, saponification and primary purification process for free lutein production from wet microalgae biomass was developed (chapter 5), representing a simple and fast approach for lutein recovery from the microalgae biomass. The extraction kinetics were modelled, and the factors affecting lutein extraction were optimized. The kinetics of integrated lutein extraction and saponification can be modelled by two parallel diffusion processes, fast and slow extraction processes. The interaction of alkali usage and solvent volume indicated that the use of alkali should correspond to the solvent volume, since the lutein extraction efficiency was more significantly related with the KOH concentration in solvent, rather than the ratio to biomass. The best conditions for extraction efficiency were with 10 minutes' pre-treatment of ultrasonication at 0.5s working cycle per second, react 0.5-1 hour in 160 mL final solvent volume of 1:3 ether/ethanol (v/v) with 2 mL 10% (w/v) ethanolic KOH.

Overall, higher lutein yield was achieved in this study than previously reported, due to the pin-pointed microalgae harvesting time, fewer operating steps, optimized extraction method, and the direct use of wet microalgae.

6.2 Recommendations

Future research should focus on the lutein productivity improvement and cost reduction.

The studied photobioreactor in chapter 3 was in a small scale, 0.34 L, scale-up of the bioreactor would allow obtaining larger quantities of the microalgae biomass and therefore

more accurate light intensity and oxygen production can be measured and the photosynthesis efficiency hence could be calculated.

In chapter 3, the RSM conditions of temperature and light stressed conditions study was limited at low temperature of 4-10°C, moderate light intensity of 125-360 μmol photons m^{-2} s^{-1}, and separate study of moderate temperate low light. If more experiment runs are allowed for temperate ranging from 4-40°C, light intensity of 25-1500 μmol photons m^{-2} s^{-1}, an integrated full spectrum study could fill in the gap between the studied ranges and provide better understanding of light and temperature for lutein production.

In chapters 4 and 5, the microalgae *C. vulgaris*, was used as a model algae to study the effects of cell disruption method. Many other strains, like *Chlorella protothecoides* or *Scenedesmus almeriensis*, are reported for good lutein content with larger cell size and less rigid cell wall, therefore it would be interesting to study the other species for lutein production, and compare with marigold flowers using the same procedure. The conflict in growth conditions of lipid and lutein production limited the efficiency of simultaneous extraction. This problem could be solved by metabolic engineering, possibly by over-expressing the PSY, PDS, BKT related-genes in the biosynthesis pathway by conventional genetic engineering or emerging transcriptional engineering methods.

As the goals in this research were different, super/sub-critical CO_2 extraction was not studied. It would be beneficial to include this method into the study as well. In addtion, the binary solvents used in this study were ethanol/ether and ethanol/hexane systems, other solvents were not examined. So some other solvents can be studied as well. Switchable solvents are also an interesting direction for future extraction process development for the easy product recovery. This latter extraction process is more environmental friendly than the conventional organic solvents, provided the less toxic solvent alternatioves can be identified or synthesised. Therefore, it would be attractive to develop a non-toxic switchable ionic liquid that would not degrade lutein.

For the production of high quality carotenoids, the concern of degradation during the production and storage should not be ignored. In chapter 5, the lutein degradation was observed. It would be a good idea to include an extra degradation term into the modeling process.

The process economic evaluation can be compared for microalgae and marigold flowers. In addition, as microalgae generates a variety of valuable products, research attempts can be given to the simultaneously production of multiple products to develop a more economically attractive and sustainable microalgae industry.

In the future continuous production processes could be developed to scale up and for cost reduction in downstream processing. The counter-current liquid-liquid extraction process can be applied and investigated for continuous lutein production. The efficiency of non-miscible binary solvent systems can also be invsetigated and compared. This can be achieved by using micellar or reverse-micellar systems the liquid-liquid extraction instead of the separate water addition step as shown earleir in Figure **5-1**. Since the micellar extraction process has been well-established for protein extraction, it would be possible to develop a similar process for lutein extraction from microalgae. The novel methods like *in situ* extraction may be interesting directions for further investigation as well due to the elimination of the energy-intensive microalgal harvesting step.

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Appendices

Appendix I Regression Analysis Data for Chapter 3

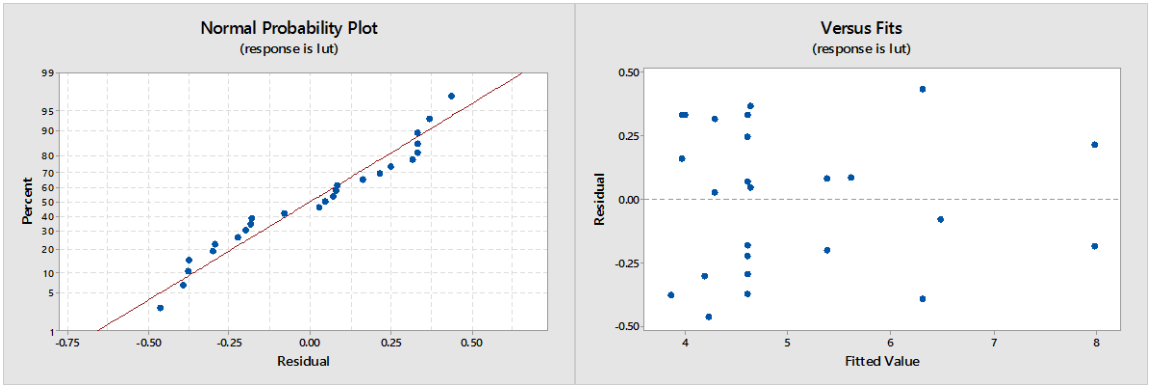

Figure S-1 Regression analysis of lutein content versus temperature, light irradiance and light cycle

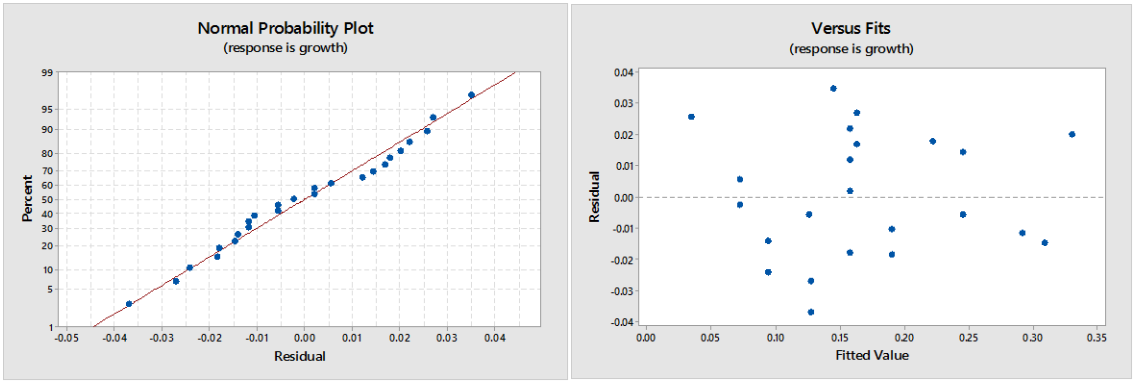

Figure S-2 Regression analysis of specific growth rate of *C. vulgaris* versus temperature, light irradiance and light cycle

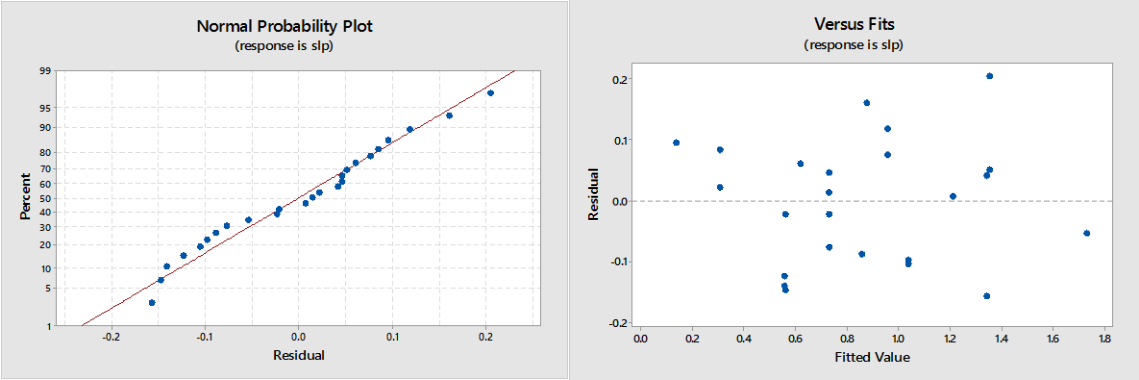

Figure S-3 Regression analysis of specific lutein productivity versus temperature, light irradiance and light cycle

Appendix II Supplemental material for Chapter 5

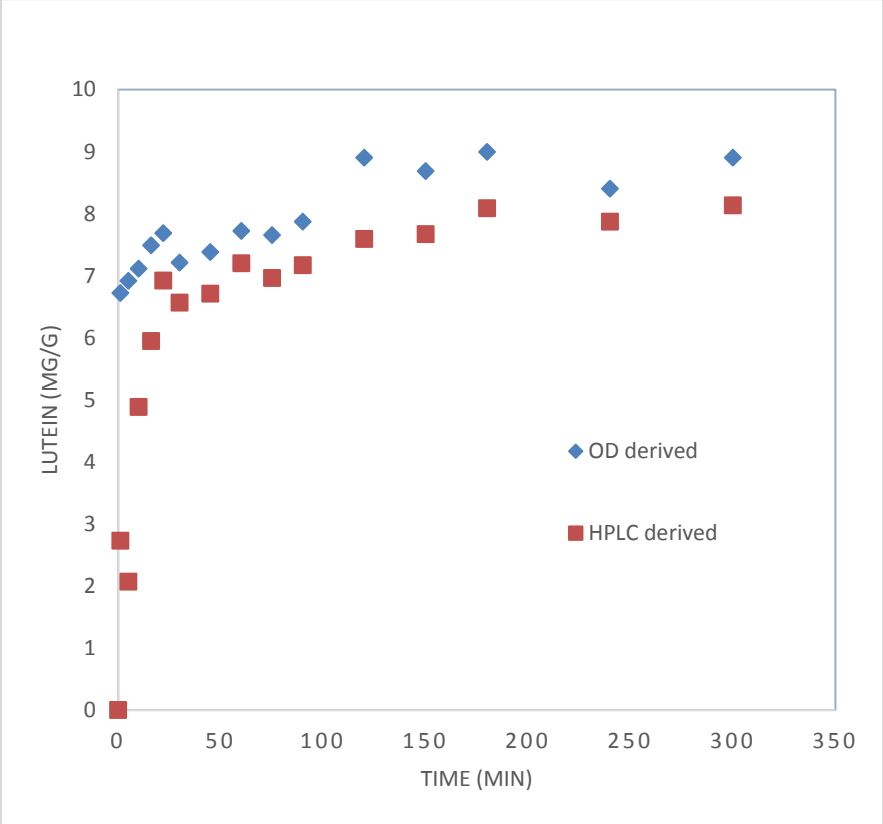

Figure S-4 The difference of HPLC-derived and OD-derived lutein content

Appendix III Examples of experimental data and photo

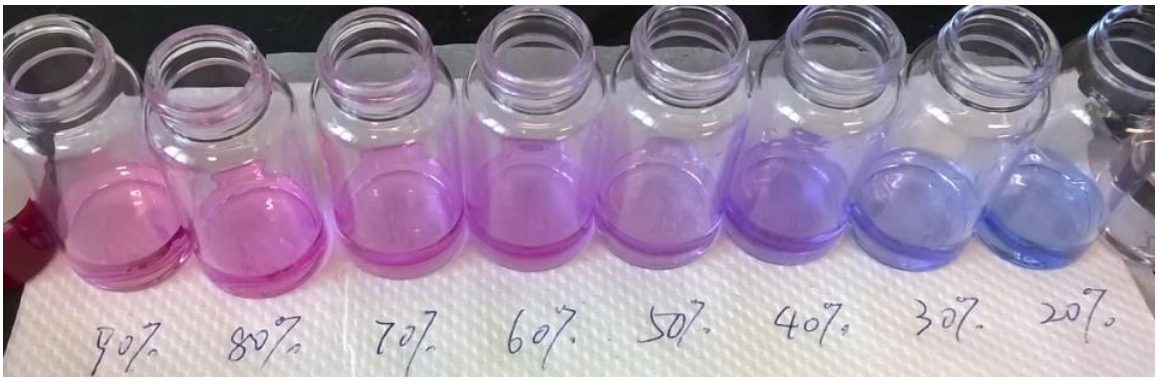

Figure S-5 The color change of Nile Red solution in different polarities. Here the solutions are 20-90% ethanol in water (v/v)

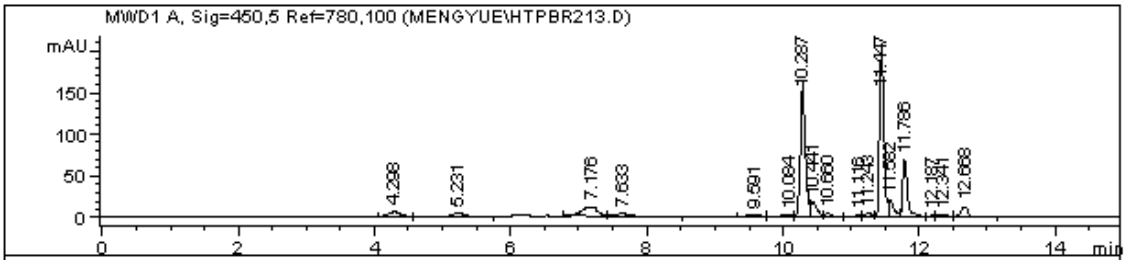

Figure S-6 HPLC profile of microalgae extract in ethanol solution, without saponification and purification, the large peaks from left to right are lutein, chl b, and chl a

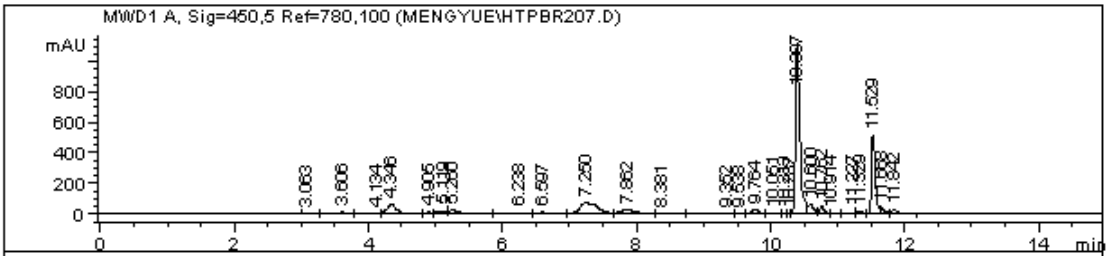

Figure S-7 HPLC profile of ethanol phase before saponification discussed in section 4.4.4, the large peaks from left to right are lutein, and chl b

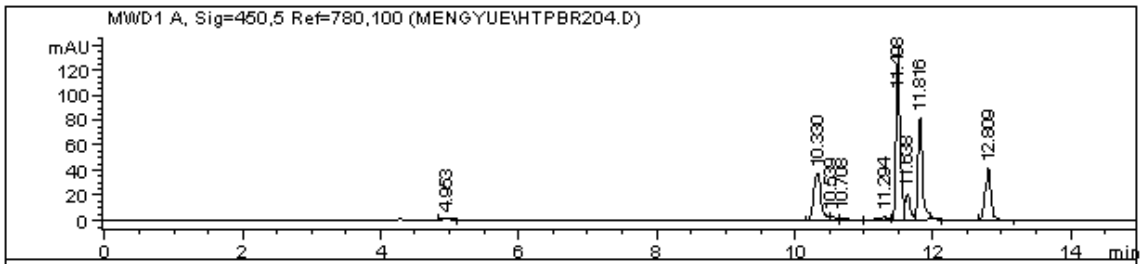

Figure S-8 HPLC profile of lipid layer as discussed in section 4.4.4, the large peaks from left to right are lutein, chl b, chl a, and beta-carotene

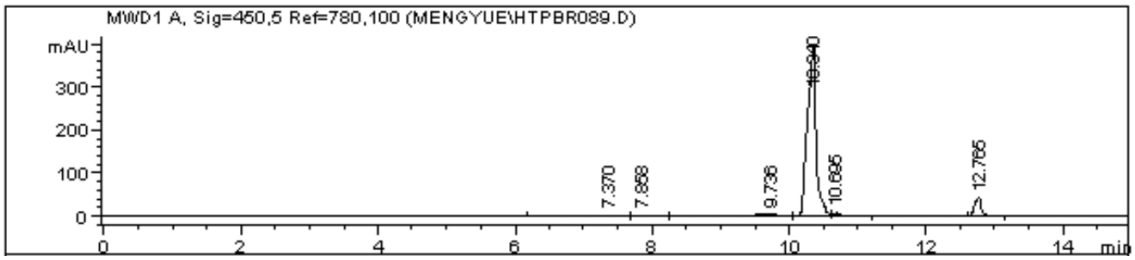

Figure S-9 HPLC profile of lutein extract after saponification and purification, the peaks from left to right are lutein and beta-carotene

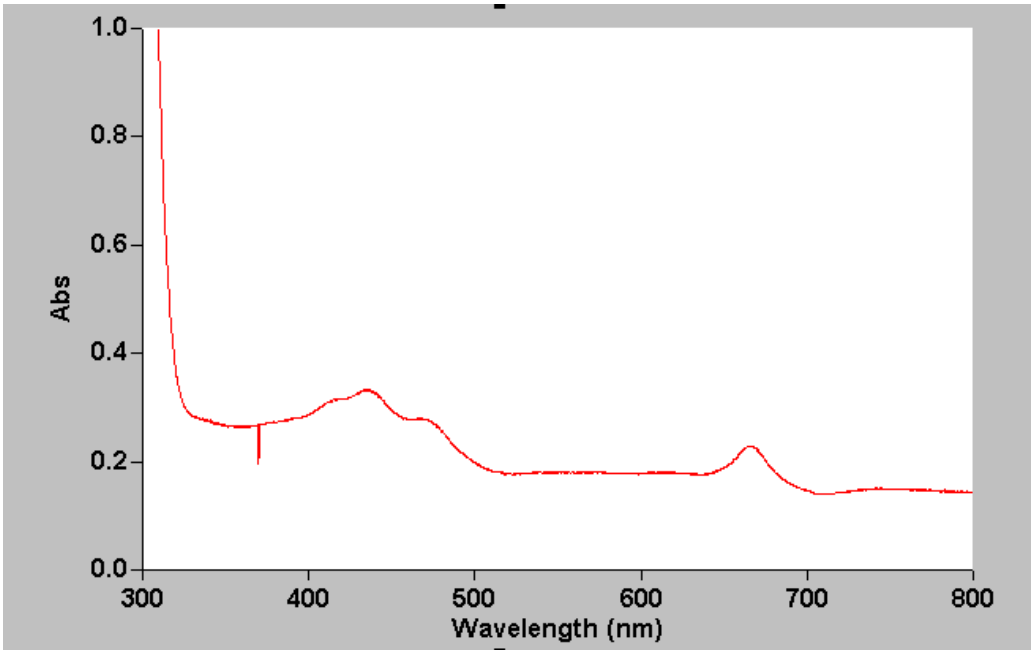

Figure S-10 Wavelength scan for *C.vulgaris* to determine the wavelength of OD measurement

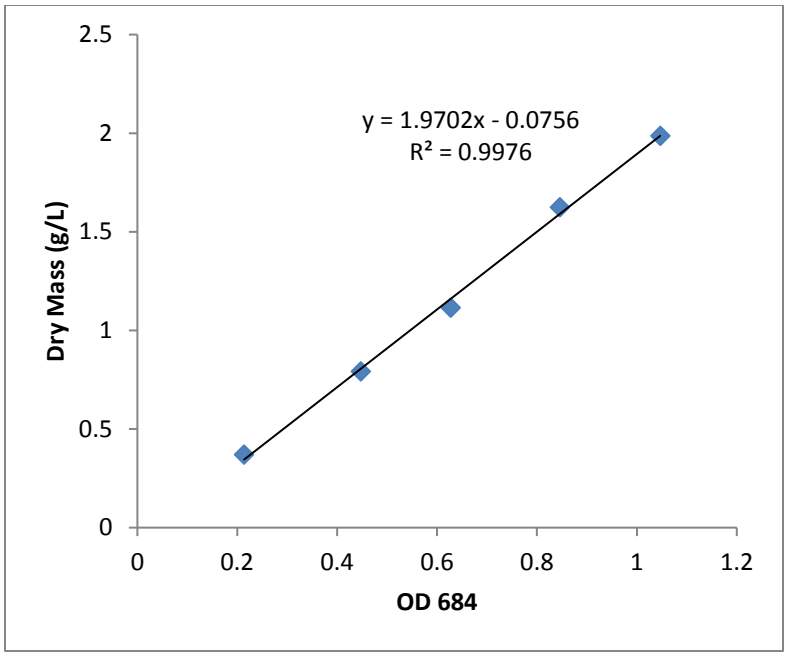

Figure S-11 Sample calibration curve for microalgae dry weight measurement (10°C)

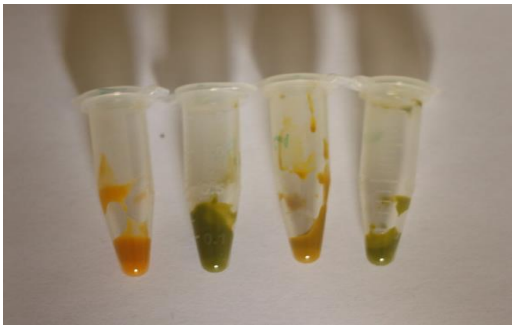

Figure S-12 Microalgae cultivated at light and temperature stressed conditions (10°C, 125 to 360 µmol photons m^{-2} s^{-1})

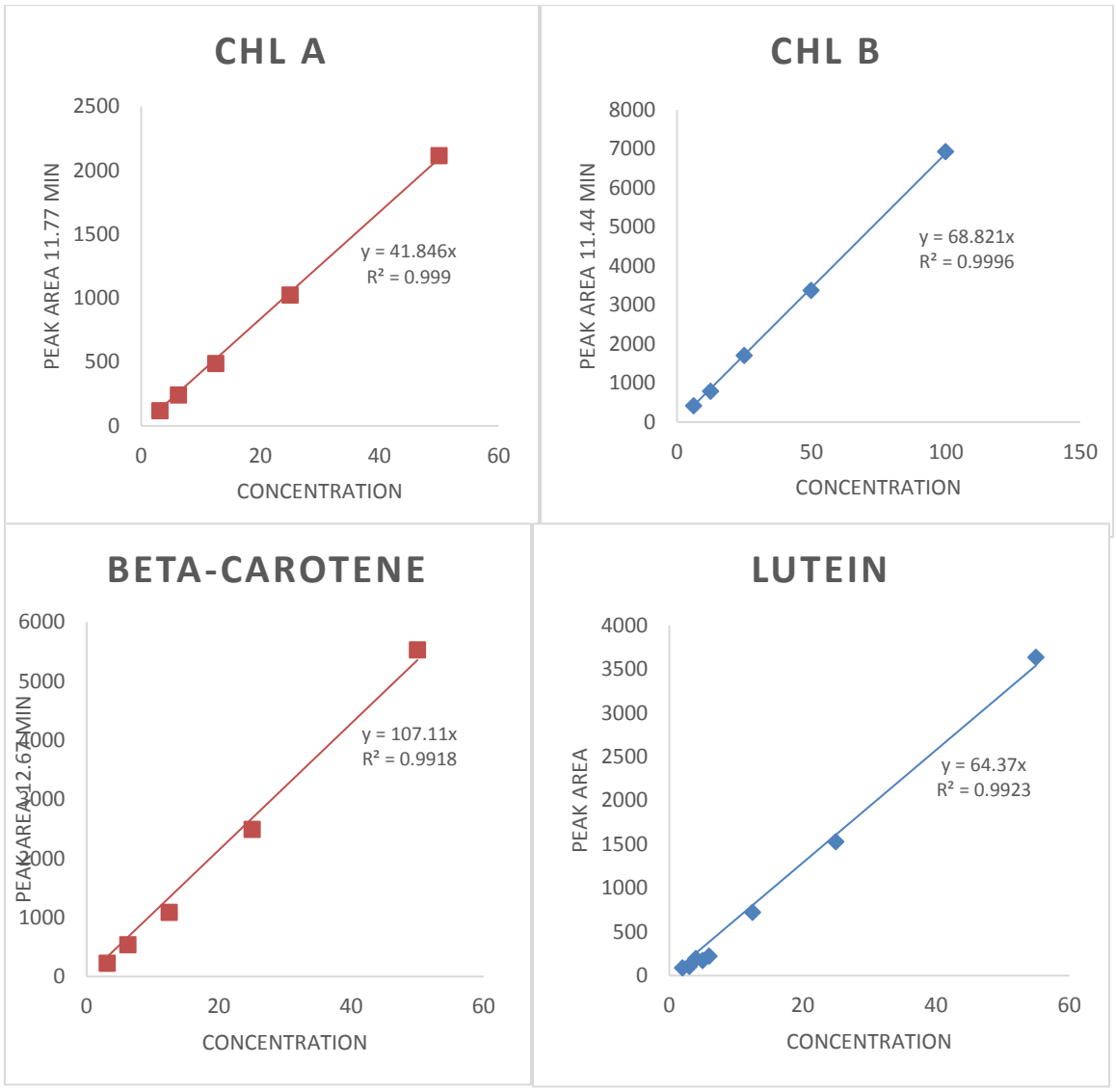

Figure S-13 Calibration curves for pigment concentration vs. peak area from HPLC

Figure S-14 Photo of lutein extraction and saponification by ether and ethanol after water addition. Left: concentrated solution, Right: 1% (v/v) sample analysis

Matlab code for Dfast, f1, Dslow, f2, AARD calculation in Chapter 5

```
function [Dfast,f1,Dslow,f2,AARD]=Fick(time,c,c0,slope,intercept,slope2)

%%(C*-c)/C*=6/pi^2*(f1*exp(-pi^2*D1*t/R^2)+f2*exp(-pi^2*D2*t/R^2))

% input pi,R, experimental result time t and c,c*, calculate f2

% from slope of ln(c*/(c*-c))vs time and D2 from the slope

% input time, c as arrays, c0 as a value

% Dslow=slope2*R^2/9.87, f2=exp(-intercept+0.498)

% input slope, intercept

pi=3.1416;R=3 %micron meter;

f2=exp(-intercept+0.498);
```

```
Dslow=slope2*R^2/9.87;

f1=1-f2;

Dfast=slope*R^2/9.87;

A=(c0-c)/c0;

E1=6/pi^2*(f1*exp(-pi^2*Dfast*time/R^2)+f2*exp(-pi^2*Dslow*time/R^2));

% AARD=1/n*E(abs((E1-A)/E1) for the model error

Er=abs((E1-A)./E1);

AARD=mean(Er);
```

Curriculum Vitae

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Mengyue Gong and Amarjeet Bassi. (2016) Carotenoids from microalgae: A review of recent developments. *Biotechnology Advances* 34.8: 1396-1412.

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Submitted:

Mengyue Gong, Xinyi Li and Amarjeet Bassi, Investigation of simultaneous lutein and lipid extraction from wet microalgae using Nile Red as solvatochromic shift probe. Submitted March, 2017 to Algal Research.

Mengyue Gong, Yuruihan Wang, and Amarjeet Bassi, Development and modelling of a single-step simultaneous extraction, saponification and primary purification process for free lutein production from wet microalgae. Submitted May, 2017 to Biotechnology and Bioengineering.

Conference Presentations:

"Investigation of the Microalgae Growth in a Tubular Tree Photobioreactor under Cold Temperatures" Mengyue Gong and Amarjeet Bassi. 64th Canadian Chemical Engineering Conference, Niagara, ON, 2014

"Investigation of kinetics of low temperature tolerant *Chlorella vulgaris* for lutein production and recovery" Mengyue Gong and Amarjeet Bassi. The International Chemical Congress of Pacific Basin Societies, Hawaii 2015

"Wet extraction of lutein and lipids from microalga by quantitative determination of polarity" Mengyue Gong, Xinyi Li and Amarjeet Bassi. Poster, 66th Canadian Chemical Engineering Conference, Quebec City, QC, 2016

"Wet extraction of lutein and lipids from microalga by quantitative determination of polarity" Mengyue Gong, Xinyi Li and Amarjeet Bassi. 19th International Conference on Bioenergy and Innovative Biorefining Systems, Osaka, Japan, 2017.