การสกัดไขมันและการผลิตไบโอดีเซลจากจุลสาหร่าย และการนำกลับของสารลูทีนที่เป็นผลพลอยได้จากกระบวนการ

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LIPID EXTRACTION AND BIODIESEL PRODUCTION FROM MICROALGAE AND RECOVERY OF FREE LUTEIN BY-PRODUCT

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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ฉัตรทิพย์ พรหมหมวก : การสกัดไขมันและการผลิตไบโอดีเซลจากจุลสาหร่าย และการนำกลับของสารลูทีนที่เป็นผลพลอยได้จากกระบวนการ. (LIPID EXTRACTION AND BIODIESEL PRODUCTION FROM MICROALGAE AND RECOVERY OF FREE LUTEIN BY-PRODUCT) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร.อาทิวรรณ โชติพฤกษ์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ. ดร.ประเสริฐ ภวสันต์ และ Prof. Motonobu Goto, 89 หน้า.

งานวิจัยนี้แบ่งออกเป็นสามส่วนโดยมีจุดประสงค์หลักเพื่อศึกษาวิธีการต่าง ๆ ในการปรับปรุงกระบวนการผลิตไบโอดีเซลจากจุลสาหร่าย โดยงานวิจัยมุ่งเน้นไปที่กระบวนการผลิตในขั้นตอนท้าย ซึ่งนับว่าเป็นขั้นตอนที่ใช้พลังงานกว่าครึ่งหนึ่งของพลังงานที่ใช้ในการผลิตทั้งหมด ในส่วนแรกของงานวิจัยเป็นการศึกษาเพื่อปรับปรุงกระบวนการในการสกัดไขมันจากจุลสาหร่าย จากการทดลอง พบว่าตัวทำละลายผสมระหว่างคลอโรฟอร์มและเมทานอลในอัตราส่วน 2:1 โดยปริมาตร สามารถสกัดไขมันได้ในปริมาณมากกว่าเฮกเซน อย่างไรก็ตามเฮกเซนเป็นตัวทำละลายที่ดีในแง่ของการเลือกสกัดไขมันที่ต้องการได้ดีกว่า การใช้เทคนิคสกัดด้วยอัลตร้าซาวด์และไมโครเวฟ ช่วยให้ผนังเซลล์ของจุลสาหร่ายถูกทำลาย การสกัดไขมันจึงเป็นไปอย่างรวดเร็วยิ่งขึ้น โดยเฉพาะอย่างยิ่งในกรณีที่จุลสาหร่ายมีผนังเซลล์ที่แข็งแรง เช่น คลอเรลล่า วูลแกริส

ส่วนที่สอง เป็นการประยุกต์ใช้ไมโครเวฟแบบพัลส์ในปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นจากไขมันสกัดจากจุลสาหร่ายคลอเรล่า และ จากจุลสาหร่ายโดยตรง เพื่อช่วยในการผลิตไบโอดีเซล จากการทดลอง พบว่าผลผลิตของไบโอดีเซลจากระบบไมโครเวฟแบบพัลส์สูงกว่าจากระบบที่มีการให้ความร้อนแบบดั้งเดิม โดยเฉพาะในระบบที่ปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นเริ่มจากจุลสาหร่ายโดยตรง ซึ่งในระบบมีทั้งการสกัดไขมันและปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นเกิดขึ้นพร้อมกัน ได้ผลผลิตไบโอเซลเพิ่มขึ้น 62% เมื่อเปรียบเทียบกับระบบดั้งเดิม ปริมาณไบโอดีเซลที่ผลิตได้ ยังขึ้นอยู่กับตัวแปรต่าง ๆ ได้แก่ ปริมาณของเมทานอลและตัวเร่งปฏิกิริยาด้วย นอกจากนี้การปรับค่ากำลังของพัลส์ไมโครเวฟยังเป็นอีกตัวแปรหนึ่งที่มีผลต่อการผลิตไบโอดีเซล จากการศึกษานี้พบว่าปฏิกิริยาทรานส์เอสเทอริฟิเคชั่นที่ 60 องศาเซลเซียส เป็นเวลา 10 นาที ที่กำลังของพัลส์ไมโครเวฟ 250 วัตต์ สามารถผลิตไบโอดีเซลได้สูงกว่าในกรณีที่ใช้กำลังไมโครเวฟสูง เช่น 500 และ 1000 วัตต์ นอกจากนี้ พบว่ากระบวนการผลิตมีประสิทธิภาพ (ปริมาณไบโอดีเซลที่ได้ต่อหน่วยพลังงานที่ใช้) สูงสุดที่กำลัง 250 วัตต์ โดยปริมาณไบโอดีเซลที่ผลิตได้ และประสิทธิภาพของการผลิต ไม่ได้ขึ้นอยู่กับกำลังของไมโครเวฟ แต่แปรผันกับความถี่และความแรงของพัลส์ซึ่งมีความสม่ำเสมอที่สุดเมื่อปรับกำลังของไมโครเวฟที่ 250 วัตต์

ส่วนสุดท้ายของงานวิจัยนี้เป็นการเสนอวิธีการที่แตกต่างออกไปจากการศึกษาในสองส่วนแรก โดยในส่วนนี้มุ่งเน้นไปที่การปรับปรุงความเป็นไปได้ทางเศรษฐกิจของกระบวนการผิตไบโอดีเซลจากจุลสาหร่ายด้วยการนำกลับสารที่มีมูลค่าสูงในลักษณะของผลพลอยได้ กล่าวคือเมื่อผลิตไบโอดีเซลจากสาหร่ายคลอเรลล่าโดยใช้ตัวเร่งปฏิกิริยาเป็นด่าง ด่างที่ใช้นี้ในขณะเดียวกันยังทำหน้าที่เป็นสารตั้งต้นในปฏิกิริยาสปอนนิฟิเคชันของลูทีนเอสเตอร์ที่ปนอยู่ในไขมันสกัดจากจุลสาหร่ายให้เปลี่ยนไปเป็นสารลูทีนอิสระที่มีมูลค่าสูงได้ โดยสภาวะที่มีการผลิตไบโอดีเซลสูงสุด สามารถทำให้ลูทีนเอสเตอร์ในไขมันสกัดเปลี่ยนเป็นลูทีนอิสระได้ทั้งหมด (2.3% โดยน้ำหนักของไขมันสกัด) นอกจากนี้ งานวิจัยนี้ยังได้เสนอวิธีการแยกลูทีนอิสระและไบโอดีเซลออกจากของผสมหลังการทำปฏิกิริยา เมื่อประเมินความคุ้มค่าทางเศรษฐศาสตร์เบื้องต้น พบว่าการนำกลับของสารมูลค่าสูงนี้มีความเป็นไปได้ที่จะทำให้การผลิตไบโอดีเซลจากจุลสาหร่ายมีความคุ้มค่าทางเศรษฐศาสตร์

ภาควิชา	วิศวกรรมเคมี	ลายมือชื่อนิสิต	
สาขาวิชา	วิศวกรรมเคมี	ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก	
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CHATTIP PROMMUAK : LIPID EXTRACTION AND BIODIESEL PRODUCTION FROM MICROALGAE AND RECOVERY OF FREE LUTEIN BY-PRODUCT. ADVISOR : ASSOC. PROF. ARTIWAN SHOTIPRUK, Ph.D., CO-ADVISOR : ASSOC. PROF. PRASERT PAVASANT, Ph.D., PROF. MOTONOBU GOTO, D. ENG. 89 pp.

The thesis is divided into three studies whose purposes are set out to determine various ways to improve the production of biodiesel from microalgae, focusing particularly on the downstream processes in which more than half of overall process energy is consumed. The first part of the thesis involved solvent extraction of lipid from microalgae. The results revealed that the mixture of chloroform/MeOH, 2:1 (v/v) could extract the highest amount of total lipid from algae, while hexane was a good solvent, concerning the selectivity for targeted lipids. In addition, application of ultrasound and microwave to the process caused cell disruption, which could then accelerate the rate of lipid extraction especially from algae with tough cell walls such as *Chlorella vulgaris*

In part 2, pulsed microwave was applied to transesterification of *Chlorella* lipid and algal biomass to enhance the production of biodiesel. Considerable enhancement in the biodiesel yield was observed for reaction under pulsed microwave over that under conventional heating. Especially for the single-step process, in which extraction and transesterification took place simultaneously, as high as 62% enhancement in biodiesel yield was found. Furthermore, in microwave irradiated reaction, the amount of MeOH and catalyst seemed to affect the biodiesel yields. Apart from reaction variables such as MeOH and catalyst amounts, biodiesel production was also found to be affected by the power settings of the pulsed microwave system. The highest biodiesel yield resulted from transesterification for 10 min at 60^{o}C was found at the power setting of 250 W instead of at higher power settings (500 W or 1000 W). The highest efficiency (biodiesel yield per unit energy) was also the highest at 250 W. It could be suggested by the characteristic power profiles that the yield and the efficiency did not correlate with the power input, but rather, with the pulse frequency and intensity during the entire reaction, which was the most uniform at 250 W.

In the last part, different route to enhance the production of algal biodiesel is taken. Herein, we emphasized on improving the economic feasibility of the biodiesel production process by producing a high-valued co-product. That is, biodiesel and valuable free lutein were demonstrated to be simultaneously produced from *Chlorella* lipid extracts. The alkali catalyst used in the transesterification of triglycerides acted as a reactant in converting lutein fatty acid esters to free lutein. A maximum biodiesel yield of 33.6% by weight of the algal lipids was obtained after a 4-h reaction with MeOH at the MeOH/biomass ratio of 16:1using 6% alkali catalyst. The excess of alkali and MeOH employed in the production of biodiesel ensured the complete saponification of all lutein fatty acid esters to free lutein, giving a maximum yield of 2.3% by weight of the algal lipids. In addition, a process for the separation of the biodiesel and free lutein products from the reaction mixture was proposed. A preliminary economic assessment was also conducted, which suggested that the process for the simultaneous production of biodiesel and lutein from *C. vulgaris* may be economically feasible.

Department : Chemical Engineering Student's Signature .....

Field of Study : Chemical Engineering Advisor's Signature .....

Academic Year : 2012 Co-advisor's Signature .....

Co-advisor's Signature .....

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Notations

D	dissipation factor
k	rate constant
A	Arrhenius pre-exponential factor
E_a	activation energy
R	gas constant
T	temperature

Greek letters

ε'	dielectric constant
ε''	dielectric loss factor

CHAPTER I
INTRODUCTION

1.1Motivations and rationale

Being a renewable fuel with low toxic emission, biodiesel has now gained much attention as a potential substitute for the depleting fossil fuel, and therefore is a solution to energy crisis and global warming problem (Atabani et al., 2012). Biodiesel is currently derived from oleaginous plant seeds such as rapeseeds, sunflower seeds, palm, coconut and others. However, to meet the global energy demand, a large land area is needed for cultivation of these crops. This poses concerns regarding the invasion of food crop area for the growing world population.

Microalgae are fast growing aquatic microorganisms that contain high lipid content comparable to, or in some species, are even higher than those of oil crops. For example, *Chlorella* sp., *Botryococcus* sp. and *Nanochloropsis* sp. contain 14-38%wt, 15-75%wt and 31-68%wt, respectively (Chisti, 2007, Xu et al., 2006). Above all, due to the need for relatively smaller cultivation land area, this microorganism is today believed to be the only resource that opens up a possibility for biodiesel to substitute the depleting fossil fuel (Atabani et al., 2012).

Algal biodiesel can be produced with the procedure similar to that used for biodiesel production from crop seed oils. The process basically consists of extraction of biomass for lipid, followed by alcoholysis of the extracted lipid which converts glycerides or fatty acids into fatty acid methyl esters (FAMEs), known as biodiesel. However, up to now, the production of algal biodiesel has not yet been practical in industrial scale due to its high input energy requirement (Ferrell and Sarisky-Reed, 2010). It has been reported that nearly half of the total algal biodiesel production cost comes from downstream processes including the extraction of lipid and transesterification (Delure et al., 2012). Therefore, the study to develop these processes toward enhancement in efficiency of the algal biodiesel production is the main focus of this thesis.

In PART 1, algal lipid extraction is our concern. Unlike extraction of crop seed oil in which mechanical press is generally used, recovery of lipid from microalgae requires solvent extraction, which takes longer time and is energy and cost intensive. In the first investigation, *Heamatococcus pluvialis* was selected as an alga model to compare the yield of lipid extracted by two commonly used solvents, hexane and the mixture of chloroform/MeOH. In addition, the quality of the crude lipid obtained from each solvent was evaluated based on biodiesel yield obtained from transesterification of each lipid. The second investigation in PART 1involved determining the effects of different cell disruption methods known to enhance extraction performance including ultrasound and microwave assisted extractions (Choi et al., 2006, Lee et al., 2010) on the lipid extraction yield. Two model algal strains with different cell structures were used in this study, namely *H. pluvialis and Chlorella vulgaris*, cultivated at Biochemical Engineering Laboratory, Chulalongkorn University. The former represented ruptured cells and the latter represented algae whose cell wall remained intact. Extraction was carried out using a selected solvent from the first investigation and the results were compared with conventional solvent extraction (maceration). PART I is presented in Chapter III.

In PART 2, emphasis was placed on production of algal biodiesel via transesterification under the microwave irradiation which has been reported by many literatures to reduce reaction time without sacrificing the production yield (El Sherbiny et al., 2010, Patil et al., 2011a, Chen et al., 2012). Nevertheless due to their simplicity and low prices, household microwave systems or those with modifications were used for the experiment in most previous studies (Chen et al., 2012, Khemthong et al., 2012, Lertsathapornsuk et al., 2008, Patil et al., 2011a), Unfortunately, these systems have limitations in that the control of power and temperature was difficult. In this study, pulsed microwave system with special designs to record and control temperature by adjusting pulse power and duty cycle was employed. Herein, the effects of key reaction parameters i.e. the amount MeOH and catalyst on the production of algal biodiesel were determined and is presented in Chapter IV. Apart from reaction conditions, effect pulse power setting was also determined. With the special feature of the microwave apparatus used in this study, efficiency of the

production from various conditions could be evaluated in terms of biodiesel yield per unit energy consumption (Chapter V).

Although microwave has been demonstrated to have advantages over conventional system, due to the complexity of the device, scaling up the system for the industrial process is still a challenge (Díaz-Ortiz et al., 2011). Accordingly, we then investigate a different route to possibly achieve the economic feasibility of the algal biodiesel production process. In PART 3 of the thesis (Chapter VI), the possibility of co-producing high value products was explored. In addition, variables affecting the production yields of biodiesel and the co-product were investigated and the process for separation of the products was proposed. Finally, the economic feasibility of the proposed process was evaluated.

1.2Objectives

The major aim of this work is to suggest 3 ways to enhance efficiency of algal biodiesel production. The study is therefore divided into 3 parts, whose objectives are as follows:

1.2.1 PART 1: Study on extraction of lipid from microalgae

1.2.1.1 To select a suitable solvents (the mixture of chloroform/MeOH or hexane) for the extraction of lipid from microalgae.

1.2.1.2 To examine the performance of techniques (maceration, UAE and MAE) applied for the extraction of lipid from microalgae at various extraction conditions.

1.2.2 PART 2: Study on algal biodiesel production via transesterification under pulsed microwave irradiation

1.2.2.1 To investigate the potential of pulsed microwave irradiation in assisting algal biodiesel production in comparison with the former conventional heating system.

1.2.2.2 To determine the effects of key reaction parameters under irradiation by pulsed microwave

1.2.2.3 To determine the effects of microwave power setting on the algal biodiesel production.

1.2.3 PART 3: Simultaneous production of algal biodiesel and high value co-product

1.2.3.1 To test the possibility of producing high value product simultaneously with algal biodiesel.

1.2.3.2 To examine the effect of key reaction parameters on the production of the co-product as well as the main product biodiesel.

1.2.3.3 To propose a method to separate the co-product from the main product biodiesel and evaluate value returned by the proposed process.

1.3 Scopes of work

1.3.1 PART 1: Study on extraction of lipid from microalgae

The study includes the effect of applying different extraction methods, solvents used and extraction condition on the lipid yield.

1.3.1.1 Hexane and the mixture of chloroform/MeOH (2:1 v/v) were used for the determination effect of different solvents on the yield of lipid extracted from a microalga, which *H. pluvialis* was used as a model.

1.3.1.2 The methods of lipid extraction carried out were maceration, UAE and MAE performed at the temperature of 30-50^{o}C and 5-30 min of extraction time. Two microalga strains including ruptured cells of *H. pluvialis* and *C. vulgaris* whose cell wall was remain intact, were chosen as models algae for this study according to the difference in their structures.

1.3.2 PART 2: Study on algal biodiesel production via transesterification under pulsed microwave irradiation

1.3.2.1 As a comparison, production of algal biodiesel was carried out in both microwave and conventional heating system. Each of which was performed by single-step (direct transesterification from the biomass) and the two-step (lipid extraction following by transesterification). Dried *Chlorella* powder (3 g) was investigated using an acid catalyst, H_2SO_4, and MeOH was used as alcohol in the reaction.

1.3.2.2 Narrowed down to only the microwave system, the effect of the amount of H_2SO_4 (0.24-1.92 mL) and MeOH (24-72 mL) on the yield of biodiesel was investigated.

1.3.2.3 The effect of pulsed microwave power setting on biodiesel yield (100 – 1000 W) was determined. For every trial, the reaction was controlled to a constant temperature of 60^{o}C and the reaction time was fixed at 10 min.

1.3.4 PART 3: Simultaneous production of algal biodiesel and high value co-product

1.3.4.1 As *C. vulgaris* is a great source of lipid and a high value carotenoid, free lutein, this stain is then used as an algal model that could produce a co-product (free lutein).

1.3.4.2 The study was carried out in a conventional heating system where algal lipid extract was introduced to simultaneous transesterification of glycerides and saponification of lutein fatty acid at a fixed temperature of 60^{o}C. Other reaction parameters i.e. the amount of KOH (0.1-8% by wt. of algal biomass), the ratio of MeOH: biomass (1:1–16:1 v/w) and the reaction time of 1-4 h were varied.

CHAPTER II
BACKGROUNDS AND LITERATURE REVIEWS

2.1 Microalgae

Microalgae are unicellular photosynthetic organisms whose sizes range from micrometer to hundreds of micrometer. They grow quickly with simple requirements e.g. light, CO_2, N, P, K, etc. in either fresh water or marine system. Major compositions of microalgae thus basically include proteins, carbohydrates and fiber, lipids, and ash, whose quantities vary with cultivation condition and the diversity of more than 50,000 existed species (Richmond, 2008). Compositions of some algae strains are shown in Table 2.1.

Table 2.1 Percentage of chemical composition in microalgae (algal dry weight basis) (Becker, 1993)

Strain	**Protein**	**Carbohydrates**	**Lipids**
Botryococcus braunii	no data	no data	29-75
Nanochloropsis sp.	no data	no data	31-68
Chaetoceros gracilis	18-25	7-11	48-58
Haematococcus pluvialis	29	19	28-40
Scenedesmus obliquus	50-56	10-17	12-14
Chlamydomonas rheinhardii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Euglena gracilis	39-61	14-18	14-20
Tetraselmis maculate	52	15	3
Porphyridium cruentum	28-39	40-57	9-14
Spirulina maxima	60-71	13-16	6-7
Synechoccus sp.	63	15	11

Due to high nutritional contents, several types of algae derived products with various applications have been developed, as summarized in Table 2.2. As human nutritional supplements, the products valid in various forms including powders, tablets, capsules, liquids or even in incorporated into beverages. For example, *Spirulina*, a helical shape microalga, is commercially cultured for the products of amino acids, vitamins and minerals. A green microalga, *Chlorella*, is found containing a great amount of anti-oxidative substances such as chlorophyll, carotenoids, lutein, and phenolic compounds. Mass production for such products has been manufactured with more than 70 companies worldwide (Spolaore et al., 2006). It is reported that *Chlorella* was annually sold in excess of $38 billion (Yamaguchi, 1996). Astaxanthin, another very active antioxidant found most abundant in *Haematococcus pluvialis* (1.5-3% of its dry weight) (Machmudah et al., 2006) has been industrially produced and marketed with the price of $2,500/kg (Hejazi and Wijffels, 2004).

Microalgae also provide high-value polyunsaturated fatty acids (PUFA), e.g. omega 3 in the forms of DHA and EPA, and omega 6 in the forms of GLA and ARA. These PUFAs are although found in fish oil, the product may give unpleasant taste due to its fishery smell and poor oxidative stability and therefore is of lower quality when compared with the microalgal products. The PUFA producing algal stains are such as *Nanocholropsis*, *Chaetoceros sp.*, *Schizochytrium imacinum*, etc.

Table 2.2 Applications of microalgae

Composition	Characteristic/Classification	Major source	Application	Price
Protein	High essential amino acids	*Spirulina* (60-71%) *Chlorella* (51-58%) *Scenedesmus* (50-56%)	Nutritional supplement	1-100 USD/kg (whole cell)
Carbrohydrate	Production of glucose, fructose and galactose is practical but polysaccharides receives most attention in the industrial context.	*Chlamydomonas Mexicana (up to 75%* *Chlamydomonas sajao*	Nutritional supplement	
Lipid	Glycerides	*Botryococcus braunii* (29-75%)	**Biofuel**	
	High value FFA			
	- EPA	*Nannochloropsis, Porphyridium*	Nutritional supplement, aquaculture	
	- DHA	*Crypthecodinium, Shizochytrium*	Nutritional supplement	
	- ARA	*Porphyridium*	Nutritional supplement, anti-inflammatory	
	- GLA	*Arthrospira*	Nutritional supplement, anti-inflammatory	
Pigment	Carotenoid (brown-orange)			
	- beta-carotene	*Dunaliella salina* (up to 14%)	Food additive (Pro vitamin A)	160–560 USD/kg
	- astaxanthin	*Haematococcus pluvialis*	Food additive, Aqualic colorant	≈ 2,500 USD/kg
	- lutein	*Chlorella, Muriellopsis, Scenedesmus*	Food additive, Aqualic colorant	500-800 USD/kg
	Chlorophyll (green)	all phototrophic oxygenic algae	Pharmaceutics and cosmetics	10-50 USD/kg
	Phycobillins (blue/red)	*Spirulina*, Cyanobacteria,	Food colorant	3,000-25,000 USD/kg

2.2 Microalgae for biodiesel production

Production of biodiesel from microalgae is expected to have advantages over that from crops owing to higher lipid content and less desire of nutrition and cultivation land area. On top of that, as microalgae doubly increase their number of cells several times every single day, the growth rate of microalgae is considered much higher compared with former biodiesel resources such as corns, palms, soy bean, sunflower seeds, jatropha, and other oleaginous land based plants. In comparison, for example, according to the biomass productivity which is here assumed to be 0.1 g (dried basis)/liter.day, if the cultivation of microalgae containing 30% wt. of lipid was taken place in one rai (1600 m^2 x 20 cm) pond, the lipid product obtained is then 9.6 kg/rai/day. This amount is considered four and twelve folds higher than that obtained from palm and jatropha, respectively. Moreover, the properties, such as viscosity, flash point, cetane number, the amount of carbon residue, etc., of biodiesel produced from microalgal lipid have been reported to meet the standard of American Society for Testing and Materials (ASTM) (Xu et al., 2006, Johnson and Wen, 2009, Krohn et al., 2011). In other words, it has qualified for utilizing as vehicle engine.

However, against many advantages of microalgae that lead to the interest of its lipid in production of biodiesel, high production cost was a major concern. In order to reduce the production cost, each step of algal biodiesel production must be taken into consideration. As illustrate in Figure 2.1, production of algal biodiesel basically includes 4 steps, as follows

- Algae cultivation
- Harvesting
- Lipid extraction
- Conversion of algal lipid into biodiesel.

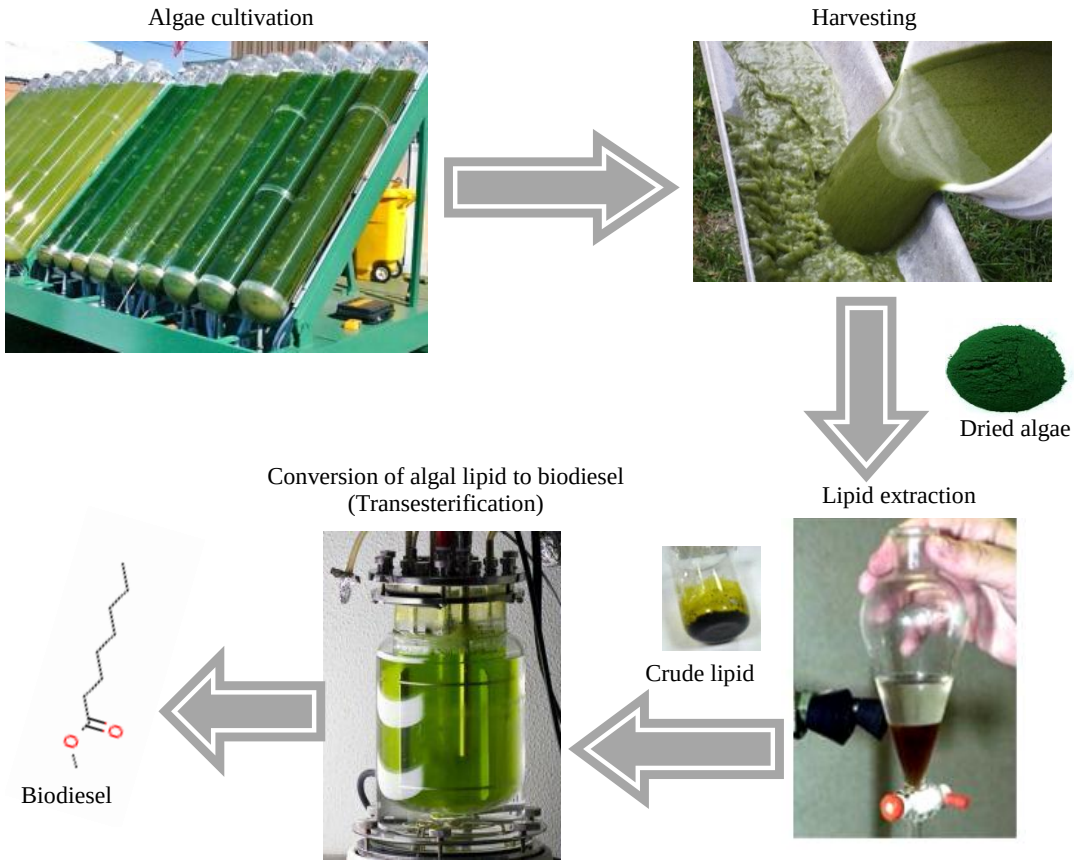

Figure 2.1 Algal biodiesel production process

Cultivation of algae has been developed for specific proposes. For biodiesel production, many researchers attempted to gain knowledge of suitable cultivation condition. These include the studies for cultivation temperature, light intensity, pH, CO_2 flow rate and nutrients where lipid could be best produced (ZHU et al., 2013, Chen et al., 2011). However, algae cultivation was limited by economic feasibility and thus cultivation under natural sunlight (outdoor cultivation) is likely to be most feasible route (Delrue et al., 2012).

Regarding the micro-size of the algae, harvesting has been one of the steps that faces difficulty. Harvesting microalgae can be carried out with several methods. Centrifugation was usually applied while methods such as filtration and economic flocculation were also developed (Ahmad et al., 2012, Vandamme et al., 2013). Nevertheless, again, limited by economic feasibility, a key to practical harvesting is

probably back to choosing self-precipitated strains. Then, after dewatering, the biomass can be dried under sun light.

Extraction of lipid and the conversion of algal lipid into biodiesel were found to consume large amount of energy (Delrue et al., 2012). As there are several ways to carry out these two steps, there are still rooms for development and thus they are attentions in this study.

2.2.1 Extraction of algal lipid

Crude lipid contains several types of lipid. The variety of components in lipid depends greatly on strains themselves and culture conditions such as, nutrient of the medium, light intensity, temperature, etc. To choose specific right strains for biodiesel production, Volkman et al. (1989) studied the compositions of lipid extracts from 10 microalgae, i.e., *Chaetoceros calcitrans and gracilis, Skeletonema costatum, Thalassiosira pseudonana, Isochrysus sp., Pavlova lutheri, Dunaliella tetioleata, Nannochloris atomus, Tetraselmis suecica and Chroomona salina*. From the results, as shown in Table 2.3, there was significant variation in lipid composition among classes and even between species within the same class, even though they were cultured with similar conditions. Table 2.3 also indicates that polar lipids, consisting

Table 2.3 Percentage composition of lipid classes in lipid extracts from 10 microalgae (Volkman et al., 1989)

Strain	HC	TG	FFA	ST	POL	Others
Chaetoceros calcitrans	0.4	8.4	11.4	6.1	72.8	0.9
Chaetoceros gracilis	1.3	34.0	14.4	6.0	44.2	-
Skeletonema costatum	0.8	1.7	8.5	1.1	84.6	1.3
Thalassiosira pseudonana	1.2	14.4	1.1	2.8	80.4	-
Isochrysis sp.	0.4	2.8	TR	0.2	83.0	13.5*
Pavlova lutheri	0.2	4.0	TR	6.3	78.3	11**
Dunalieila tertiolecta	TR	1.9	0.9	2.1	94.3	2.1
Nannochloris atomus	TR	TR	0.4	1.0	98.6	-
Tetraselmis suecica	1.8	3.3	0.8	1.9	91.5	0.7
Chroomonas salina	3.5	21.9	1.9	4.9	67.8	-

HC, hydrocarbons and wax esters; TG, triglycerides; FFA, free fatty acids; ST, sterols and alcohols; POL, polar lipids and chlorophylls. TR < 0.2%.

* Mainly C_{37}-C_{39} unsaturated methyl and ethyl ketones (Volkman et al., 1981; Marlowe et al., 1984).

** Mainly 4-methylsterols and compounds of unknown structure.

of glycolipids, phospholipids, chlorophylls and others are the most abundant types of lipid in all strains here except for *Chaetoceros gracilis* that contained a competitive amount of triglycerides and free fatty acids, the two lipid classes that can turn into biodiesel. It can be concluded that, to choose the right strains, it is necessary not only to have a good consideration on amount of total lipid, but also each lipid component whether it is really the one needed as a raw material for biodiesel production.

To gain lipid from biomass, either mechanical or chemical methods of extraction can be applied. However, as for extraction of lipid from microalgae, mechanical methods which involve compression of biomass with mechanical equipment may not be suitable regarding their tough cell walls. Hence, chemical methods using organic solvent for the extraction are more typically carried out.

Solvent extraction applies the concept of solvent solubility in making specific components in a solid sample dissolved and transferred to the phase of solvent. Organic solvents that are used for lipid extraction are normally chosen from its miscibility with lipid. Basically compounds with similar dielectric constants (listed in Table 2.4) tend to dissolve well into each other. Accordingly, a non-polar solvent i.e. hexane and slightly polar solvents such as chloroform, toluene, petroleum ether may be used.

Due to the variability of algal species and their characteristics, there is no specific lipid extraction method suitable for all strains. Accordingly, various extraction techniques have been presently tested to perform the extraction of lipid from microalgae.

Table 2.4 Dielectric constant of some solvents and lipids

Substance	Dielectric constant	Substance	Dielectric constant
water (68° F)	80.4	oil, grapeseed (61° F)	2.9
methanol (77° F)	32.6	oil, olive (68° F)	3.1
ethanol (77° F)	24.3	oil, petroleum (68° F)	2.1
acetone (77° F)	20.7	oleic acid (68° F)	2.5
n-hexane (68° F)	1.9	palmitic acid (160° F)	2.3
toluene (68° F)	2.4	stearic acid (160° F)	2.3
chloroform (68° F)	4.8	glycerol (77° F)	42.5

Simplest way to carry out lipid extraction is soaking biomass in the bulk of solvent. Heating and stirring maybe introduced to the system to increase the extraction rate. In laboratory scale, most research that intended to gain a 100% lipid recovery normally used Soxhlet apparatus for the extraction. Recently, applying techniques, i.e. ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE) were also reported to have positive results.

2.2.1.1 Ultrasonic assisted extraction (UAE)

Ultrasonic assisted extraction is another solvent extraction technique which makes use of ultrasound in enhancing extraction efficiency. Ultrasound is a sound wave with inaudible frequencies of above 20,000 hertz. This technique of applying ultrasound in breaking down cell walls is quite general in microbiology. When an ultrasonic generator releases the ultrasonic wave into liquid, it results in high pressure (compression) and low pressure (rarefaction) cycles alternately. During the rarefaction, the wave creates small vacuum bubbles. These bubbles incessantly expand until they become intolerable with the increasing energy during the high pressure cycle, resulting in great breaks, called “cavitaion”. The intenseness of this cavitation causes shear force which damages the cell walls, and the intensity of the damage depends on the frequency and the amplitude of the wave. Ultrasonic waves can also perform as an emulsifier, assisting in compatibility of lipids and solvent. The study by Ranjan et al. (2010) confirmed that there were intensive ruptures of algal biomass cell walls as a result of ultrasonic treatment, which consequently resulted in higher extracted lipid yield (Figure 2.2).

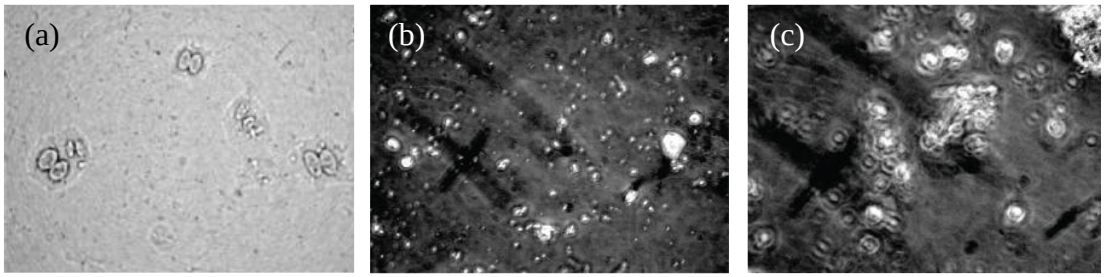

Figure 2.2 Micrograph of the algal biomass, *Scendesmus* sp. (a) original algal cells, (b) biomass after extraction in n-hexane with Soxhlet apparatus, (c) biomass after extraction in n-hexane with UAE (Ranjan et al., 2010)

2.2.1.2 Microwave assisted extraction (MAE)

Similar to UAE, MAE is another solvent extraction method used to enhance the efficiency of extraction. Microwave is a kind of radio wave with the frequency between 1 – 300 GHz. Like domestic microwave oven, as microwave system works, polar molecules containing positive and negative charges will be induced by the microwave field. The molecules are then rotated to align themselves with the alternately changing magnetic field created by the microwave generator, as shown in Figure 2.3. As the molecules rotate, they collide with other molecules nearby, resulting in heat that causes cell or tissue disintegration. The effect of microwave on extraction efficiency is dependent on polarity or dielectric constant of the solvent and the material. The more dielectric constant, the more energy molecules rotate and collide with the others. Unlike the system heated with conventional heating where heat is conducted from outside in, in system irradiated with microwave, the material and solvent within the electromagnetic field is heated simultaneously. Thus the time required for extraction by this system is much shorter than that of conductive heating. . A number of studies have recently been conducted using microwave to accelerate the extraction process as summarized in Table 2.5. As a few examples, Balasubramanian et al. (2011) reported a considerable enhancement in recoverable lipid from microalga, *Scenedesmus obliquus*, could be achieved by microwave assisted extraction at 90^{o}C for 30 min, over the simple water bath, at the same condition (76-77% versus 43-47% lipid recovery). In addition, the lipids obtained from MAE also

offer more preferable quality as they contained more glycerides and other essential lipids. In another study, Lee et al. (2010) compared the results of several extraction techniques including autoclaving, bead beating, microwave, sonication and osmotic shock, on the amount of lipids extracted from three strains, *Botryococcus sp.*, *Chlorella vulgaris*, and *Scenedesmus sp*. For all three stains, microwave was found to give the highest lipid yields which were two to three times the yields obtained with the simple non-disruptive extraction methods.

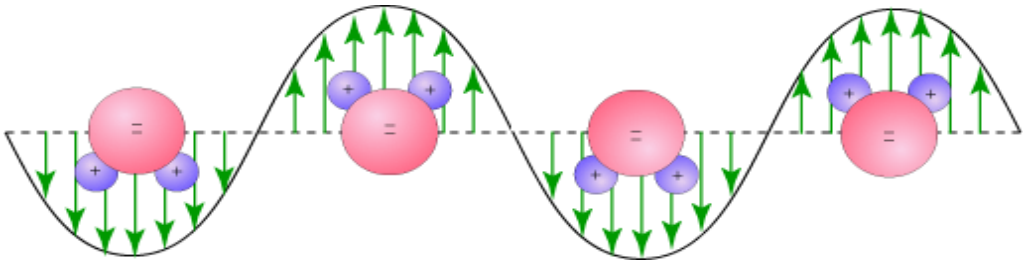

Figure 2.3 Rotation of water molecules under the influence of the electric field component in an electromagnetic wave

http://www.microwave-oven-guide.com/how-do-microwave-ovens-work.html

Table 2.5 Literature review on extraction of lipid from microalgae

Author	Strain	Pretreatment	Extraction method	Solvent	Condition	Result
Dubinsky & Aaronson, 1979	10 strains including - *Botryococcus braunii* - *Chlorella euglena* - *Chlorococcum Oleofaciens*, etc.	Dry	8 h incubation	chloroform/methanol (2:1, v/v) or with 11M HCl added to the solvent	Ambience	Lipids content in all algae were significantly increased by the addition of HCl as cat. (from 44.4 to 53% in *Botryococus*)
Volkman et al., 1989	10 strains including - *Chaetoceros gracilis* - *Dunaliella tertiolecta* - *Nannochloris atomus, etc.*	Dewatered with glass fiber filter then immediately extracted	10 min. ultra-sonication	propan-2-ol and followed with chloroform/ methanol (2:1, v/v)	Ambience	*Chaetoceros*; FA= 0.71 pg/cell *Dunaliella*; FA=7.4 pg/cell The two highest FA content strains are *Chroomona salina* no.1 (7.8 pg/cell) and *Chroomona salina* no.2 (8.2 pg/cell)
Miao and Wu, 2006	*Chlorella protothecoides*	Freeze-drying	Solvent extraction	hexane	No data	Lipid content = 55.2wt% of dry algae
Xu, et al., 2006	*Chlorella protothecoides*	Freeze-drying	Solvent extraction	hexane	Ambience	Lipid content = 55.3 wt% of dry algae
Liu, et al., 2008	*Chlorella vulgaris*	No data	Solvent extraction	methanol/chloroform (2:1, v/v)	Ambience	Lipid content = 56.6 wt% of dry algae
Zhu, et al., 2008	*Trichosporon fermentans*	Freeze-drying	Solvent extraction	chloroform/methanol (2:1, v/v)	Ambience, 1 h	Lipid content = 62.4 wt% of dry algae

Table 2.5 Literature review on extraction of lipid from microalgae (continued)

Author	Strain	Pretreatment	Extraction method	Solvent	Condition	Result
Widjaja, et al., 2009	*Chlorella vulgaris*	Freeze-drying	Solvent extraction	chloroform/ methanol (2:1, v/v)	Stirred the sample at ambient using magnetic stirrer bar for 5 h and ultrasonicated for 30 min	Lipid content = 52 wt% of dry algae
Chiu, et al., 2009	*Nannochloropsis oculata*	Freeze-drying	Solvent extraction	chloroform/ methanol (2:1, v/v)	Sonication, 1 h at ambient condition	Lipid content = 50.4 % dry weight
Lee et al., 2010	*Botryococcus sp. (1)* *Chlorella vulgaris (2)* *Scenedesmus (3)*	5 min disrupted cells with autoclaving, bead beading, microwaves and osmotic shock (48 h)	Solvent extraction	chloroform/ methanol (1:2, v/v)	Ambience	Disruption using microwaves was the method that best increased oil contents in (1), from ~8% to 28.6% in (2), from ~5% to ~10% in (3), from ~2% to ~12%
Ranjan et al., 2010	*Scenedesmus sp.*	Oven dried in 45^{o}C for 4-5 days	Solvent extraction, Soxhlet or sonication	chloroform/ methanol (2:1, v/v) or n-hexane	Ambience (except for Soxhlet extraction)	Sonication with chloroform/ methanol (2:1, v/v) gave the highest lipid yiled, 6%

2.2.2 Conversion of algal lipid into biodiesel

Lipids from many sources including plant seed oils, animal fats, and algal lipid alike, cannot be directly utilized as biodiesel regarding its high viscosity. Due to this reason, they must be converted into the form compatible with the engine. Many methods can be carried out including blending the oil with diesel fuel, microemulsion with solvent, thermal cracking and transesterification (alcoholysis) (Ma and Hanna, 1999). The last of these is the most popular method according to its ease to handle and economic processing cost.

2.2.2.1 Transesterification

As shown in Figure 2.4, transesterification is a chemical reaction where tri-glyceride reacts with alcohol forming esters. Catalyzed by base or acid, tri-glyceride is cracked into di and mono-glyceride, respectively and finally glycerin. Each cracking produces a mole of fatty acid methyl ester (if methanol is used) whose characteristic is qualified for a diesel engine. The selection of suitable catalyst can be the key to achieve high biodiesel yield in a short period of time.

Homogeneous bases, especially NaOH and KOH, have been most commonly used in industrial scale due to their fast acceleration. The mechanism of transesterification catalyzed by base is shown in Figure 2.5. In the pre-step (step 1), alkoxide ion was created from the interaction between alcohol and base. As a strong nucleophile, the alkoxide ion attacks the carbonyl group of the triglyceride generating

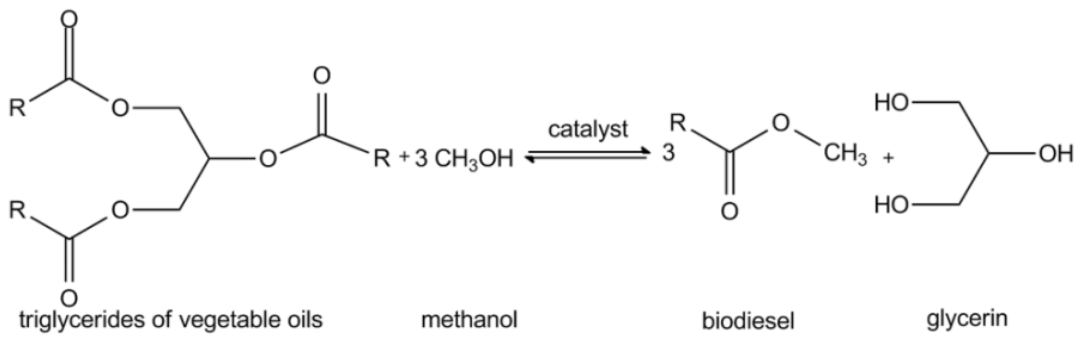

Figure 2.4 Transesterification of tri-glyceride to biodiesel

a tetrahedral intermediate (step 2) from which the alkyl ester and the corresponding anion of the diglyceride are formed (step 3). The latter deprotonates the catalyst, thus regenerating the active species (step 4), which is now able to react with a second molecule of the alcohol, starting another catalytic cycle. Diglycerides and monoglycerides are converted by the same mechanism to a mixture of alkyl esters and glycerol.

However, the use of homogenous base catalyst is limited by the amount of FFA in the reactant oil, if exist more than 0.5% (Lotero et al., 2005). That is, the base not only participates in the reaction as a catalyst but would also react with these FFA forming soap which interferes the main reaction and thus the FAME yield could be lowered. These high FFA resources are such as used cooking oil, non-edible oil e.g. jatropha, karanja, sea mango and also microalgal oil. In that case, acids such as H_2SO_4

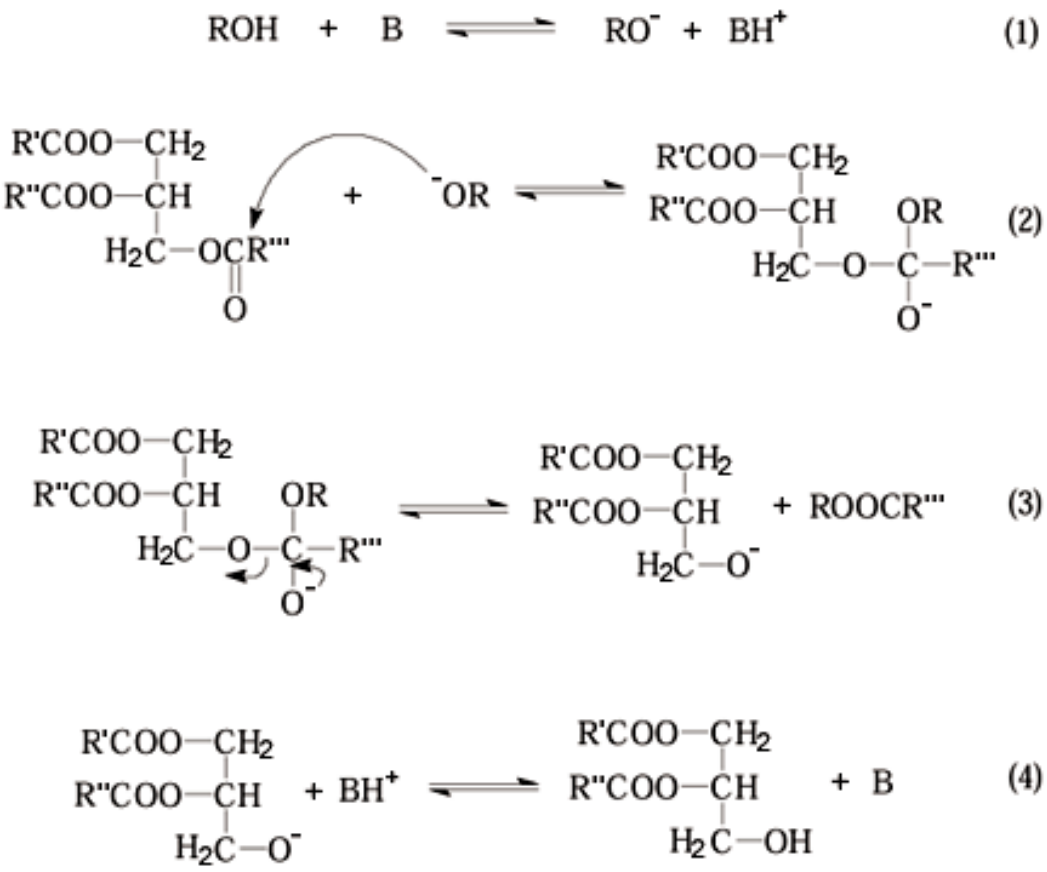

Figure 2.5 Mechanism of base-catalyzed transesterification (Schuchardt et al., 1997)

and HF were likely to be more suitable due to their ability in converting both glycerides and FFAs (via esterification) into FAME. The mechanism of acid-catalyzed reaction is shown in Figure 2.6. Firstly, proton from a strong acid interacts with carbonyl oxygen of triglyceride forming an intermediate (step 1). This intermediate possess high electrophilicity of the adjoining carbon atom, making it more susceptible to nucleophilic attack. In step 2, alcohol attacks the protonated intermediate forming tetrahedral structure. Lastly, proton immigrates and breakdown the tetrahedral intermediate leaving a di-glyceride and alkyl ester. Repeated twice, the reaction finally produces 3 esters and a glycerine. As described by the mechanism, unlike base, acid takes indirect route in catalysis and thus the reaction takes 4,000 times as long (Lotero et al., 2005).

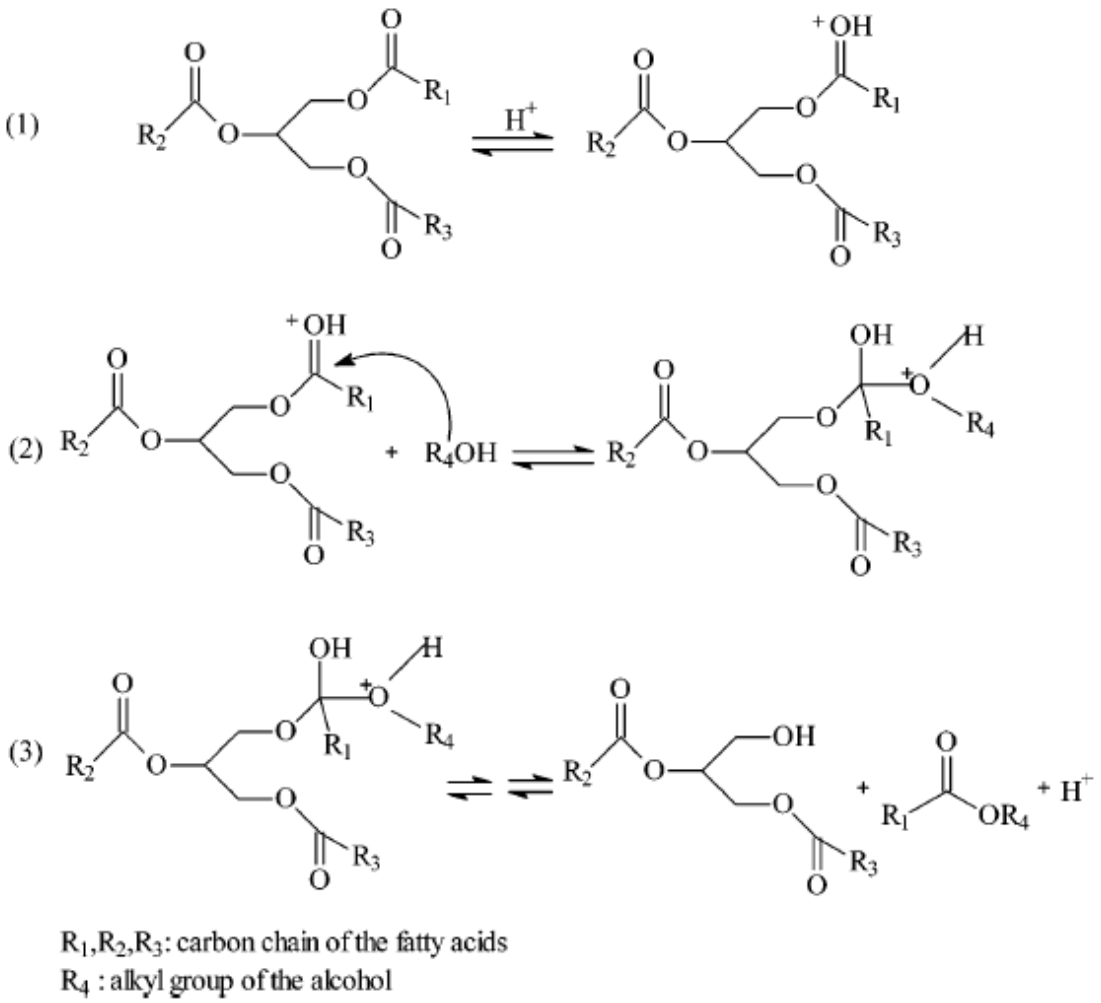

Figure 2.6 Mechanism of acid-catalyzed transesterification (Lotero et al., 2005)

For microalgae, the production of biodiesel from this new feedstock has faced hindrances. The reason why biodiesel from microalgae was claimed to be idealistic (Razon and Tan, 2011) is that the energy input to produce algal biodiesel is greater than the output energy. Based on economic analysis of algal biodiesel production by Delure et al. (2012), downstream processes, including lipid extraction and transesterification, accounted for nearly half of energy input for the entire process. To reduce energy input, many researchers conducted studies on direct transesterification where the energy-intensive algal lipid extraction step could be omitted. In other words, this method allows both oil soluble solvents such as hexane, chloroform, petroleum ether etc. (Johnson and Wen, 2009) and methanol as an alcohol to simultaneously perform their roles in extraction and transesterification, respectively. In many studies, this direct biodiesel production was reported to have comparable and even better efficiency comparing with the former two-step biodiesel production (Johnson and Wen, 2009, Li et al., 2011). The presence in better high yield of biodiesel regarding this one-step method was mentioned to be because of its reduction of lipid loss that may occur during the extraction process (Johnson and Wen, 2009, Li et al., 2011, Koberg et al., 2011).

Alternatively, techniques such as supercritical fluid, ultrasound and microwave might be applied to assist the reaction process as there are a number of successful research evidences for their potential in accelerating transesterfication of glyceride from vegetable. For instance, without catalyst, performing the reaction at supercritical condition, glyceride could be converted to biodiesel in short periods of time (10-30 min) (Demirbas 2002, Yujaroen et al., 2009, Patil et al., 2011b). In addition, microwave was also reported to be an efficient way of shortening time to only 5-15 min for the complete transesterification which, as a result, considerably reduced the energy consumption in the production of biodiesel. The study by Kim et al. (2011a) reveals that energy consumption for the microwave heating in esterification of oleic acid was only 67% of that for conventional heating system due to the significantly reduced reaction time. Literature review regarding algal biodiesel production is elucidated in Table 2.6.

Table 2.6 Literature review on biodiesel production from microalgae

Author	Microalgae	Method	Solvent/Alcohol/Catalyst	Condition/Variable	Result
Johnson and Wen, 2009	*Schizochytrium* limacinum (freeze-dried or paste) (1 g)	two-step - oil extraction; Bligh & Dyer's - transesterification; no data	solvent: chlorofrom:methanol (2:1 v/v) solvent (4 ml): chloroform, alcohol (3.4 ml): methanol catalyst (0.6 ml): H_2SO_4	room temperature, 15 min 90^{o}C, 40 min	- optimum condition: single step tranesterification of dry biomass using chloroform as a solvent - highest yield achieved '72.69 %of algal oil
		single-step	solvent (4 ml): hexane, chloroform, petroleum ether or none alcohol (3.4 ml): methanol catalyst (0.6 ml): H_2SO_4	90^{o}C, 40 min	
Patil et al., 2011	*Nanochloropsis sp.* (paste) (4 g)	single-step - 100 ml reactor supercritical methanol	solvent: none alcohol: methanol catalyst: none	- pressure = 1200 psi - temperature = 240 - 260^{o}C - time = 10 - 30 min - biomass:alcohol (w/v) = 1:4 - 1:12	- optimum condition: temperature = 255^{o}C time = 25 min biomass:alcohol (w/v) = 1:9 - highest yield achieved '90 %of algal oil
Wahlen et al., 2011	*Chaetoceros gracilis* (freeze-dried or wet (by adding water to the dired one) (0.1 g)	single-step - microwave	solvent: none alcohol: methanol, ethanol, 1-butanol, 2-methyl-1- propanol or 3-methyl-1butanol catalyst: H_2SO_4	- temperature = 60 - 110 ^{o}C - time = 25 - 150 min - %catalyst = 1.2 - 2.4 - moiture content in the algae = 20 - 400%w/w	- optimum condition: temperature = 90^{o}C time = 125 min %catalyst = 2.0 moiture content in the algae = 0 - highest yield achieved 33% of biomass

Table 2.6 Literature review on biodiesel production from microalgae (continued)

Author	Microalgae	Method	Solvent/Alcohol/Catalyst	Condition/Variable	Result
Li et al., 2011	*Nanochloropsis sp.* (65 ^{o}C oven dried) (1 g)	two-step - oil extraction; soxhlet	solvent = methanol/methylene dichloride (2:1 v/v)	65^{o}C, 4 h	- optimum condition: single step tranesterification %catalyst = 10 ratio of methanol to methylene dichloride = 3:1 v/v - highest yield achieved 28% of biomass
		- transesterification; a flask equiped with reflux condenser	solvent: none alcohol: methanol catalyst: Mg-Zr base (10 wt%)	65^{o}C, 4 h - biomass:alcohol = 1:4, 1:8, 1:12 (w/v)	
		single-step - soxhlet extractor	alcohol/solvent = methanol/methylene dichloride	65^{o}C, 4 h - %catalyst = 2 - 15 - ratio of methanol to methylene dichloride (v/v) = 1:2, 1:1, 2:1, 3:1, 4:1	
Patil et al., 2011	*Nanochloropsis sp.* (50-60 ^{o}C oven dried) (2 g)	single-step - microwave (400 W)	solvent: none alcohol: methanol catalyst: KOH	- time = 3 - 9 min - %catalyst = 1 - 3 - biomass:alcohol (w/v) = 1:9 - 1:15	- optimum condition: time = 4 min %catalyst = 2 biomass:alcohol = 1:12 w/v - highest yield achieved 77% of algal oil

2.2.2.2 Microwave assisted reaction

In conventional heating systems, heat is generated and conducted through the vessel towards the medium. In microwave systems, on the other hand, heat is generated by the oscillation of molecules exposed to the electromagnetic field and transferred from the inside out resulting in a more rapid heating. This is illustrated in Figure 2.7 where the entire material was rapidly and simultaneously heated.

For a material to be heated under microwave irradiation, it must possess a dipole moment. A dipole is sensitive to electromagnetic fields which, in a typical microwave system, change direction at the rate of about two point four five billion times per second. As a results, the molecules of such material will rotate to align themselves with the changing field. Owing to this high frequency, the rotation of the dipole lags behind the changes of the field and thus phase differece occurs. This phase difference causes energy to be lost from the dipole by molecular friction and collisions, giving rise to dielectric heating (Lidström et al., 2011).

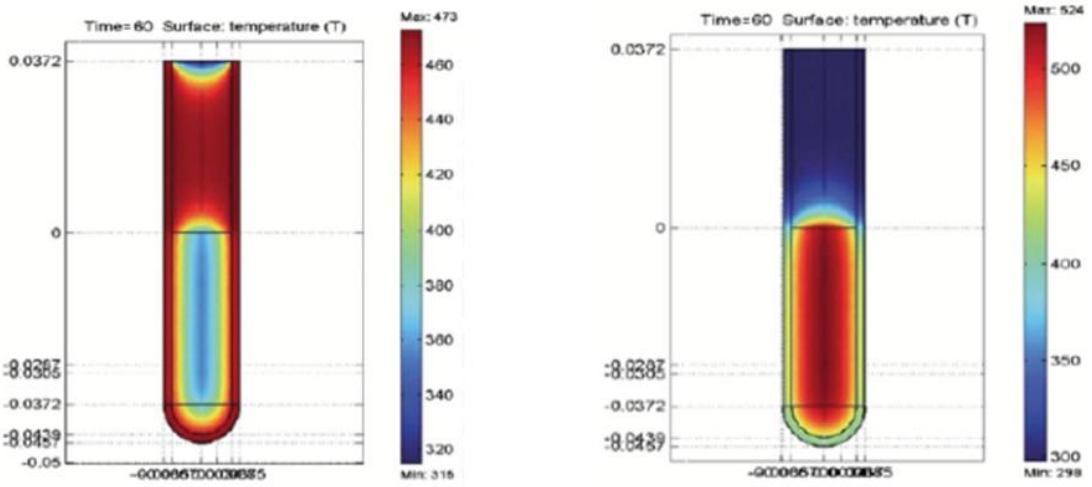

Figure 2.7 The temperature profile after 60 sec as affected by microwave irradiation (left) compared to treatment in an oil bath (right). (Schanche, 2003)

Different solvent and material have different interaction with microwave. The efficiency of a substance in absorbing and converting energy into heat is decribed by Equation 2.1

$$D = \varepsilon'' / \varepsilon' \tag{2.1}$$

where D is the dissipation factor which indicates material capability of absorbing microwaves. ε' or dielectric constant indicates the ability of a molecule to be polarized and ε'' denotes dielectric loss factor representing the efficiency of the microwave conversion in heat. The dielectric constant and the dissipation factor of some solvents are listed in Table 2.7. As an example, although dielectric constants of ethanol and acetone are in the same range, ethanol has a much higher dissipation factor and thus its temperature increases more rapidly under microwave irradiation.

Table 2.7 Dielectric constants and loss tangent values for some solvents relevant to organic synthesis

Solvent	Dielectric constant (ε')[a]	Dissipation factor (D)[b]
Hexane	1.9	0.020
Chloroform	4.8	0.091
Acetic acid	6.1	0.174
Ethyl acetate	6.2	0.059
THF	7.4	0.047
Dichloromethane	9.1	0.042
Acetone	20.7	0.054
Ethanol	24.6	0.941
Methanol	32.7	0.659
Acetonitrile	37.5	0.062
DMSO	45	0.825
Water	80.4	0.123

[a] Condition at room temperature and under the influence of a static electric field.
[b] Values determined at 2.45 GHz and room temperature.

The use of microwave in accelerating chemical reactions has long been investigated. Many publications reported satisfactory results on the application of microwaves in organic synthesis, polymer synthesis, catalyses, nucleation and crystallization (Adnadjevic and Jovanovic, 2011), and many other processes including biodiesel synthesis via transesterification (Motasemi and Ani, 2012). According to Arrhenius's equation (Equation 2.2), reaction rate constant can be described by

$$k = Ae^{-E_a/RT} \tag{2.2}$$

where k is the rate constant, A is the Arrhenius pre-exponential factor, E_a denotes activation energy, R is gas constant and T is temperature. It is implied from Equation 2.2 that the reaction rate can be enhanced by two effects, the effect regarding to the elevation of reaction temperature, which is called the thermal effect and that regarding to the increase in A or the decrease in E_a, which is called the non-thermal effect.

- **Thermal effect**

Thermal effect of microwave on accelerating chemical reactions has been described in various ways in which microwave affect the temperature of reaction system.

Rapid heating

As previously described, the first thermal effect involves the capability of microwave in giving quick increase in temperature. As the reaction temperature is attained much earlier by microwave heating, the total run time for the reaction to proceed for the same product yield then becomes significantly shorter, compared with conventional system. This effect plays a vital role especially for the reactions that take place at very high temperatures. For example, as shown in Figure 2.8, the heating rate of the mixture of ammonium diurante and U_3O_8 can be increased by the increase in power input. 30% of power input reaches 800 K in approximately 120 s while 10% takes twice as long and by conventional heating system (0% power input), such high temperature was not even close to attained in 300 s

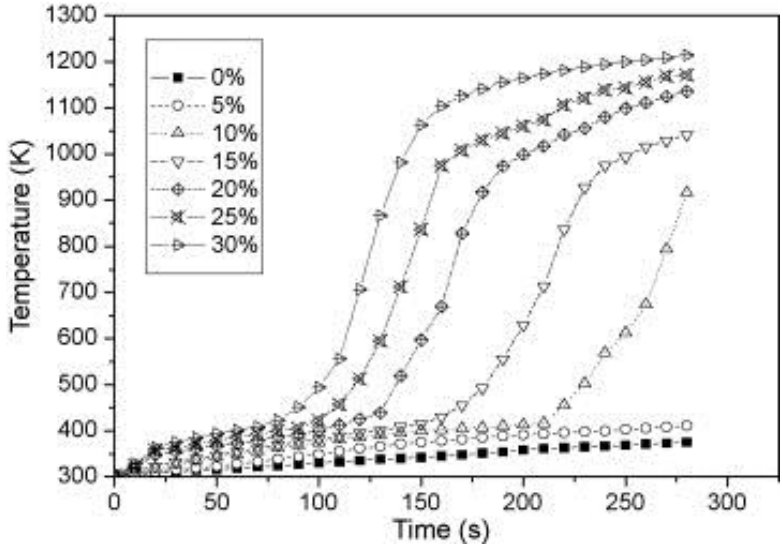

Figure 2.8 Temperature rise characteristics of the mixture of ammonium diurante and U_3O_8 in microwave field (Liu et al., 2010)

Super heating

This effect is relevant to the elevation of boiling point of polar solvents under the exposure of microwave irradiation. For example, the boiling point of heptanol which normally is 176°C was observed to be 200°C under 150 W microwave irradiation and yet increase with the increase of the power input to 200 W, as shown in Figure 2.9. As such, for the system that the temperature is not controlled, the

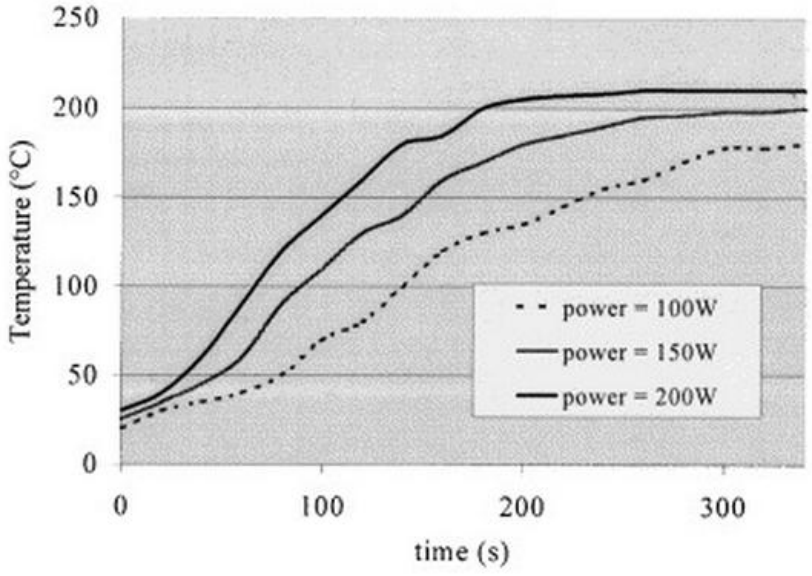

Figure 2.9 Profiles of temperature increase of heptanol when subjected to microwave irradiation (Perreux and Loupy, 2001)

reaction may take place at the higher temperature, compared with conventional heating system and thus resulting in faster reaction. Other solvents were also tested and were reported for the similar result, as shown in Table 2.8.

Hot spot (localized heating)

This effect is mostly mentioned in the reaction where solid catalyst is used. In the study by Yuan et al., (2009), sulfuric acid was used to catalyze biodiesel production via transesterificaion. The acid was introduced into the system either in its original liquid form blended with the medium or impregnated onto the active carbon and used as heterogeneous catalyst (H_2SO_4/C). The reaction took place both on internal and external surface of the catalyst. As sulfuric is a very good microwave absorption substance, hot spots could be formed on H_2SO_4/C. The temperature of these hot spots is considerably higher that of its environment and thus resulted in faster reaction, compared with another system where liquid catalyst was employed.

Table 2.8 Boiling points of some typical polar solvents (°C) at normal conditions and under microwave exposure (Perreux and Loupy, 2001)

Solvent	Normal conditions	MW exposure	Difference
Water	100	105	5
1-Butanol	117	138	21
2-Butanol	98	127	29
Methanol	65	84	19
1-Pentanol	136	157	21
1-Heptanol	176	208	32
Acetone	56	89	33
Ethyl acetate	77	102	25
Tetrahydrofuran	67	103	36
Acetonitrile	82	120	38

- **Non-Thermal effect**

In many studies where the reaction temperature was kept constant, the observed enhancement in the reaction rate was believed to be caused by the decrease in activation energy or/and the increase in pre-exponential value in Arhenius's equation (Adnadjevic, B. and Jovanovic, 2011). The decrease in the activation energy was reported to be a result of electrostatic energy stabilization (Lewis et al., 1992). However, the concept of energy stabilization seems counterintuitive since most effects of microwave radiation are associated with the vibration of bonds and movements of ions. Hence, this is still a controversial issue regarding the difficulty in investigation. Another possible reason of the decrease in activation energy was proposed by Asakuma et al. (2011). The study reveals the change in dipole moment of triolein after pre-treated by microwave. Based on theoretical computation, less activation energy is needed for this pre-treated triolein to convert to methyl oleate when reacted with methanol in transesterificaiton process.

The pre-exponential factor indicates the probability of molecular collisions. As these molecules vibrate in response to microwave, the probability of them to collide with each other increases and thus the pre-exponential factor increases.

2.3 Co-product from algal biodiesel production; Lutein

Apart from improving process efficiency, another way to enhance the process economics is to recover high value co-products. Among nutritious compounds in microalgae, carotenoid pigments, especially lutein were found to exist in quite interesting amount. This yellow pigment has been reported to provide many health benefits, such as preventing coronary, heart and very outstanding eye and vision disease. (Sowbhagya et al., 2004). Its prices are as high as 570-790 USD/kg (prices from Changsha Winner Bio-tech Co., Ltd., Changsha, China; Changsha Sunfull Bio-Tech Co. Ltd., Changsha, China; Xi'an Aladdin Biological Technology Co., Ltd., Xi'an, China). When compared to lutein from marigold which is currently known as the most economically feasible natural source of lutein, the extremely rapid growth

rate of algae could provide many times greater lutein productivity as shown in Table 2.9.

2.3.1 Chemistry of lutein

A yellow pigment lutein is a classified as a kind of xanthophyll, as shown in Figure 2.10, and is one of 600 known naturally occurring carotenoids. The presence of the long chromophore of conjugated double bonds (polyene chain) provides the distinctive light-absorbing properties. The polyene chain is susceptible to oxidative degradation by light or heat and is chemically unstable in acids. The two hydroxyl groups, one on each side of the molecule are believed to play a critical role in their biologic function. Lutein is a lipophilic molecule and is generally insoluble in water. On the other hand, this pigment is well soluble in organic solvent such as, THF, chloroform and ethyl ether (Craft et al., 1992)

Lutein in nature mostly exists in diesterified forms called lutein fatty acid eater. The structure of lutein fatty acid esters comprises of two fatty acids occupying the sites of its hydroxyl groups. Sowbhagya et al. (2004) reported that the major lutein fatty acid ester in marigold is lutein palmitate, while others include dimyristate, myristate palmitate, palmitate sterate, and distearate.

Table 2.9 Comparison of lutein productivity from marigold and microalgae

Biomass	Lutein content (mg/g dry biomass)	Lutein productivity (g rai^{-1} day^{-1})	Comparison with marigold lutein productivity (time)	Reference
Marigold	14.0	27	1	Palumpitug et al., 2011
Muriellopsis sp.	5.5	336	12	Del Campo et al., 2000
	4.3	288	11	Del Campo et al., 2001
		1,728	63	Del Campo et al., 2001
	4-6	160	6	Blanco et al., 2007
		2	0.06	Blanco et al., 2007
Scenedesmus almeriensis	5.5	1,176	43	Sanchez et al., 2008
	4.5	464	17	Sanchez et al., 2008
Chlorella protothecoides	4.6	2,400	88	Wei et al., 2008
Chlorella zofingiensis	3.4	816	30	Del Campo et al., 2000
Chlorococcum citriforme	7.2	6,048	221	Del Campo et al., 2000
	7.6	4,032	147	Del Campo et al., 2000

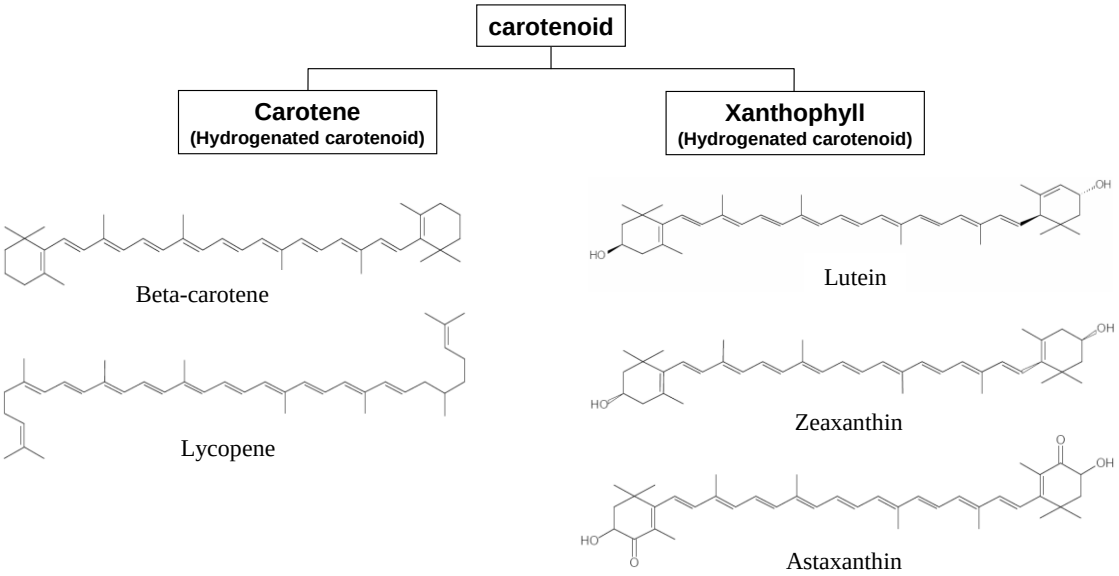

Figure 2.10 Classification of carotenoids

2.3.2 Free lutein and its separation

Lutein in ester form can simply be extracted using organic solvents such as ethanol (Shibata et al., 2004), tetrahydrofuran (THF) (Khachik et al., 2001) and hexane, with the latter seems to extract the highest amount of lutein due to its non-polar characteristics (Ausich et al., 1997). Elaborately, lutein esters can also be extracted with supercritical CO_2 with or without co-solvents (Palumpitug et al, 2011).

However, only lutein in its free form could be taken up by human (Khachik et al., 2001). Therefore, to obtain such high value product, saponification using alkali solution, i.e. NaOH and KOH solution in alcohol, must be taken place to turn lutein fatty acid esters to free lutein. The saponification reaction is depicted in Figure 2.11, which describes the conversion of the lutein fatty acid esters to free lutein, obtaining fatty acid salt or soap as a by-product. It is noted that as lutein is a heat sensitive substance, the reaction was usually carried out at a mild temperature range, 40-60^{o}C, to avoid degradation at elevated temperatures.

Separation and purification of free lutein can be carried out with several techniques. For example, Li et al., 2002 carried out the separation of *Chlrorella* free lutein by partitioning into two phase system and the latter anti-solvent precipitation.

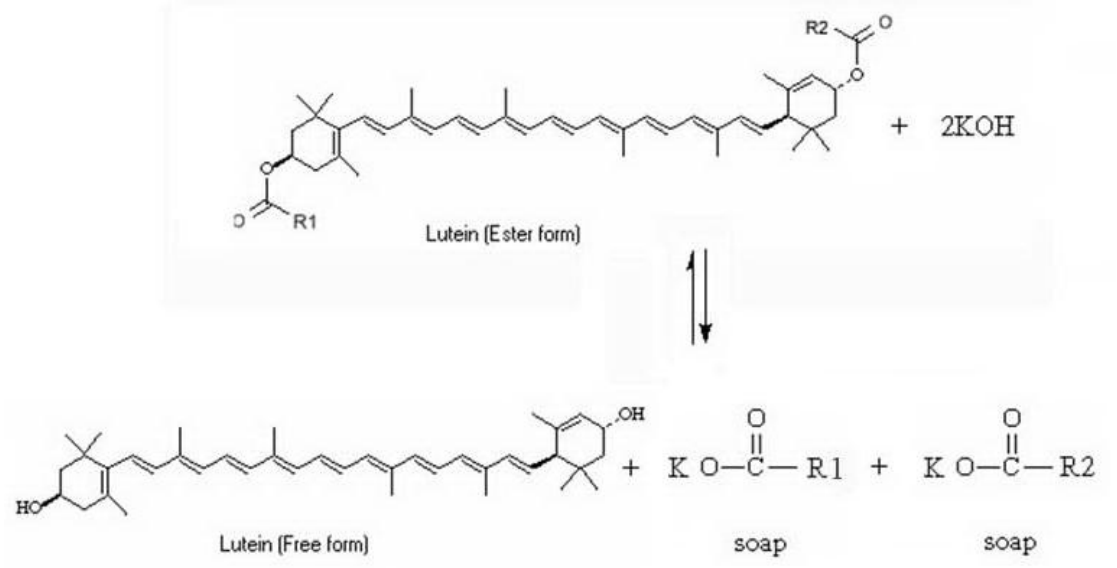

Figure 2.11 Saponification of lutein fatty acid esters

This method involves the selection of two suitable solvents. One of which, ethanol, well dissolves free lutein while one another, hexane, which is immiscible with the first solvent, hardly dissolve free lutein but is excellent in dissolving impurities. Once the phase containing impurities was discarded, free lutein was recovered by adding an anti-solvent to reduce solubility of free lutein in ethanol and thus the free lutein precipitated. The method yielded 85-91% free lutein with the purity of 90-98%. Other ways of purifying marigold free lutein are such as crystallization and chromatography. By adjusting water/ethanol ratio used as solvent (2:0.5 as reported by Vechpanich et al., 2011) as much as 99% of lutein crystals with 86% purity could be obtained after 30 min of crystallization. On the other hand, silica gel chromotography yielded 60% of free lutein at 97% purity with the mixture of 70:30 hexane/ethyl acetate as a mobile phase (Boonnoun et al., 2012).

CHAPTER III
EXTRACTION OF LIPID FROM MICROALGAE

3.1 Introduction

Among all the steps involved in algal biodiesel production, extraction of algal lipid is considered the most costly step requiring considerable amount of input energy. Improvements in extraction process would therefore be translated to the key steps to improve the overall efficiency of entire biodiesel production process. Extraction of algal lipid normally involves the use of some organic solvents. The mixture of chloroform and MeOH (2:1 v/v) (C/M) and hexane were most commonly employed (Halim et al., 2012). However, the yield of algal lipid in many studies often reported as the weight ratio of the crude extract and the dry biomass. In fact, the crude extract cannot all turn into biodiesel as the solvents might dissolve also some impurities. In the first part of this chapter, the suitability of using C/M and hexane for algal lipid extraction was evaluated using *H. pluvialis* as a model alga species. Moreover, due to the variety of structures of microalgae, there is no single method that can be used to recover lipid from all strains. For strains with thin cell walls, lipid could be extracted easily merely by soaking the algae into a suitable solvent while for some others, the lipid may be contained within the cells, enveloped by tough cell walls. For the latter case, the cell wall is needed to be cracked *a priori* and thus modifications to solvent extraction may be necessary. In the second part of this chapter, various solvent extraction methods were investigated including ultrasound assisted extraction (UAE), and microwave assisted extraction (MAE). The resulting extraction yields were then compared with that obtained with the conventional method, maceration. In this part, two strains with different cell structures were used as models for algal lipid extraction. *H. pluvialis* powder represented a microalga with broken cell wall and *C. vulgaris* represents the algal cells possessing intact though cell walls.

3.2 Materials and methods

3.2.1 Biomass and sample preparation

In powder form, *H. pluvialis* was purchased from Cyanotech Corporation, Hawaii Ocean, Science and Technology Park, USA. *C. vulgaris* was cultivated in a bubble column photobioreactors, with diameter of 75 cm, 170 liter, at the Department of Chemical Engineering, Chulalongkorn University, Thailand under outdoor conditions (24–32^{o}C and a diurnal illumination cycle at the intensity of 0–100 klux). The cultivation medium, purchased from Pathumthani Inland Fisheries Research and Development Center, Patumthani, Thailand, was composed of fertilizers including 10 mg/l of triple super phosphate ($Ca(H_2PO_4)_2$), 120 mg/l of ammonium phosphate ($(NH4)_3PO_4$), urea ($CO(NH_2)_2$) and lime. Aeration was applied at the rate of 10 l/min. The microalga was harvested on Day 4 (at the end of log phase growth) and was dewatered by 8000 rpm continuous-flow centrifugation (a disc centrifuge, Alfa Laval DX203B-34, Spain). The resulting alga paste was subsequently lyophilized (FreeZone Freeze Dry System (-50^{o}C), USA), and the dry *Chlorella* powder was then stored at 4^{o}C until use.

3.2.2 Effect of different solvents on algal lipid extraction

3.2.2.1 Lipid extraction

H. pluvialis powder (1 g) were extracted using a Soxhlet apparatus. Either hexane (Fisher Scienctific Ltd.) or C/M, the mixture of chloroform (RCI labscan Ltd.) and MeOH (Mallinckrodt Chemicals Inc.) with the ratio of 2:1 (v/v) was employed as solvent. The solvent was heated to vaporize with the cycle rate of approximately 9 min/round. The extraction was carried out for 4 h or until the colorless extract was observed. After the extraction, the solvent was removed using a rotary evaporator. The remaining crude lipid was kept overnight in a desiccator to ensure the complete absence of the solvent and moisture before measured for its weight.

3.2.2.2 Lipid composition analyses

Analyses of lipid composition were included in order to select the suitable solvent for microalgal lipid extraction. The analyses were carried out following the standard method of AOAC (2005). That is, 2 g of crude lipid extracted by hexane and C/M from previous section were saponified by 25 ml of 0.5 M methanolic NaOH. Subsequently, 300 mg of the saponified lipid sample was treated with 8 ml of BF_3-methanol and boiled for 2-4 min. Thereafter, 2-3 ml of petroleum ether (60^oC) was added to resultant solution to dissolve the esters. Sufficient amount of saturated NaCl aqueous solution was then added to allow the FAMEs to float to the top of the flask, which was collected for chromatographic analysis GC-MS.

3.2.3 Effect of extraction methods on algal lipid extraction

Various extraction methods including maceration, UAE and MAE were investigated for extraction of lipid from microalgae. *H. pluvialis* and *C. vulgaris*. A solvent, used in this investigation was chosen from the study of solvent type (Section 3.1.2). The procedure and the conditions examined for each method are provided as follows. For all methods, after the extraction, the biomass debris was filtered out using Whatman filter paper, No.5 (2.5 µm). Subsequently, the solvent was removed following the procedure described in section 3.1.2. The potential of each extraction method is presented in terms of lipid recovery where 100% equals to the amount of lipid extracted by Soxhlet apparatus, or defined by Equation 3.1

$$\%\ \text{lipid recovery} = \left(\frac{\text{amount of lipid extracted by each method}}{\text{amount of lipid extracted by Soxhlet extraction}} \right) \times 100 \qquad (3.1)$$

3.2.3.1 Maceration

Dry microalgae (1 g) were charged into a 250 ml Erlenmeyer flask added with 100 ml of solvent. The extraction was proceeded under constant temperatures, 30^oC or

40^{o}C, controlled by a water bath. Stirring was provided manually at the beginning, followed by maceration through stagnant liquid for 5-30 min.

3.2.3.2 Ultrasonic assisted extraction, UAE

The biomass and the selected solvent (1:100 w/v) were introduced into a flask which was then rapidly placed into a 40 kHz ultrasonic bath, Crest Ultrasonics. The extraction was conducted at room temperature (30^{o}C), 40^{o}C or 50^{o}C for 5-30 min.

3.2.3.3 Microwave assisted extraction, MAE

The experiment was carried out using a microwave extractor, MARS 5, CEM Corp. (Mathews, NC, USA). Biomass and the selected solvent with the ratio of 1:100 (w/v) were charged into the vessel. The effects of extraction time and temperature on the amount of lipid extracted were studied at 5-30 min, 40-50^{o}C, respectively while the maximum power of microwave was kept constant at 300 W.

3.3 Results and discussion

3.3.1 Effect of different solvents on algal lipid extraction

As shown in Table 3.1, the amount of the crude extract obtained from the two solvent are greatly different. C/M gave higher yield of crude extract, 38.9% by weight of algal biomass while 20.3% was obtained from the extraction by hexane. This is because some neutral lipids found in cytoplasm are bound with polar lipid in a

Table 3.1 Amount of crude lipid extracted with hexane and C/M and amount of biodiesel obtained from corresponding extracted lipids.

Solvent	Amount of crude extract (% by wt of algae)[a]	Amount of biodiesel (% by wt of crude lipid)[b]	Amount of biodiesel (% by wt of algae) (a x b)/100
C/M	38.9	33.3	13.0
hexane	20.3	44.3	9.0

complex form. This complex is attached strongly with protein in cell membrane by hydrogen bond. A non-polar solvent such as, in this case, hexane interacts with the lipid complex with van der Waals force which is not strong enough to destroy the hydrogen bonding between the lipid and cell membrane protein. A polar solvent such as MeOH, is on the other hand capable of disrupting the lipid–protein associations by forming hydrogen bond with the polar lipid in the complex (Halim et al., 2012). Another possible reason is that a corrosive solvent such as chloroform has potential in damaging cell wall (Ranjan et al., 2010, Araujo et al., 2013). As the cell wall is weakened, the lipid could be extracted more readily.

After transesterification, chromatographic analysis shows that 33.3% and 44.3% of the crude lipid extracted with C/M and hexane, respectively, could be converted to biodiesel. This indicates that although smaller amount of crude extract was obtained with hexane, it is a better solvent in terms of selectivity for targeted lipid such as mono-, di- and tri-glycerides (see Appendix A). However, when multiplying these percentages of converted biodiesel with the amount of crude lipid, C/M gave higher amount of biodiesel by weight of algae than hexane (13% Vs 9% by weight of algae) and thus was the selected solvent for further studies.

3.3.2 Effect of extraction methods on algal lipid extraction

In this experiment, the effects of ultrasound and microwave were investigated for lipid extraction of two algal strains: *H. pluvialis* and *C. vulgaris*. The performance of each method was evaluated by means of lipid recovery[1]. As shown in Figure 3.1, the result indicates that for the short period of UAE (5-10 min), the amount of lipids recovered slightly increased with increasing temperature, from 30 to 40^{o}C. On the other hand, the lipid yield decreased with increasing UAE duration (from 15 to 30 min). It is possible that ultrasound accelerated the oxidation reaction of some fatty acids (Metherel et al., 2009). Thus, the decrease of lipid recovery was possibly due to

[1] a 100% recovery is defined by the amount of algal lipid extracted by Soxhlet extraction manufacturing process of powder production. Hence, there was no cell wall resistance and the rate of extraction would therefore be mainly affected by the solute solubility in the solvent.

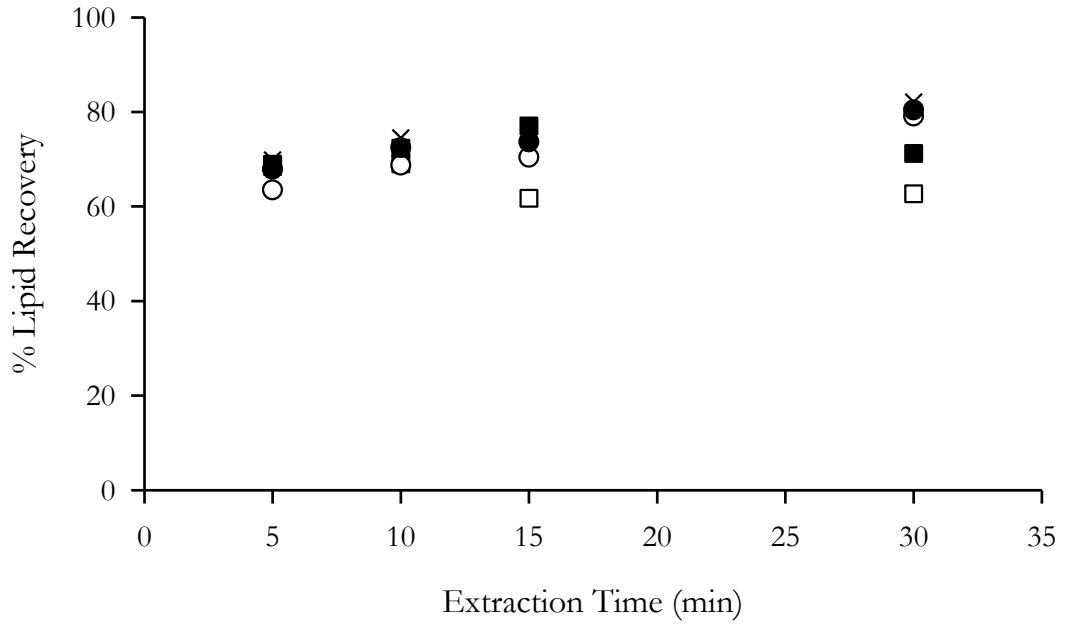

Figure 3.1 Comparison of %lipid recovery from *H. pluvialis* using three different extraction methods, maceration at 30 (○) and 40°C (●), UAE at 30 (□) and 40°C (■) and MAE at 40 (×) (Replication of experiments was carried out for selected conditions. The standard deviations of %lipid recoveries vary from ± 0.2 to 3.5.)

the damage of some substance in the cells when applying ultrasound for longer duration. Although it is likely that MAE gave the highest extraction rate in this study and the rate tended to increase with the increase in extraction temperature, only slight benefit was found from the method. This was possible that the purchased *H. pluvialis* used in this study was already cracked during the manufacturing process of powder production. Hence, there was no cell wall resistance and the rate of extraction would therefore be mainly affected by the solute solubility in the solvent. The above hypothesis was supported by the lipid extraction results of the cultivated *C. vulgaris*, whose cell wall remained intact. Herein, the same three methods from previous study including maceration UAE and MAE were investigated. As shown in Figure 3.2, it is obvious that such method without applying cell disruption techniques as maceration resulted in the slowest extraction rate. Although the increase in maceration temperature might help enhance the extraction, the percentage of lipid recovery is likely to be limited at approximately 70%, even after the extraction time as long as

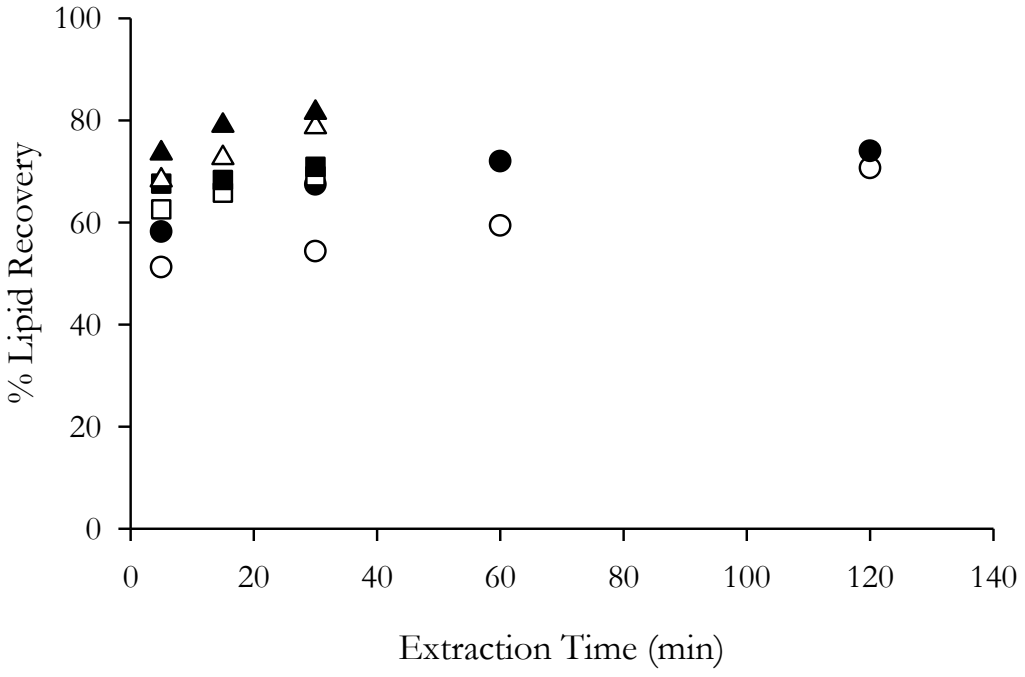

Figure 3.2 Comparison of %lipid recovery from *C. vulgaris* using different extraction methods: maceration at 40 (○) and 50 (●) ^{o}C, UAE at 40 (□) and 50 (■)^{o}C and MAE at 40 (△), 50 (▲) ^{o}C (Replication of experiments was carried out for selected conditions. The standard deviations of %lipid recoveries vary from ± 0.2 to 3.5.)

120 min. On the other hand, both UAE and MAE resulted in better extraction as the time required to reach the same amount of lipid was shortened. The better lipid yield obtained in UAE is due to the cavitation shear force that breaks the cell wall mechanically. This then facilitates the transfer of lipids from the cell into the solvent. Comparing among these two techniques, MAE is likely to be favorable for extraction of lipid from *C. vulgaris* as it resulted in higher lipid recovery, even when operated at lower temperature. The maximum lipid recovery in this study (82%) was obtained from MAE for 30 min at the extraction temperature of 50^{o}C. The reason of the greatest yield is because, while exposed to microwave, the media (MeOH) including compositions in the microalgae or even the cell itself were oscillated as an attempt to align themselves with the rotating electromagnetic field caused by microwave. This resulted in friction and subsequently, intramolecular heat was generated, causing pressure effect rupturing the cell wall (Choi et al 2006) and thus the lipid was extracted more easily.

3.4 Concluding remarks

In summary, for the extraction of lipid from microalgae, C/M gave the highest amount of extracted crude oil, while hexane was found to be a better choice in terms of providing more desirable content of glycerides. The selection of extraction method depends greatly on cell morphology such as the existence of the cell walls. For example, for strains whose cell walls were previously damaged, neither UAE nor MAE gave additional benefits. In this case, lipid extraction could be processed by an energy saving method like maceration. On the other hand, for strains with tough cell wall such as *C. vulgaris*, without any prior cell disruption, as little as 70% of lipid was recovered even when extra time was given. In this case, comparing among the three methods in this study, MAE provided most satisfactory lipid recovery. Therefore, detailed study on algal biodiesel production under microwave irradiation was carried out and the results are be presented in subsequent chapters.

CHAPTER IV
ENHANCEMENT OF *CHLORELLA* SP. BIODIESEL PRODUCTION BY PULSED MICROWAVE

4.1 Introduction

The process to produce biodiesel from microalgae basically consists of extraction of biomass for lipid, followed by alcoholysis of the extracted lipid which converts glycerides or fatty acids into fatty acid methyl esters (FAMEs), known as biodiesel. To significantly enhance the efficiency of algal biodiesel production, apart from improving the high energy consuming process like that of lipid extraction, Johnson and Wen (2009) proposed to combine extraction and chemical reaction to produce biodiesel into a single-step. That is, algal biomass, catalyst and alcohol (which acts both as an extraction solvent for lipid and a reactant) are introduced into the system in which these two processes occur simultaneously. By this combined process, the yield of biodiesel was found to be comparable to that obtained from traditional method while time was reduced by half, and thus the operating cost was expected to be considerably lowered

Apart from the single-step process, biodiesel production under microwave irradiation has been evaluated as the other mean to shorten the reaction time. In several studies, application of microwave resulted in higher biodiesel yield, compared with conventional and ultrasound assisted methods carried out for the same duration (Koberg et al., 2011 and Patil et al., 2011). Due their availability, household microwave systems or that with modifications (Lertsathapornsuk et al., 2008, Patil et al., 2011, Khemthong et al., 2012) are usually employed. To control microwave power to the load, these household microwave systems use time slicing method where maximum power is applied for time interval, proportional to the power set by the user. For example, if 25% power is set on microwave oven rated at 1000 W, the device supplies maximum power, 1000 W for 25% of the total time. The typical low cost magnetron employed in such system causes inhomogeneous field of microwave.

Therefore reliable controls of microwave power and temperature are not possible in these systems.

Less often used on the other hand, pulsed microwave provides a means to maintain a desired system temperature by adjusting the duty cycle. Koberg et al. (2011) applied pulsed microwave to the production of biodiesel from *Nanochloropsis* sp. and showed that, at a selected condition pulsed microwave system gave higher biodiesel yield compared with a conventional conduction heating system and system with ultrasound irradiation for both in single and two-step methods. In a recent study (Kim et al., 2011b), pulsed microwave system was demonstrated to improve the conversion of triolein to biodiesel over the continuous microwave system. However, the use of pulsed microwave systems with the production of algal biodiesel is still limited. Particularly, the effects of various operating variables on biodiesel yield obtained with pulsed microwave irradiated reaction have not been much informed.

Based on the results from the previous chapter in which microwave was found to enhance lipid extraction, and on its well documented ability to accelerate chemical reactions (Adnadjevic and Jovanovic, 2011), in this study, pulsed microwave was applied to H_2SO_4 catalyzed transesterification of algal biomass with MeOH to enhance the biodiesel yield. A green microalga, *Chlorella* sp. was chosen as a model alga, due to its satisfactory lipid productivity and good environmental tolerance. Firstly, both for the two-step (extraction-transesterification) and the single-step processes, FAMEs yields obtained with pulsed microwave irradiation were compared with those obtained with conventional conduction heating at a selected condition. Secondly, in the system irradiated with pulsed microwave irradiation, the effects of the amount of MeOH and H_2SO_4 on FAMEs yields were examined.

4.2 Materials and methods

4.2.1 Biomass and sample preparation

The liquid culture of *Chlorella* sp. was purchased from Pathumthani Inland Fisheries Research and Development Center, Pathumthani, Thailand. The culture was

dewatered by 8000 rpm continuous-flow centrifugation (a disc centrifuge, Alfa Laval DX203B-34, Spain) to obtain paste-like algal sample. The algal paste was then lyophilyzed at -40^{o}C for 24 h to dry powder, and was stored at 4^{o}C until use.

4.2.2 Comparison of FAMEs yields from systems irradiated with pulsed microwave and conventional heating

Comparisons of FAMEs yields obtained with reactions at 60^{o}C for 10 min under conventional heating and under microwave irradiation were made for both two-step and single-step esterification. In the two-step process, extraction of 3 g of algal biomass was carried out first to obtain the lipid, followed by transesterification with 72 ml of MeOH and 0.48 ml of H_2SO_4 catalyst. While in a single-step process, the cells, MeOH and the catalyst were charged directly into the reaction system. The methods of extraction and transesterification in systems with conventional heating and microwave heating are described below.

4.2.2.1 Lipid extraction

Lipid from 3.0 g of lyophilized *Chlorella* powder was extracted for 4 h following the procedure described previously in Section 3.2.2.1. Subsequently, the extracted lipid underwent transesterification, either under conventional conduction heating or microwave irradiation.

4.2.2.2 Transesterification under conventional conduction heating

In the two-step biodiesel production, MeOH, H_2SO_4 catalyst (conc.) (Wako, Japan) and the extracted crude algal lipid obtained from 3 g dried algae were charged into a vessel equipped with a condenser. In a single-step process on the other hand, instead of extracted lipid, dry biomass was directly charged into the system together with MeOH and H_2SO_4 catalyst.

In either case, the reaction mixture was then heated by a conventional conduction heater and agitation was provided at 300 rpm by a magnetic stirrer. As

soon as the temperature reached the set point of 60^{o}C, timing was started and the reaction was allowed to take place for 10 min, after which the reaction mixture was then allowed to cool to room temperature. For the two-step process, chloroform and water were then added directly into the reaction mixture at the ratio of mixture:chloroform:water of 10:10:9. For the single-step process on the other hand, algal cells were first filtered out of the reaction before the addition of the chloroform and water. After shaken vigorously, the mixture was centrifuged at 2000 rpm for 10 min, resulting in separation into two phases. MeOH and other polar impurities dissolved in water forming the upper phase, while FAMEs, FFAs and other neutral lipids dissolved in the chloroform bottom phase which was analyzed for FAMEs content by gas chromatography (GC).

4.2.2.3 Transesterification under pulsed microwave irradiation

The pulsed microwave assisted transesterification was investigated with the apparatus, schematically shown in Figure. 4.1. The multimode microwave system (Shikoku Instrumentation Co., Ltd., Japan), 2.45 GHz consisted of an oven (a)

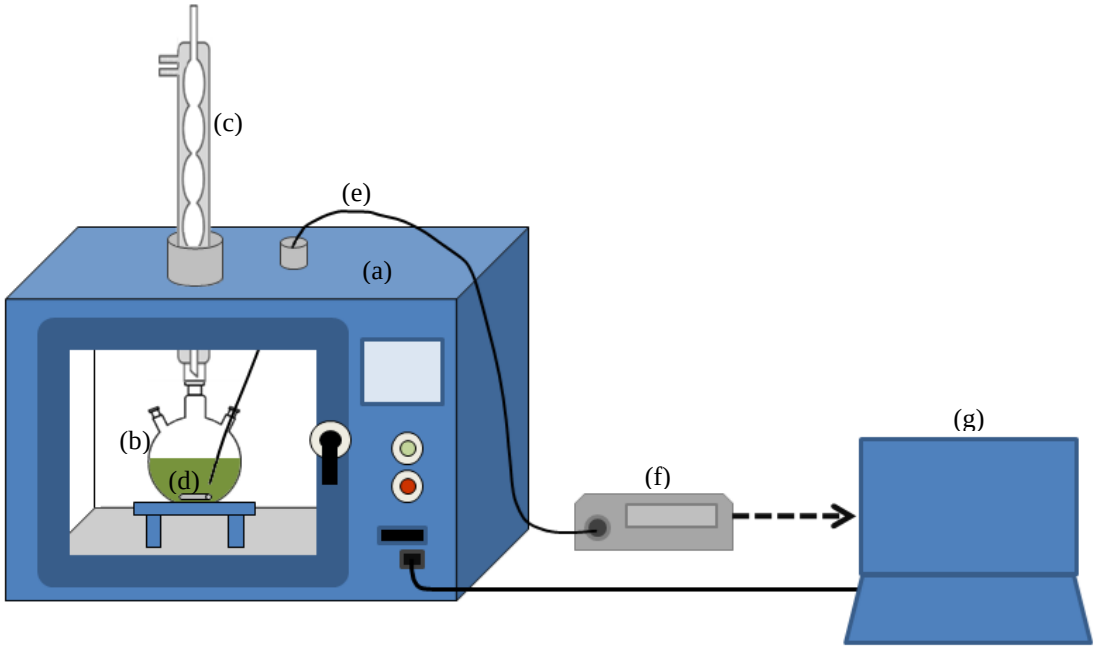

Figure 4.1 Schematic diagram of pulsed microwave apparatus for biodiesel production

containing a magnetron that generates energy. Transesterification in pulsed microwave system was conducted by charging MeOH and of H_2SO_4 (conc.) into a round bottom glass flask (b) containing extracted crude lipid (for the two-step method) or 3 g of whole algae cell (for the single-step method). The flask was equipped with a condenser (c) with 2-4^oC water flowing through to maintain the volume of liquid in the system. A regular magnetic bar (d) was placed into the flask to provide 300 rpm stirring to the system. The reaction temperature was measured with a fiber optic sensor (e) connected to a display (f). The maximum microwave power was set at 500 W with the duty cycle of 20%, and the reaction temperature was controlled at 60^oC.The software SLP-C35, Yamatake Corp. (g) was applied to monitor the microwave power and reaction temperature profiles as shown in Figure 4.2. During the ramping period, pulsed microwave was supplied with the maximum power (500 W). As soon as the set point temperature was attained, the system was automatically adjusted to supply just sufficient power only to maintain the temperature (60^oC). To prepare the sample for the analysis with GC for FAMEs content, chloroform and water were then added to the reaction mixture following the procedure described in Section 4.2.2.2

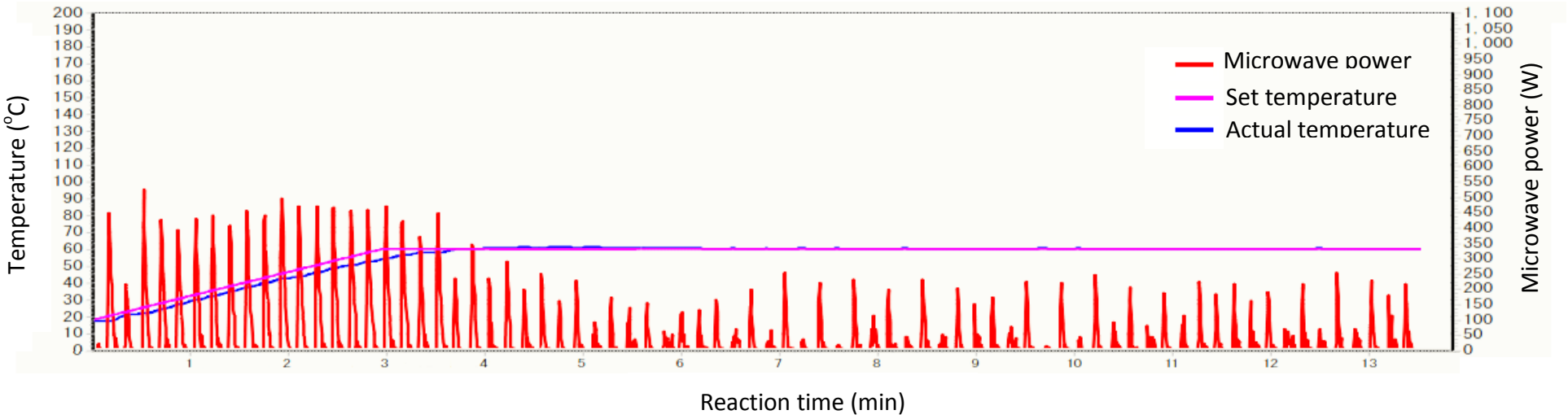

Figure 4.2 Temperature and power profiles of pulsed microwave system (power ≤500 W and 60°C reaction temperature)

4.2.3 Effect of amount of MeOH and H_2SO_4 catalyst on microwave assisted transesterification

The effect of the amount of alcohol and catalyst on the production of algal biodiesel was investigated under pulsed microwave irradiation with the maximum power of 500 W both for the single and two-step processes. The reactions were carried out at 60^{o}C for 10 min following the procedure described in Section 4.2.2.3. The range of MeOH amount studied was 24-72 ml and that of the catalyst amount was 0.24-1.92 ml, respectively.

4.2.4 GC Analyses for FAMEs

The samples were analyzed for the amounts of FAMEs using GC (Shimadzu GC-14B), equipped with a flame ionization detector (FID) with the injection volume of 0.1 µl. Separation of the sample products was achieved by a capillary column (CP-FFAP CB, 25 m (length), 0.32 mm (ID), 0.3 µm (film thickness), Varian, California. Injector and detector temperatures were set at 270^{o}C and 300^{o}C, respectively. The elution temperature program started with an initial temperature of 100^{o}C which was held for 5 min prior to linear ramping with the rate of 10^{o}C/min to the final temperature of 250^{o}C. The final temperature was then held for 20 min, making the total run time of 40 min. The amount of biodiesel was quantified based on standard mixture of 5 FAMEs found in most biodiesels including palmitic, stearic, oleic, linoleic and linolenic acid methyl ester (Wako Japan). In addition, heptadecanoaic acid methyl ester (Wako Japan) was used as an internal standard. The FAME yield was determined from following Equation 4.1.

$$\text{FAME yield}\ (\%) = \left(\frac{\sum \text{weight of the 5 FAMEs in sample}}{\text{weight of crude lipid}\ ^{1}} \right) \times 100 \qquad (4.1)$$

[1] In case biodiesel was produced via a single-step process, this is the weight of crude lipid if the biomass were to be extracted for 4 h by Soxhlet apparatus, which is taken to be the lipid weight averaged over the extracted weights from the experiments carried out with the two-step process.

4.3 Results and Discussion

4.3.1 Comparison of FAMEs yields obtained under microwave irradiation and conventional conduction heating

As shown in Figure 4.3, FAMEs yields produced by two-step and single-step and methods under conventional heating were comparable. For both two-step and single-step processes under pulsed microwave irradiation, higher FAMEs yields were obtained compared with those obtained with the conventional heating system despite the shorter total time used for the reaction (12.7 min versus 25 min) For the two-step process, 17% increase in FAMEs yields was resulted with the system under microwave irradiation, while 62% increase in FAMEs yields was found for the single-step process. Microwave has been known to enhance various chemical reactions by both thermal and non-thermal effects (Jacob and Chia, 1995). By the thermal effect,

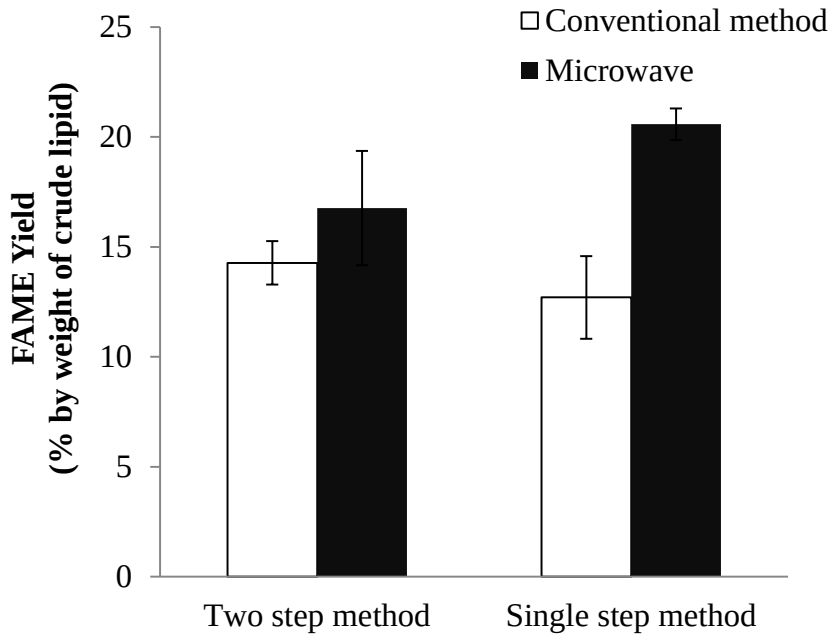

Figure 4.3 Comparison of FAMEs yields obtained with 10 min reaction at 60°C under microwave irradiation and conventional heating
(3 g of biomass, 72 ml of MeOH and 0.48 ml of H_2SO_4).

microwave causes quick rising of the temperature of the whole reaction mixture as heating occurred as a result of the friction loss during rapid rotation of the molecules in the electromagenic field (Barnard et al., 2007, Manco et al., 2012, El Sherbiny et al., 2010). While in the conventional heating system on the other hand, heat transfers from the heat source by conduction through the wall of vessel to the reaction mixture from outside-in. The non-thermal effect of microwave on acceleration of chemical reactions has also been described in various studies (Jacob and Chia, 1995, Yuan et al., 2009, Binner et al., 1995, Asakuma et al., 2011). When exposed to microwave irradiation, molecules in the reaction system vibrate with high frequency, leading to the more probability of molecular collisions which was directly proportional to rate constant in Arrhenius equation, thus the reaction proceeded faster (Binner et al., 1995). In biodiesel production in particular, MeOH was believed to quickly reorient in response to microwave, thus the two-tier structures of the interface of MeOH and lipid were destroyed. As a result, the solubility of MeOH and algal oil was improved giving higher possibility for the two reactants to encounter (Yuan et al., 2009). Moreover, as the molecules of triglyceride tried to align themselves with the rotating microwave field, they isomerized to different forms. These isomers were demonstrated in their study to have lower dipole moments, and thus lower activation energy when transesterified to FAMEs (Asakuma et al., 2011).

The greater enhancement of FAMEs yields (62% enhancement) for the single-step process by microwave was probably related to the enhancement in the algal lipid extracted (Lee et al., 2010, Prommuak et al., 2012, Balasubramanian et al., 2011) other than the effect of microwave on the reaction. Oscillations of components in the reaction mixture under the field of microwave caused friction, and subsequently, intramolecular heat was generated, causing pressure effect that ruptured the cell wall (Choi et al., 2006).

It is noted that the rather low FAMEs yield in this study, compared with that obtained with purified vegetable oil in most literatures was due to the nature of the crude algal extract. Reported by Chia et al. (2013), *Chlorella* crude lipid extracted by the mixture of chloroform and methanol contained 15-60% of tri-glyceride and a major content of polar lipids such as phospholipid (20-35% by weight of crude lipid) which were unreacted. Apart from that, other impurities including free fatty acid

(FFA) (Hatano et al., 1982) and pigments such as chlorophyll and carotenoids (Gouveia et al., 1996) were also reported to be present in the crude lipid. However, the yield lies within the range of biodiesel yields reported in literatures such as in Montes D'Oca et al. (2011) and Li et al. (2011) for transesterification of lipid from *Chlorella pyrenoidosa* and *Nannochloropsis* sp., respectively.

4.3.2 Effect of MeOH amount

Figure 4.4 shows the effect of the amount of MeOH on FAMEs yields obtained with the single and two-step processes carried out with a fixed amount of catalyst of 0.48 ml and 3 grams of biomass. For the two-step process, FAMEs yields were found to decrease with increasing amount of alcohol despite the fact that the reversed was expected. This was probably because the amount of MeOH used in this study (24 ml) was already far in excess of the stoichiometric amount. Increasing the MeOH amount rather, caused the catalyst dilution, resulting in decreased FAMEs yields.

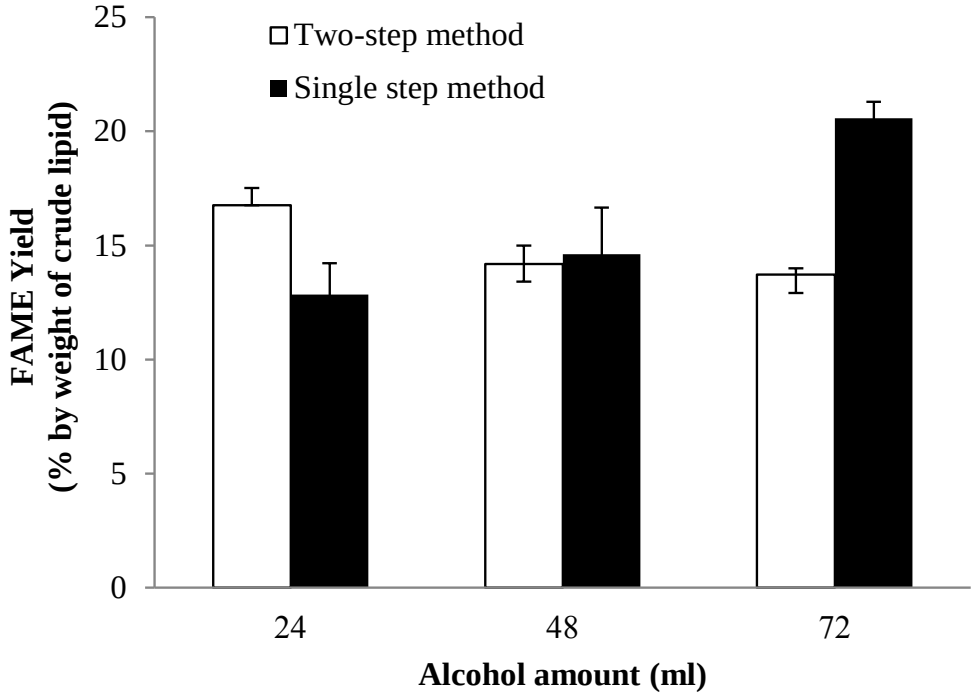

Figure 4.4 Effects of MeOH amount on FAMEs yields (with 0.48 ml of H_2SO_4)

On the contrary, for the production of biodiesel carried out with the single-step method, FAMEs yields increased with increasing amount of MeOH. This was probably because the system involved two simultaneous mechanisms: extraction and reaction. Although increasing the amount of MeOH within the range of this study might not directly enhance the reaction, it was likely to help enhance lipid extraction as it provides greater concentration gradient between algal biomass and the bulk liquid, and thus greater driving force to lipid mass transfer.

4.3.3 Effect of catalyst amount

The effect of catalyst amount on FAMEs yields shown in Figure 4.5 indicates that the FAMEs yields increased with increased amount of catalyst, for both the two-step and single-step processes. It is noted that, not only H_2SO_4 participates in the reaction as a catalyst, but the fact that it is a strong acid which ionizes into H+, which interacts with microwave, causes the solution to be heated and increases the probability for the molecules to encounter and react rapidly (Li et al., 2012). It is noted that at the lowest amount of catalyst used in this study (0.24 ml), both single

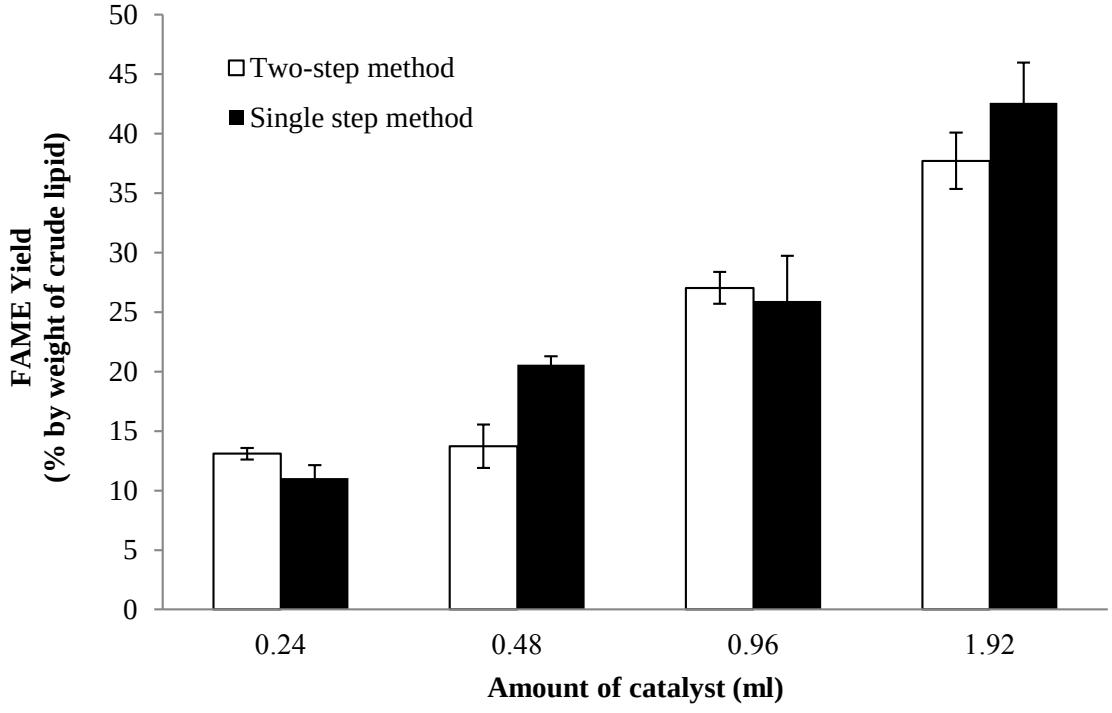

Figure 4.5 Effects of catalyst amount on FAMEs yields (with 72 ml of MeOH)

and two-step methods gave comparable FAMEs yields. When the amount of catalyst was doubled (0.48 ml), the effect on the two-step method was almost negligible.

As described in the previous section, since high amount of MeOH (72 ml) was employed, the catalyst amounts of 0.24 and 0.48 ml might be diluted by MeOH, thus the reaction could not take place efficiently from 0.24 to 0.48 ml in the single-step process on the other hand, the increase in FAMEs yield was more pronounced. For a single-step process, not only H_2SO_4 had the effect on the reaction, but as a strong acid, H_2SO_4 could cause cell wall disruption, and thus enhanced algal lipid extraction as well (D'Oca et al., 2011).

4.4 Concluding remarks

Under pulsed microwave irradiation, esterification could be enhanced in both two-step and single-step processes, compared with reaction carried out at the same conditions under conventional heating. In the single-step process, microwave also helps breaking microalga cell wall resulting better extraction yield, then higher amount of available oil for the reaction. With the single-step method, the increase in the MeOH amount increased the FAMEs yield while in case of the two-step method, resulted in decreased yield. On the other hand, increasing the amount of acid catalyst gave increased FAMEs yields in both the two-step and single-step processes. It can therefore be concluded from the results that the reaction yield could be greatly affected by the various systems (single or two-step) and the reaction conditions employed. As there are still a limited number of literatures related to production of biodiesel from algae under pulsed microwave, further information regarding the optimization of other parameters that might affect the efficiency of the production is yet necessary. This issue is presented in the following chapter.

CHAPTER V
EFFECT OF PULSED MICROWAVE POWER ON TRANSESTERIFICATION OF *CHLORELLA* SP. FOR BIODIESEL PRODUCTION

5.1 Introduction

In the last chapter, the advantage of pulsed microwave over the conventional system and the effect of key reaction parameters on the production of biodiesel were demonstrated. Apart from these variables, microwave power has also found to be one of the key factors affecting the reaction yield (Chen et al., 2012, Khemthong et al., 2012).

As mentioned in Chapter IV, due to their availability, household microwave systems or those with modifications are usually employed in most studies (Chen et al., 2012, Khemthong et al., 2012, Lertsathapornsuk et al., 2008, Patil et al., 2011, Encinar et al., 2012). One limitation of this system for use to determine the effect of power on the biodiesel yield however is because the typical low cost magnetrons employed in these systems cause inhomogeneous field of microwave. Furthermore, the simplistic control systems available on these units only usually allow a limited number of power steps and do not allow reliable control of heating schedules and reaction temperatures (Kappe, 2004). As a consequence, reaction temperature increased with increased power setting.

Fortunately, specially designed microwave systems today feature built-in direct temperature control of the reaction mixture with the aid of fiber optic probes or IR sensors and software that enables real-time temperature/pressure and other process variable feedback control by regulation of pulsed microwave power output. This system not only allows accurate control of power to the load, but the heat up rates and etc. (Kappe, 2004). Kim et al. (2011b) employed such a specially designed microwave system and demonstrated that esterification of oleic acid irradiated with pulsed

microwave resulted in higher biodiesel yield than the one conducted under continuous microwave irradiation.

The aim of this study was to determine the effect of pulsed microwave power settings on biodiesel yields. Here, the system equipped with software that allows real-time temperature and power profiles to be monitored and recorded was employed to provide heating to the direct transesterification of *Chlorella* sp. biomass for biodiesel production. The pulsed microwave system used would allow accurate evaluation of the efficiency of the process i.e. yield of biodiesel per unit energy consumed.

5.2. Materials and Methods

5.2.1 Biomass and Sample Preparation

The dry alga biomass obtained from the preparation described in Section 4.2.1 was also used in this investigation.

5.2.2 Microwave assisted biodiesel production

In the microwave apparatus described in Section 4.2.2.3, the experiment was conducted using 72 ml of MeOH and 0.48 ml of H_2SO_4 for the direct transesterification from 3 g of algal biomass. The reaction temperature was controlled at 60^{o}C by automatic adjusting of microwave power. The effects of power settings at 100, 250, 500, 750 and 1000 W on algal biodiesel production were investigated. At all power settings, microwave was supplied in a pulse mode with the duty cycle of 20%. The software SLP-C35, Yamatake Corp. was used to report the real-time profiles of microwave power and reaction temperature. The process after the reaction was followed the method in Section 4.2.2.2 and the analysis for FAMEs content in each sample by gas chromatography (GC) were carried out following the method in Section 4.2.4. From the power profiles, energy consumptions were calculated by integrating the areas under the power profiles.

5.3 Results and discussion

5.3.1 Microwave power and temperature profiles

As shown in Figure. 5.1, for all power settings, the profiles of microwave power and temperature can be divided into two periods: the ramping period and the holding period in the first period, microwave was supplied with the maximum power until the desired temperature (60^{o}C) was attained. Subsequently, in the second period, the system supplied only sufficient power to maintain the set temperature. It can also be seen from Figure 5.1, the power setting of 100 W, the system could not reach the desirable temperature of 60^{o}C (only 35^{o}C was attained). As the maximum power was adjusted to higher levels, the time it took for the reaction system to reach the set temperature became considerably shorter, in other words, the heating rate increased with increased power setting. In addition, it should be noted that, not only the peak intensities were lowered during the holding period, the pulse frequencies (the numbers of pulses per minute) also became smaller, despite the set duty cycle of 20%. The pulse frequencies during the ramping and holding periods at various power settings are summarized in Table 5.1. It can be seen from this table and the power profiles in Figure 5.1 that at the lower maximum power settings ($\leq$500 W), the power levels and pulse frequencies did not differ considerably between the ramping and the holding periods. As higher maximum power settings increased further (750 and 1000 W), the power levels as well as the pulse frequencies differ considerably between the two periods.

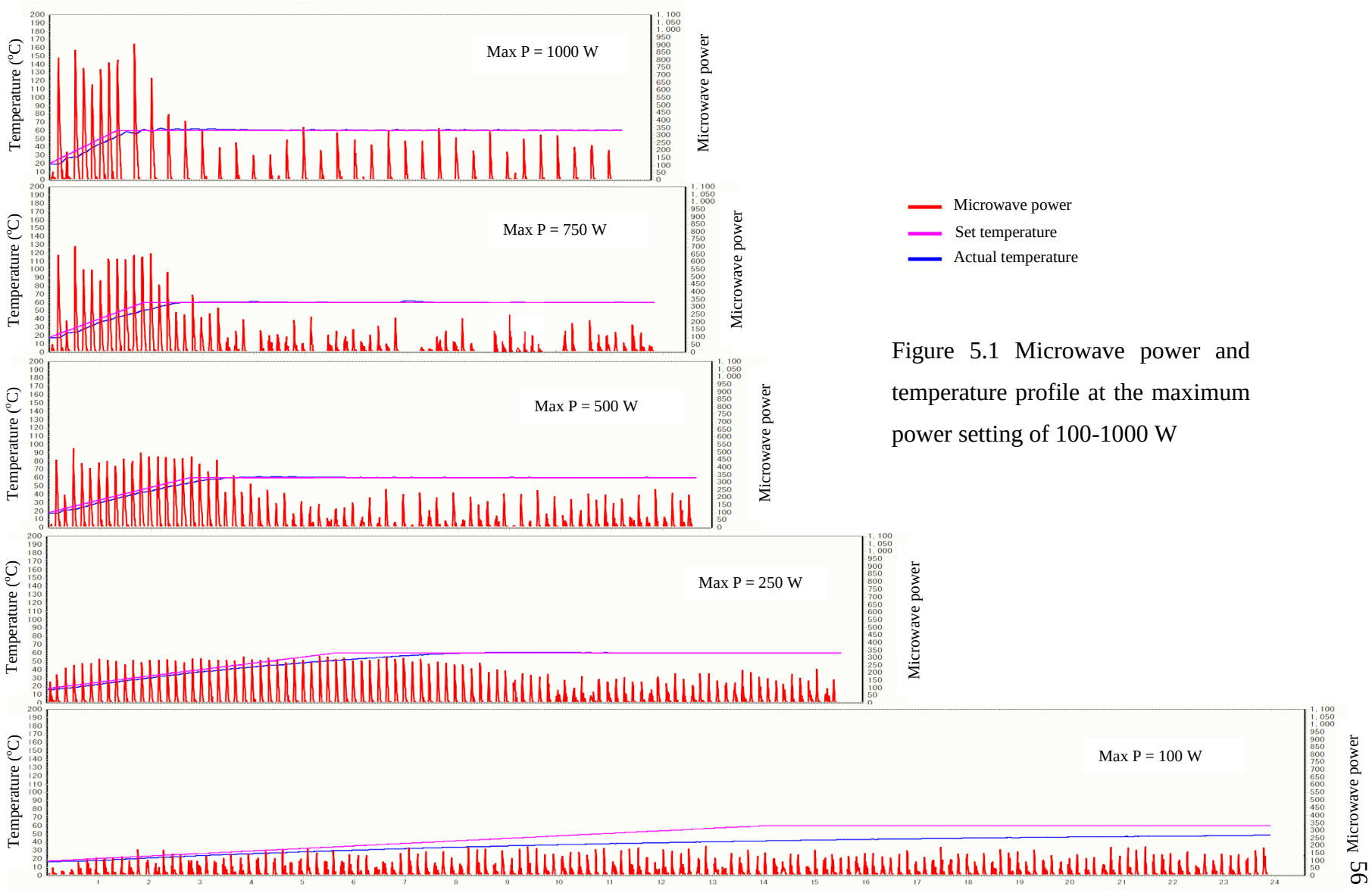

Figure 5.1 Microwave power and temperature profile at the maximum power setting of 100-1000 W

Table 5.1 Pulse frequency from various maximum microwave power setting

Max Power (W)	Number of pulses/min (frequency)	
	Ramping	Holding at 60^{o}C
100	6.2	6.0
250	6.4	5.9
500	6.3	5.9
750	6.7	5.2
1000	6.9	2.9

5.3.2 Effect of power setting on FAMEs yield

The FAME yields obtained from the reaction under various microwave power (at 60^{o}C and 10 min holding time) are summarized in Table 5.2. The highest FAMEs yield of 24.3% by weight of crude lipid was found at the power setting of 250 W. The yield was, however, comparable to that obtained for the reaction irradiated by pulsed microwave at the power of 100 W despite the fact that at this condition the set temperature 60^{o}C could not be reached. The reason for rather high yields at 100 W was probably due to the extended heating (ramping) time (13.8 min) during which the reaction was continuously taking place although at lower temperature. Nevertheless, FAMEs yield per unit time (based on the total reaction time) was the lowest in this case, and therefore this condition cannot be recommended as a suitable power setting. At the highest power setting of 1000 W, the lowest FAMEs yield was obtained. Although the total reaction time was the shortest, the yield per unit time was still as low as that achieved with the maximum power of 100 W. FAMEs yield per unit time is the highest for the power setting of 500 W, followed by that at 250 W. Alternative to FAMEs yields per unit time, the efficiency of the reaction could be determined based on the amount of energy used produce a fixed amount of biodiesel. Integration of peak area gives the energy supplied by the microwave to the reaction system. With this, FAMEs yield per unit energy could be determined and the results are summarized in last column of Table 5.2. This value was the highest when the maximum power setting of pulsed microwave was 250 W, suggesting that the reaction

Table 5.2 FAME yield and energy consumption from each maximum microwave power setting

Max Power (W)	Duration (min)			FAME yield[b] (% by wt of crude lipid)	FAME yield per unit time[b/a] (% by wt of crude lipid/min)	Energy Consumption (kJ)			Energy Consumption per unit time[c/a] (kJ/min)	FAME yield per unit energy (% by wt of crude lipid/kJ)[b/c]
	Ramping	Hold at 60°C	total[a]			Ramping	Hold at 60°C	total[c]		
100	13.8	10	23.8	23 ± 0.7	0.97	35.3	26.5	61.8	2.6	0.37
250	5.5	10	15.5	24.3 ± 2.3	1.57	23.3	22.2	45.5	2.9	0.53
500	2.7	10	12.7	20.6 ± 0.7	1.62	26.1	37.3	63.4	5.0	0.32
750	1.8	10	11.8	15.3 ± 2.0	1.30	24.9	33.1	58.0	4.9	0.26
1000	1.3	10	11.3	11 ± 0.1	0.97	21.2	36.5	57.7	5.1	0.19

occurred most efficiently at this power setting. Except for 100 W, the values seemed to decrease with increasing power setting indicating lower efficiency at higher power setting.

Considering these results together with the pulse data from the previous section (Figure 5.1 and Table 5.1), the more uniform the pulse frequency as in the case of 100 W and 250 W, seemed to be correlated with higher yields. When higher power setting was used, the system automatically adjusted the pulse rate despite the set of 20% cycle, and when the pulse frequency was reduced considerably for the case of 1000 W, considerably lower yield was resulted. This result may be supported by the study by Asakuma et al. (2011) in which the non-thermal effects of microwave on transesterification of triglyceride was described. That is, when exposed to microwave radiation, molecules of tri-glyceride tried to align themselves with the rotating magnetic fields. The molecules then collided with other molecules nearby and caused not only friction heating, but also isomerization of the molecules to different forms. The resulting isomers were demonstrated to have lower dipole moment that results in less activation energy when transesterified to FAMEs and thus the reaction occurred rapidly. Nevertheless, the molecules remained in this form only momentarily and reverted back to the original form without applied microwave irradiation. Hence, more numbers of pulses per unit time could probable keep the isomer stay more active. As seen from Figure 5.1 (with an exception of data at 100 W since the set temperature could not be achieved), at the power setting of 250 W, the pulse frequency was most uniform and the difference in peak intensities between the ramping and the holding period was minimal, the resulting FAMEs yield per unit energy was the highest. The results seemed to contradict with that reported by Kim et al. (2011b), which suggested that pulsed microwave with higher power setting but lower duty cycle would results in greater biodiesel. The different findings could be due to the differences in the reaction system as well as the microwave system that was used. The pulse period in the system employed by Kim et al. (2011a) was much shorter (in μsec) than that of the system used in the present study. Therefore, the molecules may still stay isomerized even when irradiated with high intensity pulses at lower duty cycle.

5.4 Concluding remarks

Production of biodiesel from *Chorella* sp. under pulsed microwave irradiation was found to be greatly affected by the pulsing characteristic, which in turns depends on the power setting of the system. Based on the results in this study, higher algal FAMEs yield was obtained when the power was set to the low levels, where the pulses appeared to have lower intensities but have higher and uniform frequencies. At these lower power settings energy consumption per unit biodiesel produced were lower compared with that resulted by the higher power settings. The results from this study indicated that with use of pulsed microwave, there are still rooms for improvements for the efficiency of the algal biodiesel production. By adjusting the power setting, the process efficiency can be optimized. Nevertheless, determining the suitable scheme to supply the microwave power to a reaction could very well be a system specific problem. Thus more studies will be needed when a system of different designs is used.

CHAPTER VI
SIMULTANEOUS PRODUCTION OF BIODIESEL AND FREE LUTEIN FROM MICROALGAE

6.1 Introduction

Due to the excessive cost for algal biodiesel production, it has been suggested that the overall economic feasibility of biodiesel production from microalgae is largely dependent on the selection of a suitable microalgal strain. Desirable traits include not only an alga's rapid growth and high lipid content but also its environmental hardiness and the possibility of simultaneously generating valuable co-products. Some current research has even emphasized that economically feasible production of microalgal biodiesel will be achieved if only high value co-products are explored (Wijffels and Barbosa, 2010).

Regarding the possible capacity of *Chlorella* species to produce high-value co-products in particular, cultivation of these algal species to harvest the lipids for biodiesel synthesis has gained a surge of interest in recent years (Widjaja et al., 2009, Prommuak et al., 2012). Besides their high lipid content (14–56% by dry weight) (Gouveia and Oliveira, 2009), *Chlorella* is also one of the major sources of the naturally occurring carotenoid lutein (2–4 mg/g of dry biomass) (Wu et al., 2007, Machmudah et al., 2006) The compound has great capability of preventing cancers as well as eye and heart diseases (Sowbhagya et al., 2004); its price is as high as USD 570–790 per kilogram (prices from Changsha Winner Bio-tech Co., Ltd., Changsha, China; Changsha Sunfull Bio-Tech Co. Ltd., Changsha, China; Xi'an Aladdin Biological Technology Co., Ltd., Xi'an, China). Indeed, *Chlorella* species have long been cultivated for the production of biomass (for protein) and high-value products (Converti et al., 2009, Shi et al., 2000) and, as a consequence, the economical harvesting and processing methodologies for these products are already well established, regardless of biodiesel production (Scott et al., 2010). Although a number of studies have recently been conducted on the production of biodiesel from *Chlorella*

lipids, we believe our investigation into the simultaneous production of both biodiesel and free lutein from *Chlorella* microalgae to be the first study of its kind.

In this chapter, we sought first to isolate algal lipids from *Chlorella vulgaris* which are rich in lutein fatty acid esters (LFE) and then to transesterify them using an excess of alkaline MeOH, yielding fatty acid methyl esters (FAME) known as biodiesel. These conditions were deemed suitable also for the saponification of LFE into free lutein, which then allows the production of biodiesel and free lutein as co-product in a single step. Furthermore, the effects of key reaction parameters on the yields of biodiesel and free lutein were investigated, including the amount of catalyst, the biomass-to-alcohol ratio, and the reaction time. In addition, a possible process for the separation of the biodiesel and lutein products from the reaction mixture is proposed. Finally, a preliminary evaluation of the economic feasibility based on the experimental results is presented.

6.2 Materials and methods

6.2.1 Biomass and sample preparation

Dried powder of *C. vulgaris* was prepared following the method previously described in Section 3.1.1

6.2.2 Feasibility study on the simultaneous transesterification and saponification of *C. vulgaris* lipids

The experiment was carried out using a two-step (extraction-transesterification) method. *Chlorella* algae powder (3 g) was extracted with C/M for 4 h in a Soxhlet apparatus following the method described in Section 3.2.2.1. The crude lipid was then charged into a glass vessel equipped with a condenser, along with a solution of KOH (6% by dry weight of the biomass) (Wako, Japan) in MeOH, with a ratio to dry algae of 16:1. The system was then heated to 60°C under agitation at 350 rpm with a magnetic stirrer, and was kept at this temperature for 4 h, after

which it was cooled to room temperature. Chloroform and water were then added into the reaction mixture at a ratio of mixture/chloroform/water of 10:10:9, and the mixture was shaken vigorously. The mixture was then centrifuged (2000 rpm, 10 min) resulting in separation into two phases. MeOH and polar impurities such as glycerol dissolved in the water forming the upper phase, and the lower chloroform phase was retained for the analysis for the FAME and free lutein contents.

6.2.3 Effects of varying reaction parameters on the biodiesel and free lutein yields

The procedure described in Section 6.2.2 was repeated in subsequent experimental runs, each one varying in transesterification duration (either 1, 2, 3, or 4 h), amount of KOH (in the range of 0.1–8.0% by dry weight of the algae), or MeOH to biomass ratio (1:1, 8:1, 12:1, and 16:1 v/w).

6.2.4 Analysis method

Biodiesel yield was determined by gas chromatographic analysis previously described in Section 4.2.4. The samples were also analyzed for free lutein content using high performance liquid chromatography (HPLC). The HPLC analyses were carried out using the method reported by Roberta et al. (1998) with modifications. Of sample, 20 µL was injected into a Lichrocart C-18 column equipped with a Diode Array Detector Module 335 (detection wavelength 450 nm). Chromatographic separation was obtained with a gradient system of acetonitrile/MeOH 9:1 (v/v) as solvent A (HPLC-grade acetonitrile; Sigma-Aldrich, Germany) and ethyl acetate as solvent B. Solvent B was run with a linear gradient at a flow rate of 1 ml/min from 0 to 100% over 30 min.

6.3 Results and discussion

6.3.1 Feasibility of the simultaneous production of biodiesel and free lutein from *C. vulgaris* lipids

The transesterification of a *C. vulgaris* crude lipid extract with MeOH at the MeOH/biomass ratio of 16:1 and 6% KOH catalyst by algal weight resulted in a reaction product that contained FAME with a yield of 33.6% by crude lipid weight (or 4.7% by algal weight). In addition, the analysis of this sample revealed that the composition of the FAME produced in this study was similar to that reported by Lee et al. (2010). Specifically, methyl palmitate (37.7wt %) and methyl linoleate (34.6 wt %) were found to be major components. Other than these, methyl linolenate (15.6wt %), methyl oleate (9.7wt %), and methyl stearate (2.4wt %) were also detected.

Apart from the predominant FAME, the same sample was also analyzed by HPLC for the expected free lutein. The sample was found to contain free lutein with a yield of 2.0% by weight of algal lipids or 3.0 mg free lutein/g of algae. Figure 6.1 a shows the chromatogram of the crude extract, which reveals the peak of free lutein at the retention time of 9.863 min and a group of lutein esters at a later time. After the reaction (Figure 6.1 b), a decrease in the peak intensity of LFE was observed while the peak intensity of free lutein (retention time of 9.956 min) had increased. This attests the occurrence of saponification of LFE during the production of algal biodiesel, and thus free lutein was coproduced.

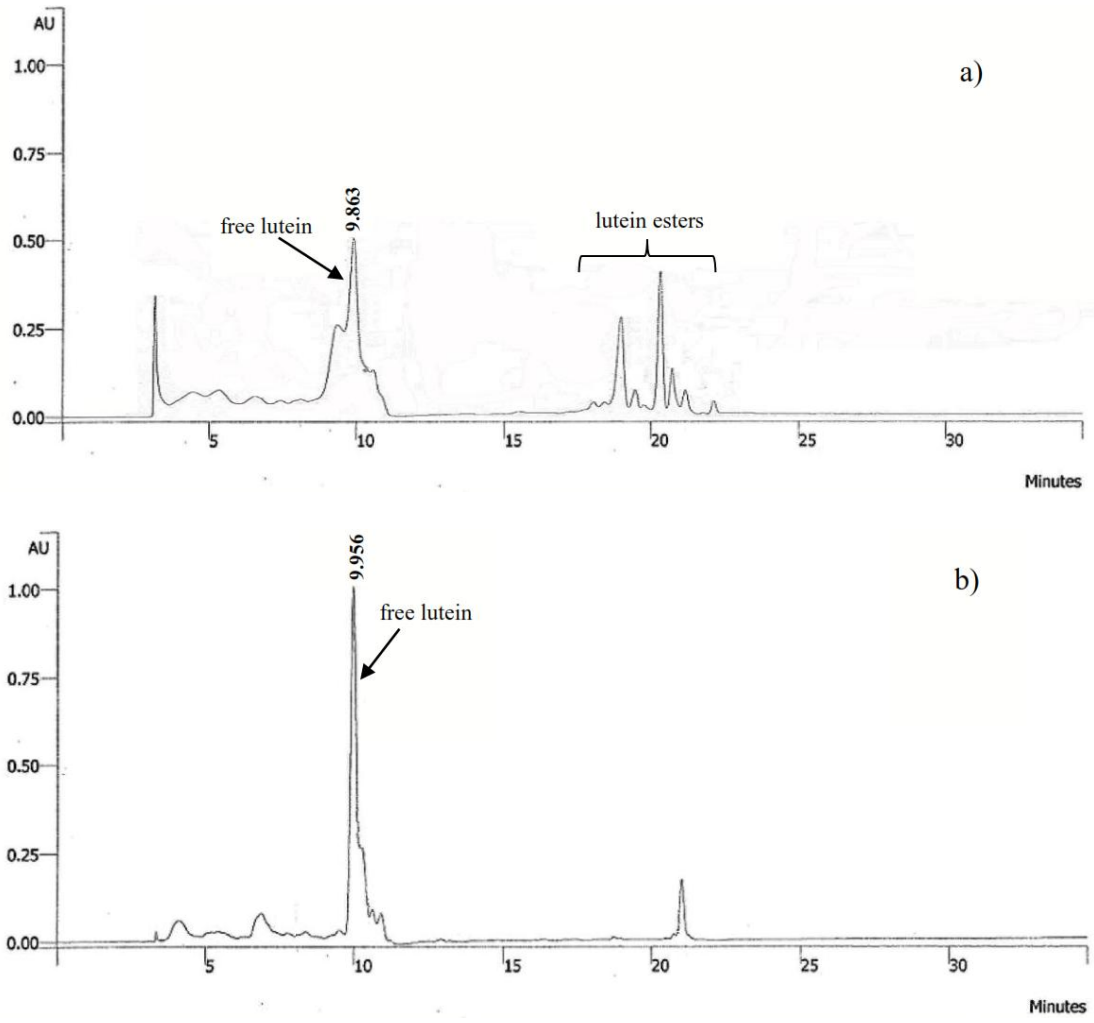

Figure 6.1 HPLC chromatograms of (a) the crude lipid extract,
(b) the product after alkali reaction (6% KOH, 16:1 MeOH/biomass, 4 h)

6.3.2 Effects of reaction parameters on the biodiesel and free lutein yields

6.3.2.1 Effects of reaction time

The effects of the reaction time on the biodiesel and free lutein yields are shown in Figure. 6.2. The FAME yield increased with the reaction time from 1 to 4 h, while the free lutein yield increased similarly from 1 to 2 h but remained almost constant thereafter. This leveling off could simply be a consequence of 2 h being sufficient to release the entire free lutein content of the lipids in the sample (Wu et al., 2007).

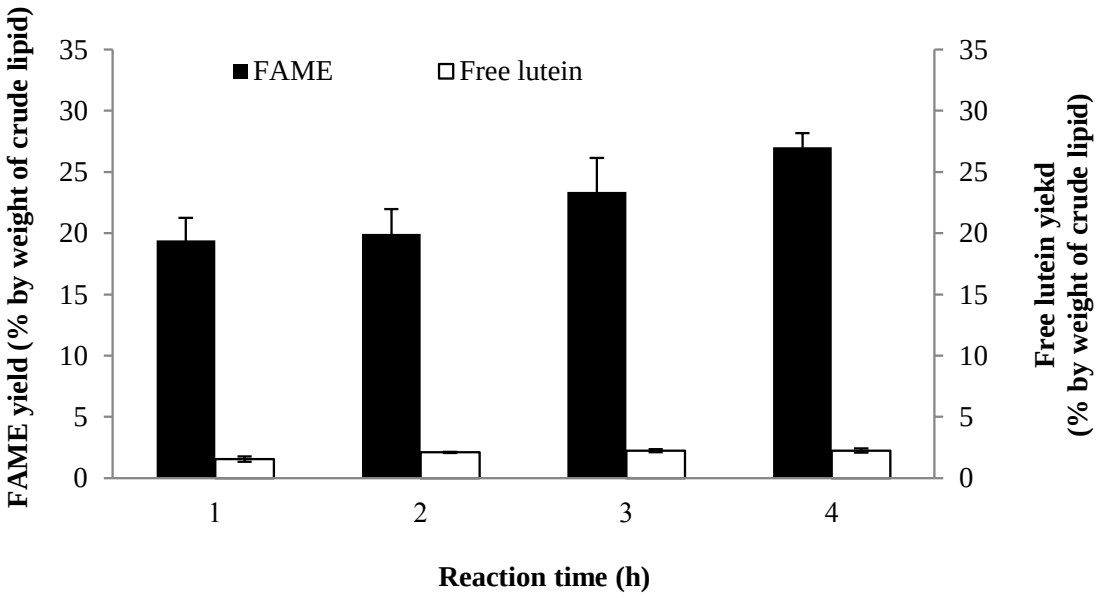

Figure 6.2 Effect of the reaction time on the biodiesel and free lutein yields (4% KOH, 16:1 MeOH/biomass)

6.3.2.2 Effects of the alcohol-to-biomass ratio

Employing a fixed reaction time (4 h) and catalyst amount (4 %), the results shown in Figure. 6.3 reveal that an increase in the MeOH-to-biomass ratio from 8:1 to 16:1 resulted in only a slight increase in the biodiesel yield. This is probably because this surplus of MeOH was far greater than that in the theoretical ratio required for biodiesel conversion. Specifically, the theoretical molar ratio for the conversion of triglycerides is 3:1, which is equivalent to a MeOH-to-biomass (v/w) ratio of only 1:100. This was calculated based on the assumption that the total lipid content of dry algae is, on average, 14 wt% and that only 50% of this lipid is suitable for biodiesel production. It should be noted that no biodiesel product was observed with the smallest ratio of MeOH/biomass of 1:1 used in this study, despite the fact that this ratio is considerably higher than the theoretical ratio (1:100). This result could be attributed to poor mixing in the highly viscous reaction system.

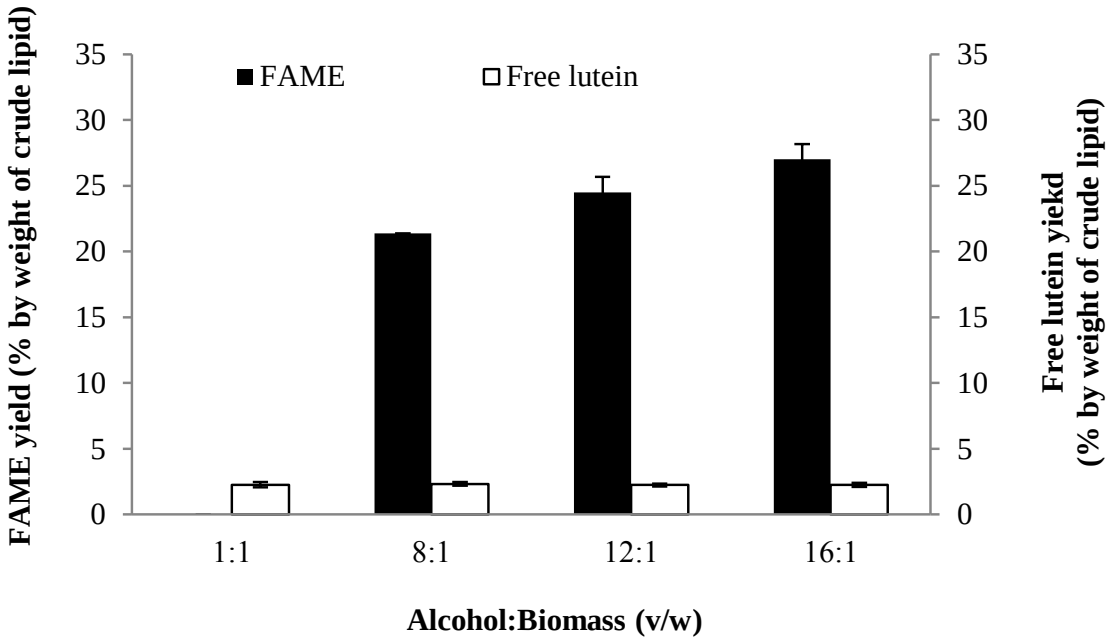

Figure 6.3 Effect of the MeOH/biomass ratio on the biodiesel and free lutein yields (4% KOH, 4 h)

On the other hand, the free lutein yields obtained at any ratio of MeOH/biomass in this study did not differ significantly. In fact, the yield decreased slightly with increasing MeOH amount, probably due to KOH dilution by the excess MeOH slowing down the reaction.

6.3.2.3 Effects of the catalyst quantity

The effects of varying amounts of KOH catalyst on the biodiesel yields were investigated by fixing the reaction time (4 h) and the alcohol-to-biomass ratio (16:1). Figure 6.4 illustrates the increase in the biodiesel yield as the concentration of catalyst was raised from 0.1 to 6%. However, the yield thereafter dropped on further introduction of catalyst (8 %). This decrease could arise from partial saponification of the FAME produced (forming water and amphipathic ‘soap’ salts), thereby competing with the desired transesterification reaction.

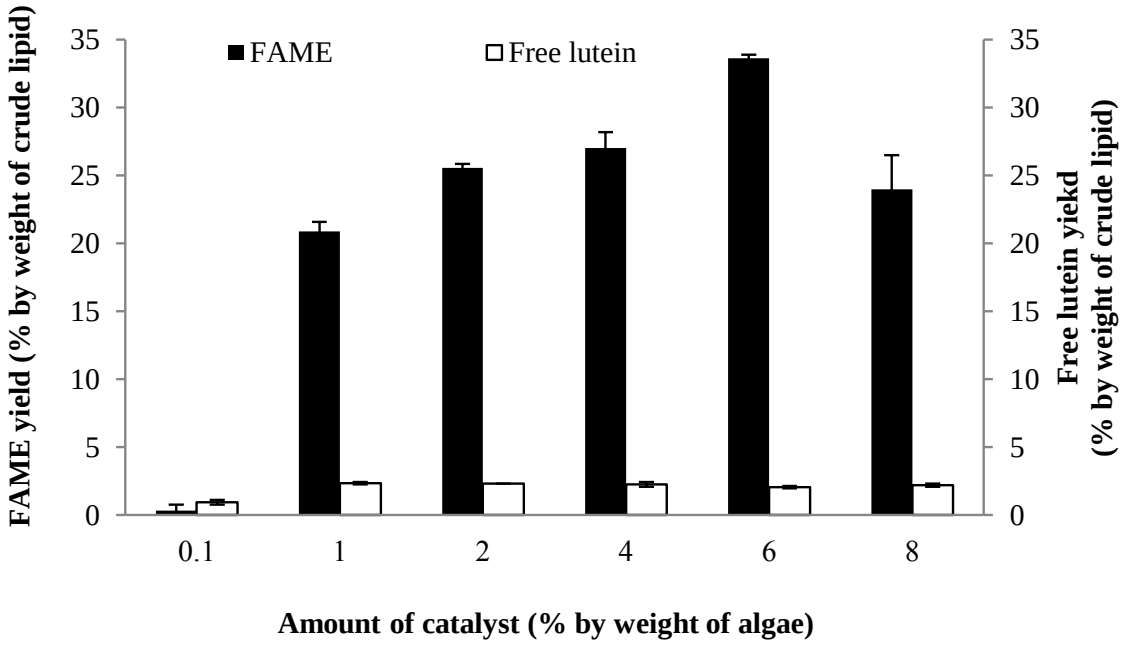

Figure 6.4 Amount of catalyst on biodiesel and free lutein yields (16:1 methanol:biomass, 4 h).

The amount of alkali catalyst also seems to have an effect on the free lutein yield. The use of 0.1% KOH was found to yield very little free lutein, despite the fact that this quantity still provides a stoichiometric excess of KOH with the potential to completely transesterify all lutein esters in the algal lipid sample. One possible reason for the low free lutein yield is that part of the KOH instead preferentially participated (as catalyst) in the transesterification of triglycerides. Another reason may be based on interferences with the reaction caused by existing impurities in the *Chlorella* crude extract, such as free fatty acids (FFA) (Hatano et al., 1982) and pigments such as β-carotene, chlorophyll, and astaxanthin (Gouveia et al., 1996). As more KOH (1%) was introduced, the free lutein yield increased from 0.9 to over 2.3% by weight of algal lipids, after which further KOH (in the range of 2–8%) did not appear to significantly affect the yield. Based on the results shown in Figure 6.4, 1% KOH is likely to be sufficient for the complete saponification of LFE from *Chlorella* biomass.

6.3.3 Proposed product separation process

Although we demonstrated the possibility to simultaneously transesterify *Chlorella* lipid extracts and to saponify their LFE to produce biodiesel and free lutein, for achieving technical and economic feasibility of the entire process, the further downstream processing must also be taken into consideration. Various processes have been proposed to isolate and purify lutein extracted from marigold and *C. vulgaris* after saponification, such as by recrystallization (Vechpanich and Shotipruk, 2011), chromatography (Li et al., 2001), and partitioning in two-phase systems (Li et al., 2002). The last of these techniques was the simplest and the most economical method: 85–91% of free lutein with 90–98% purity could be recovered just by partitioning the extract of saponified *C. vulgaris* in various two-phase systems, followed by anti-solvent precipitation (Li et al., 2002). Given similar components in the saponified lutein extract and in the reaction mixture in our study (KOH solution in MeOH, free lutein, biodiesel, soap, glycerol, and unreacted glycerides), a process modified from that described in Li et al. (2002) is proposed for the separation of biodiesel and free lutein from the reaction mixture (Figure 6.5).

As shown in the diagram, the separation process begins with the addition of a proper organic solvent that dissolves biodiesel and free lutein (such as dichloromethane or chloroform) to the reaction mixture (Li et al., 2002, Craft, 1992). Water is then added, which causes the system to form two phases. It should be noted that, unlike in a typical biodiesel production process, water should not be added directly to the reaction before the addition of the organic solvent. This is necessary to avoid the formation of a thick layer caused by the interaction between soap, water, and glycerides present in the reaction mixtures, which would complicate the separation process. The system of chloroform/mixture/water at a volume ratio of, e.g., 10:10:9 forms two phases: the top MeOH/water-rich phase containing KOH, soap and glycerol and the bottom chloroform-rich phase containing free lutein, biodiesel and unreacted glycerides. The bottom phase can then be collected and, after evaporation of the solvent, the mixture of free lutein, biodiesel, and unreacted glycerides is obtained. Isolation of the two main products (biodiesel and free lutein) can then be

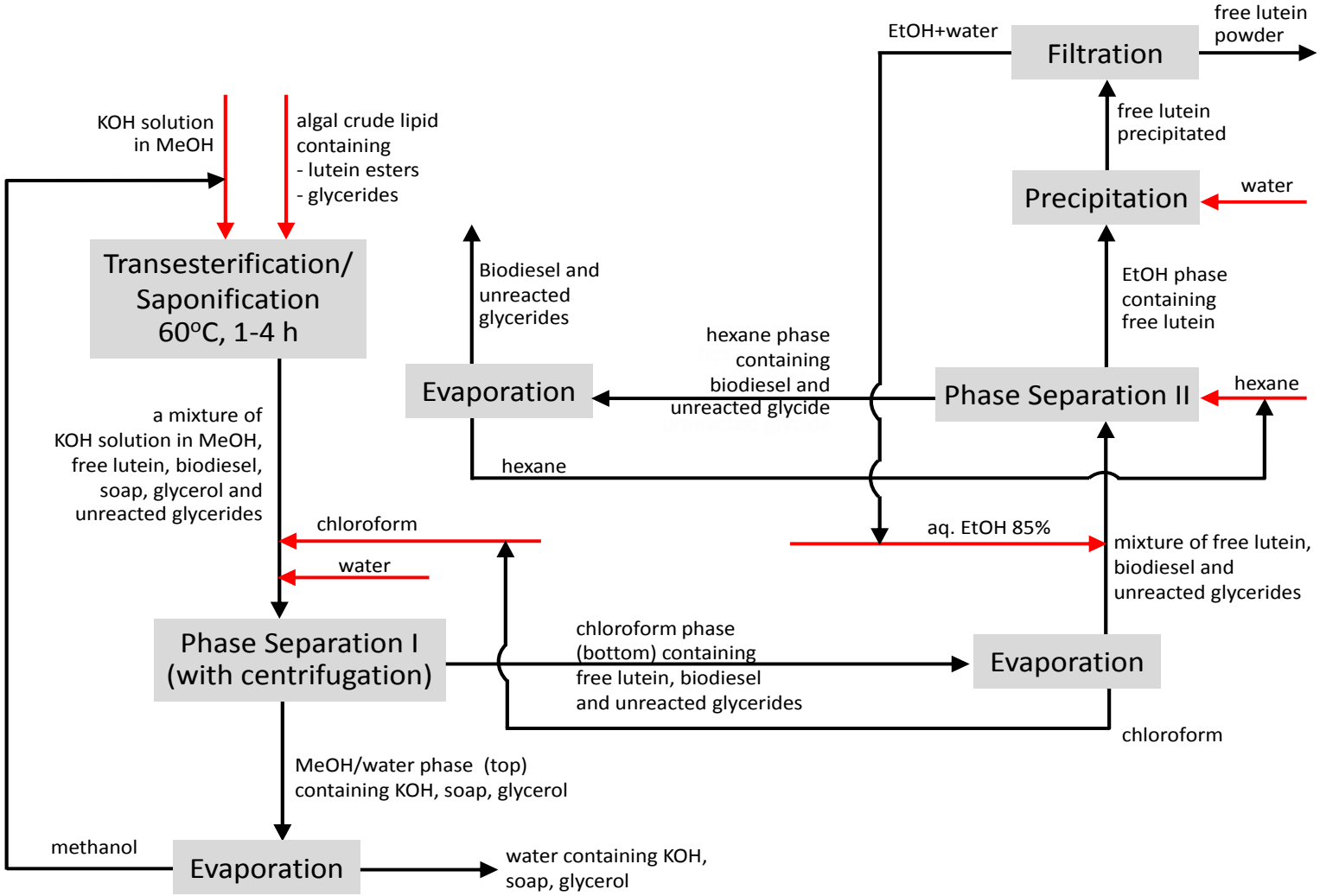

Figure 6.5 Process flow diagram of product separation

achieved in another two-phase system. Redissolving the mixture first in an 85% aqueous EtOH solution followed by the addition of hexane at a hexane/aqueous EtOH solution ratio of 2:1 results in phase separation into the hexane-rich phase containing biodiesel and unreacted glycerides and the EtOH/water-rich phase containing free lutein. Free lutein in the EtOH/water phase can be recovered by precipitation with further addition of water as an anti-solvent. Subsequently, a fine powder of free lutein can be obtained by filtration. Our preliminary investigation using a model mixture of free lutein and palm FAME to represent biodiesel indicated that partitioning of the mixture in a hexane/aqueous EtOH solution system followed by anti-solvent precipitation of free lutein as described above gave nearly 100% recovery of the biodiesel and free lutein. Assuming that the solvents used in the process can be recycled, the additional cost to the process proposed in Figure 5.6 compared with that of conventional biodiesel production would largely be the cost of the additional process equipment, such as for phase separation, precipitation and filtration of the free lutein, and for additional units needed for the evaporation and recovery of organic solvents (chloroform and hexane).

6.3.4 Preliminary economic feasibility assessment

As a potential source of lipid for biodiesel production, microalgae have gained increased attention in recent years, and several studies on the economic feasibility of their biodiesel production process have been published (Pienkos and Darzins, 2009, Delrue et al., 2012). In most of these studies, the production of algal biodiesel is reported to be uneconomical. Delrue et al. (2012) reported the production cost of algae-derived biodiesel to be as high as USD 2.9–5.0 per kilogram[1], which was considerably higher than that of a current commercial biodiesel of only USD 1.63 per kilogram. Their sensitivity analysis indicated that the cost of production varies with the methods of cultivation (raceway or photobioreactor), dewatering/drying

[1] The production cost was the sum of the operating cost i.e. utilities, labor and other costs at 0.9% of the capital cost – and the fixed cost. The fixed cost was calculated from the depreciable capital cost, consisting of 55% of the capital cost for the general maintenance, storage, engineering and spare parts costs, license fees, initial expenses at 2% of the capital cost, and process start-up cost at 25% of the operating cost. The calculation was based on 20-year annuities, an 8% discount rate, and 7% of the capital cost per year for maintenance cost, taxes, insurances, and business expenses.

(centrifugation, belt filter press, solar drying or bed drying), and lipid extraction (n-hexane or dimethylether). Based on the 14% lipid content in algal biomass determined in this study, the biodiesel yield of 20–34% by weight of the crude lipids was calculated to be equivalent to 3–5% by weight of the biomass. A similar calculation resulted in a yield of free lutein co-product of approximately 0.15–0.35% by weight of the biomass, which is equivalent to 0.03–0.12 kg of free lutein produced per 1 kilogram of algal biodiesel. Taking the price of free lutein to be between USD 570–790 per kilogram, our results suggest that approximately USD 17–95 worth of free lutein could potentially be produced with each kilogram of biodiesel. Despite the higher production cost due to additional equipment required for the separation process, the high price of free lutein nevertheless would justify the economic feasibility of the simultaneous production process described in this work.

6.4 Concluding remarks

The results of this study show that, apart from the main product biodiesel, free lutein can be obtained as a co-product, by the simultaneous transesterification and saponification of *C. vulgaris* lipids. The maximum FAME yield (33.6%) was achieved from a 4 h reaction with alcohol/biomass 16:1 v/w and 6% alkali catalyst. Under these conditions, all LFE was de-esterified to the more valuable free form. Separation of the biodiesel and free lutein from the reaction mixtures can be carried out by sequential partitioning in various liquid-liquid two-phase systems, followed by anti-solvent precipitation of the free lutein. Based on our preliminary evaluation, it can be concluded that the process for the simultaneous production of biodiesel and free lutein could be achieved technically and economically. Nevertheless, an investigation into process optimization, such as minimizing the soap formation that lowers the products yields and improving the product separation process, should be in the focus of future studies.

CHAPTER VII
CONCLUSIONS

7.1 Overall conclusions

In PART 1, the study on algal lipid extraction, the mixture of chloroform/MeOH was found to be better than hexane in terms of the obtaining higher lipid yield, but was less suitable in terms of purity of the targeted lipids. Use of UAE and MAE did not have a benefit to algal lipid extraction if the algae cell wall has already been destroyed. For algae with intact cell walls, MAE gave the highest lipid yield.

The results in PART 2 of the study indicate that microwave also assisted the production of algal biodiesel, especially when applied with the single-step method where lipid extraction and transesterification were performed simultaneously. Furthermore, in microwave irradiated reaction, the amount of MeOH and catalyst seemed to affect biodiesel yields. In the pulsed microwave system, different power settings resulted in different pulse intensity and frequency which significantly affected the yield of biodiesel and also the efficiency of the process. With too low maximum power setting (100 W), the reaction temperature could not be attained. The suitable maximum power setting was found to be 250 W. At this condition, the power profile seemed to be the most uniform and possessed the highest pulse frequency which resulted in the highest biodiesel yield per unit energy. As the maximum power setting was increased to higher levels, the pulse intensities were higher but pulse frequencies were lower and less uniform. At these conditions, the biodiesel yields per unit energy tended to decrease.

Finally, in PART 3, improving the economic feasibility of the biodiesel production process by producing a high-valued co-product was emphasized. That is, biodiesel and valuable free lutein were demonstrated to be simultaneously produced from *Chlorella* lipid extracts. The alkali catalyst used in the transesterification of triglycerides acted as a reactant in converting lutein fatty acid esters to free lutein. The

excess of alkali and MeOH employed in the production of biodiesel ensured the complete saponification of all lutein fatty acid esters to free lutein. The separation of the co-product from the biodiesel could be achieved by solvent partitioning and the subsequent anti-solvent precipitation. Finally, the preliminary economic assessment shows feasibility of the algal biodiesel production.

7.2 Summary of contributions

In the midst of controversy over whether biodiesel from microalgae would be a breakthrough or just an idealistic energy, this work reveals potential ways to enhance algal biodiesel production which may lead this new fuel to practicality. Information from the study on extraction of lipid, one of the most energy consuming steps, could help select suitable solvent, extraction methods and conditions for specific strains.

As another option, the lipid extraction combined with the transesterification step, and with the assistance by pulsed microwave irradiation, was investigated. The combination of these two steps could draw out solutions to reduce the overall time, energy, material (solvents) consumption and the cost from installation of extraction unit. The study on effect of key reaction parameters on the production of biodiesel yield informs suitable amounts of H_2SO_4 and MeOH where biodiesel could be best produced under microwave irradiation system. In addition, with the ability of the apparatus to record temperature and power profile, the energy consumption per unit of biodiesel from each setting of maximum microwave power was revealed. This then contributes to information on how to control the microwave system efficiently and thus operating cost could be minimized.

One of the outstanding advantages of producing biodiesel from microalgae over the crops is that the algae can provide not only the biodiesel, but they also contain many high-value products. This issue has been very well aware of, but the attempt to bring about such benefit has not yet been on current research trend. This work could be one of the very first that emphasized this advantage. The study on simultaneous production of biodiesel and free lutein, the proposed separation method

and the preliminary economic feasibility assessment could be examples that clearly show the possibility of this concept.

7.3 Recommendations for future research

7.3.1 To fetch out clearer benefit from the development on extraction and transesterification process in this study, economic analysis may be further carried out. The analysis could be divided into two scenarios where the first is the evaluation of the cost in production when lipid extraction unit is included and the second is that when the single-step (simultaneous extraction and transesterification) is carried out. Additionally, both scenarios maybe sub-divided into two cases, with or without the pulsed microwave irradiation.

7.3.2 Based on the great performance of pulsed microwave in the production of algal biodiesel, either when applying for the step of lipid extraction or the transesterification, the development of the apparatus for industrial scale maybe of further consideration. This includes the modification of the apparatus to a continuous system and the scaling up.

7.3.3 Not only lutein, *C. vulgaris*, also contains significant amounts of other pigments such as β-carotene, chlorophyll as well as high value fatty acids such as ω-3 and ω-6. Even the cell debris which is residual from lipid extraction still contains a large amount of protein. To return more value to algal biodiesel production, these high-value products should be taken into consideration and sought for recovery. In addition, other algal strains with different cell composition may also be investigated.

7.3.4 Due to the limited amount of algal biomass, the proposed method for the separation of free lutein from the main product (biodiesel) in this study was tested using a model mixture of methyl palmitate and marigold lutein. For the future work, this method of separation should be tested with the actual system mixture. Also, suitable conditions including the ratio of the mixture to the solvents and anti-solvent precipitation temperature should be investigated.

7.3.5 Soap formation (caused by the co-production of free lutein) not only hinders the production of the main product, biodiesel, but it also does make the

product separation difficult. Investigation into process optimization, such as minimizing the soap formation should therefore be considered as a future research.

7.3.6 The amount of FAMEs obtained from each experiment in this study was reported in terms of %yield by weight of the algal crude lipid. Due to the fact that the algal crude lipid contained not only the reactants (glycerides) but also major contents of impurities such as phospholipids and FFA, the reported yields were rather low. Therefore, conversion should be determined and accordingly, glyceride content in the crude lipid is needed to be explored. However, from the method of GC analysis used in this study as an attempt to find the glyceride content, the peaks in the obtaining chromatograms (Appendix A) are not clearly separated. The adjusting of GC condition or modification of analysis method to determine this glyceride content is then recommended as a future work.

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APPENDIX

APPENDIX A

The algal crude lipid was analyzed for the appearance of glycerides by GC-MS. The chromatograms are shown in Figure A-1.

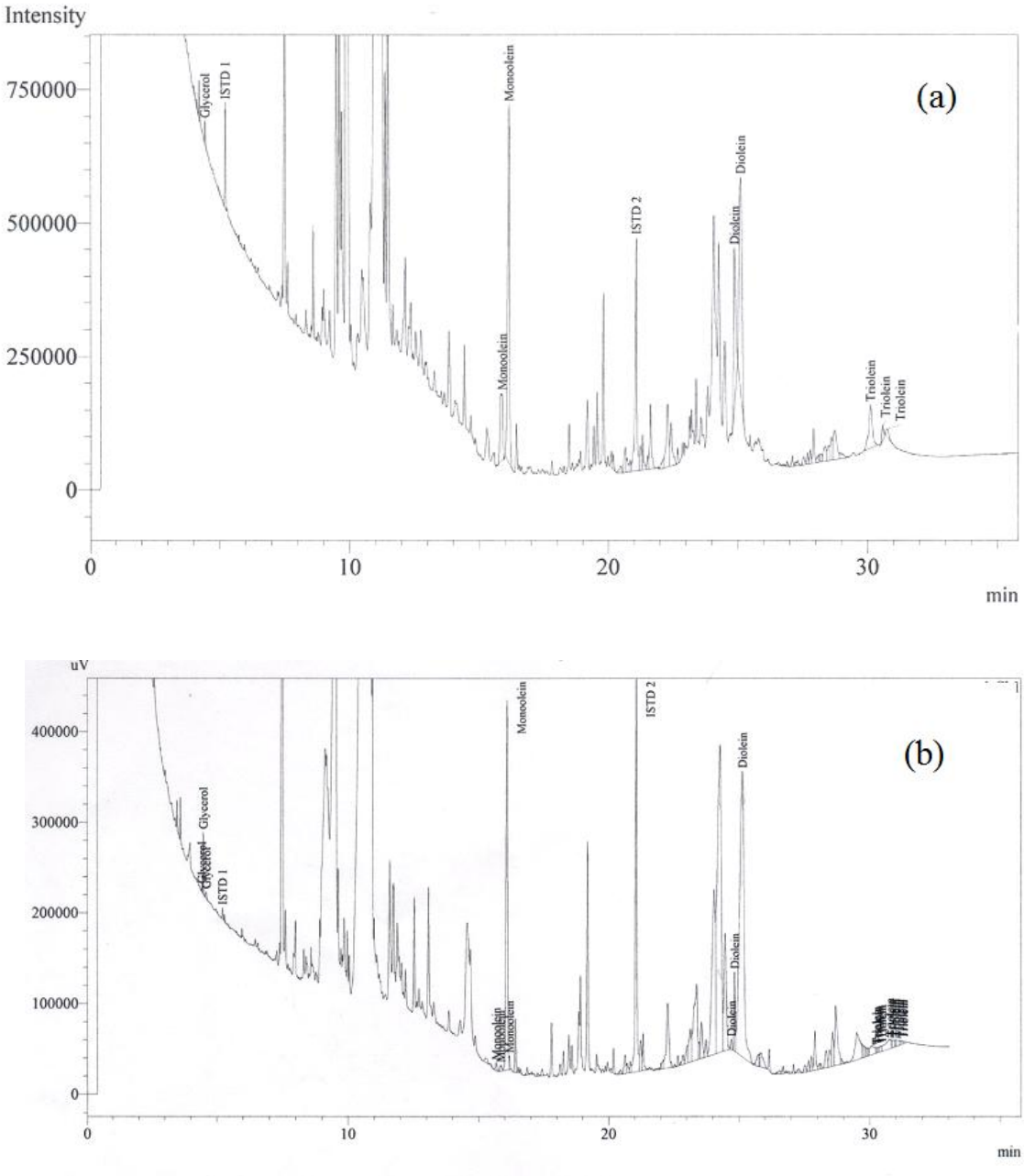

Figure A-1 Chromatogram of crude algal lipid extracted by
(a) hexane and (b) chloroform/MeOH (2:1 v/v)

It can be seen from the Figure that the peaks of glycerol and other polar impurities appeared at the retention time of 3-12 minute following by the non-polar lipids such as mono-, di- and tri-glyceride, respectively. Although non-polar glycerides appeared on the chromatograms of the crude algal extracts by both solvents: hexane (Figure A-1 (a)) and the mixture of chloroform and methanol (Figure A-1 (b)), the latter is likely to extract more polar impurities.

BIOGRAPHY

Miss Chattip Prommuak was born on May 4, 1982 in Nonthaburi, Thailand. She received a Bachelor's Degree of Chemical Engineering from the Faculty of Engineering, King Mongkut's Institute of Technology Ladkrabang in 2004. She completed the requirements for a Master's Degree in Chemical Engineering at the Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University in 2007. Chattip continued pursuing her Ph.D. degree in the 2009. During the Ph.D. program, she was granted by Thailand Research Fund through The Royal Golden Jubilee Ph.D. Program (RGJ-TRF) in collaboration with Chulalongkorn University.